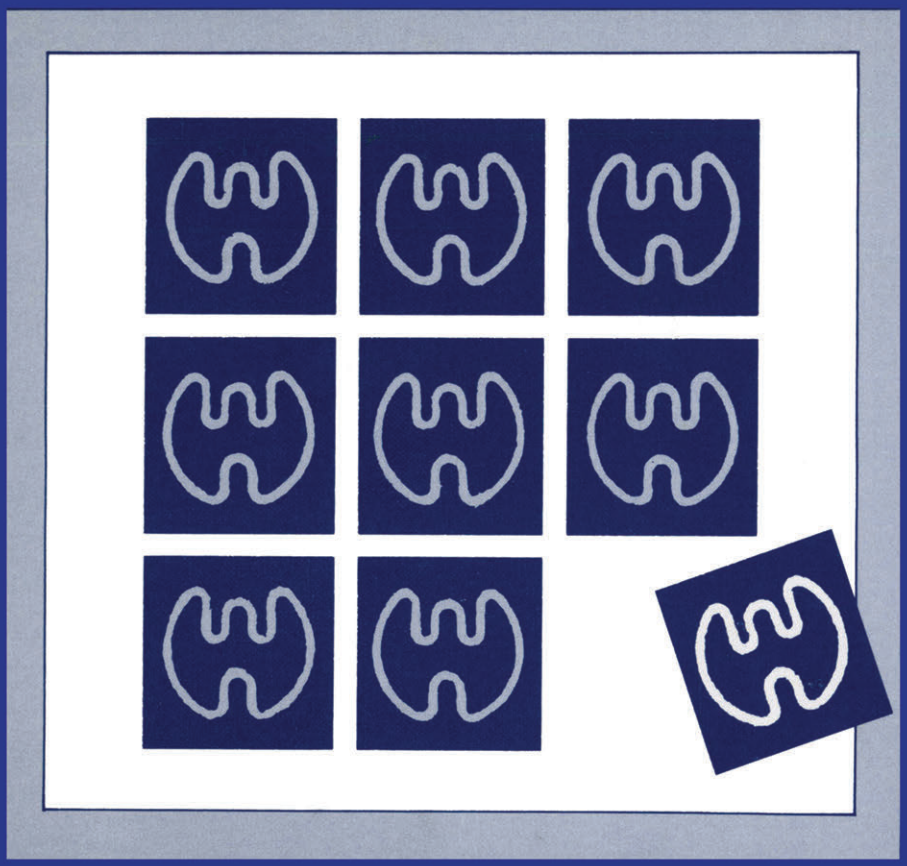

Frontiers in Thyroidology

Edited by Geraldo Medeiros-Neto
and Eduardo Gaitan

Frontiers in Thyroidology

Volume 1

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FOREWORD

"Frontiers In Thyroidology" assembles the prize lectures, symposia, and papers presented during the Ninth International Thyroid Congress in São Paulo, Brazil in September, 1985. This book represents the State-of-the-Art in thyroid studies with its continuous expansion into the areas of immunology, molecular biology, clinical and environmental medicine. As its name also indicates, the material compiled in this volume defines important questions to be answered in basic and applied aspects of the thyroid. So, this book clearly demonstrates the rewards, excitement, and challenges posed by the thyroid gland. Once again, the thyroid gland, through its unique and important functions and implications in health and disease, has been able to congregate scientists throughout the world to communicate their findings, to share their expertise and experiences, and to strengthen their bonds of friendship and commitment to make a better world through their work.

This publication consists of 309 papers in 19 different topics. The prize lectures and symposia review and update our knowledge in basic and clinical aspects of exceptional interest in the thyroid field. "Frontiers in Thyroidology" adds to a series of publications from previous international thyroid meetings which includes: "Advances in Thyroid Research", London, 1960; "Current Topics in Thyroid Research", Rome, 1965; "Further Advances in Thyroid Research", Vienna, 1970; "Thyroid Research", Boston, 1975, and "Thyroid Research VIII", Sydney, 1980.

We would like to express our gratitude to Mrs. P. Langford and Drs. L.L. Braverman, L.J. DeGroot, J.T. Dunn, R.H. Lindsay, J.M. Montalvo, K.C. Neill, J. Robbins, P.G. Walfish, and J.F. Wilber for their expert and generous assistance in reviewing and editing the manuscripts for this volume. Their help has added significantly to the final presentation of the book. We would also like to thank Dr. David Evered, Director of the CIBA Foundation, for housing, once again, the members of the Program Organizing Committee in London, May, 1985. We are also grateful to Mrs. Silvia Gabay who organized the manuscripts received during the 9th ITC, Mrs. Suzanne Clay at the University of Mississippi Medical Center who generously provided administrative assistance and arranged facilities for word processing, and to Mrs. Carol Boone who with expertise and dedication undertook the tremendous task of retyping and organizing all manuscripts for final publication.

September, 1986
Geraldo A. Medeiros-Neto
Eduardo Gaitan

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THYROTROPIN-RELEASING HORMONE ACTION IN THYROTROPIC CELLS: MECHANISM OF STIMULATION OF THYROID-STIMULATING HORMONE SECRETION

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Production of pituitary thyrotropin (thyroid-stimulating hormone; TSH), the major modulator of secretion of L-triiodothyronine ($L\text{-}T_3$) and L-thyroxine ($L\text{-}T_4$) from the thyroid gland, is, in turn, regulated by the circulating thyroid hormone level. Superimposed on the pituitary-thyroid feedback system is a modulation by the central nervous system that appears to be mediated, in large part, by hypothalamic thyrotropin-releasing hormone (TRH). The studies described herein were undertaken to define the intracellular events that mediate TRH stimulation of the thyrotroph, in particular, TRH stimulation of TSH secretion.

In order to study the molecular mechanisms involved in transducing the signal of TRH-receptor interaction at the cell surface into regulators of intracellular processes, it is necessary to employ a homogeneous population of thyrotrophs so that the intracellular events monitored can be presumed to occur uniformly in all the cells. A thyrotropic cell culture system that is responsive to physiological concentrations of thyroid hormones and to TRH was developed for these studies (1-3). Pituitary thyrotropic tumors were induced in mice of the LAF_1/J strain by ablation of the thyroid gland with radioiodine as originally described by Furth and his colleagues (4); the pituitary tumors were then serially transplanted into similarly radiothyroidectomized mice. Short-term suspension cell cultures of thyrotropic (TtT) cells were established after enzymatic dispersion and selective attachment techniques and incubated in Ham's F-12 medium supplemented with 15% horse serum and 2.5% fetal bovine serum. Electron micrographs of cells in suspension culture showed that they have morphological traits characteristic of thyrotrophs in hypothyroid animals. The cultures appeared morphologically to be comprised of a homogeneous population of epithelial cells containing TSH secretory granules and were shown to synthesize and secrete TSH.

The binding of many hormones and neurotransmitters to plasma membrane receptors appears to be coupled to stimulation of secretion partly by an elevation of cytoplasmic free calcium ion concentration ($[Ca^{2+}]_i$) (5). Stimulus-induced elevation of $[Ca^{2+}]_i$ is caused by release of intracellular calcium into the cytoplasm or by increased influx of extracellular Ca^{2+} into the cytoplasm, or both (6). In some cells, binding of a calcium-mobilizing stimulus to its receptor causes a rapid enhancement of the hydrolysis of phosphatidylinositol 4,5-bisphosphate [$PtdIns(4,5)P_2$] by a phospholipase C (or phosphodiesterase) to yield inositoltrisphosphate

($InsP_3$) and 1,2-diacylglycerol, and an increase in the level of unesterified arachidonic acid (7,8). It has been proposed that $InsP_3$, 1,2-diacylglycerol and arachidonic acid serve as intracellular mediators to activate secretion. $InsP_3$ appears to act to mobilize calcium from an intracellular pool to elevate $[Ca^{2+}]_i$. 1,2-Diacylglycerol may exert its effects by activating a calcium- and phospholipid-dependent protein kinase (protein kinase C). Arachidonic acid may stimulate secretion by directly affecting exocytosis. It appears that this general model of signal transduction is applicable to TRH stimulation of TSH secretion. In this report, I review the data from studies in my laboratory that support this hypothesis.

The effect of TRH on $[Ca^{2+}]_i$ was studied using the fluorescent Ca^{2+} chelator, Quin 2, trapped within the cytoplasm of TtT cells (9). In control cells incubated in medium containing 1.5 mM Ca^{2+}, a physiologic concentration, resting $[Ca^{2+}]_i$ was 110 nM. TRH (1 μM) caused a rapid elevation of $[Ca^{2+}]_i$ to 700 nM followed by a sustained elevation to 240 nM. Previous studies in which fluxes of radiocalcium were measured (10,11) and in which cellular membrane-bound Ca^{2+} was monitored with the fluorescent probe, chlortetracycline (12), provided evidence that the elevation of $[Ca^{2+}]_i$ was caused by both release of Ca^{2+} from intracellular stores and stimulation of Ca^{2+} influx. This was confirmed in studies with Quin 2. In fact, there appears to be a temporal sequence of rapid release of intracellular Ca^{2+} that comprises the major component of the rapid peak elevation of $[Ca^{2+}]_i$ followed by enhanced influx of extracellular Ca^{2+} that causes the sustained elevation of $[Ca^{2+}]_i$. The mechanism whereby TRH stimulates influx of extracellular Ca^{2+} appears to involve a TRH-induced, transient depolarization of TtT cells, which we measured with the lipophilic cation triphenylmethylphosphonium ion (13), that opens "Ca^{2+} channels" in the plasma membrane.

The effects of TRH on lipid metabolism in TtT cells were studied. The effects of TRH on the levels of $PtdIns(4,5)P_2$ and $InsP_3$ were measured in cells labeled with [^{3}H]inositol to isotopic steady-state. Under these conditions of labeling, changes in ^{3}H-radioactivity reflect changes in the masses of these compounds. TRH caused a rapid decrease in the content of [^{3}H]$PtdIns(4,5)P_2$. Simultaneously, there was a rapid increase in the level of [^{3}H]$InsP_3$ (9). Because $InsP_3$ can only be formed in mammalian cells by hydrolysis of $PtdIns(4,5)P_2$, these data demonstrate that an early effect of TRH in thyrotropic cells is stimulation of the phospholipase C-mediated hydrolysis of $PtdIns(4,5)P_2$. In cells labeled with [^{3}H]arachidonic acid to isotopic steady-state, TRH stimulated a transient increase in the content of unesterified [^{3}H]arachidonic acid and of 1,2-[^{3}H]diacylglycerol (14).

Berridge (7) proposed that $InsP_3$ may function as the intracellular mediator to release Ca^{2+} from a cellular pool(s). The ability of $InsP_3$ to release Ca^{2+} was studied in a preparation of detergent-permeabilized rat pituitary mammotropic (GH_3) cells in order to expose intracellular Ca^{2+} pools to $InsP_3$ (15). $InsP_3$ caused a very rapid loss of previiously sequestered Ca^{2+} from a nonmitochondrial pool within permeabilized cells. The effect of $InsP_3$ was specific, as there was no effect of other inositol sugars at similar or even higher concentrations. These findings are consistent with the notion that $InsP_3$ is the intracellular messenger generated after TRH binding to its receptor that causes mobilization of Ca^{2+} from a pool within pituitary cells, perhaps within the endoplasmic reticulum. These studies have not as yet been performed in TtT cells; however, similar findings would be expected.

Many previous studies have shown that agents that elevate $[Ca^{2+}]_i$ stimulate TSH secretion (for review, see 16). Recently, it was shown that phorbol esters, which presumably act as simulators of 1,2-diacylglycerol to activate protein kinase C, stimulate TSH secretion (17). In TtT cells,

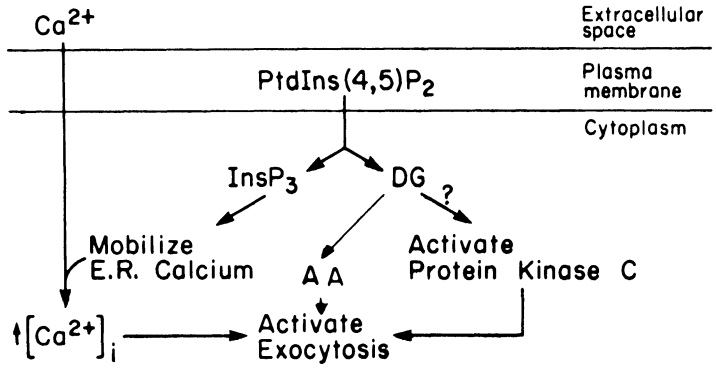

Fig. 1. Model of TRH stimulation of TSH secretion.

arachidonic acid added exogenously also caused TSH secretion (14). Hence, three products of rapid changes in thyrotrope metabolism caused by TRH are potential mediators of stimulated secretion of TSH.

The following model may be proposed for the intracellular events involved in TRH stimulation of TSH secretion (Fig. 1). The binding of TRH to its plasma membrane receptor stimulates the hydrolysis of PtdIns(4,5)P_2 to yield $InsP_3$ and 1,2-diacylglycerol, and there is a concomitant increase in unesterified arachidonic acid. These three agents serve subsequent mediator functions. $InsP_3$ causes the release of Ca^{2+} from a nonmitochondrial pool to rapidly elevate $[Ca^{2+}]_i$. TRH also causes depolarization of the cell surface leading to opening of Ca^{2+} channels, enhanced Ca^{2+} influx, and continued elevation of $[Ca^{2+}]_i$. The elevation of $[Ca^{2+}]_i$ couples stimulus to secretion. The elevation of $[Ca^{2+}]_i$ may activate exocytosis directly or through phosphorylation of proteins involved in the exocytotic process via activation of Ca^{2+}- and calmodulin-dependent protein kinase(s), or both. Concomitant with the effects of $InsP_3$ and of elevation of $[Ca^{2+}]_i$, phosphorylation of proteins involved in the exocytotic process may be stimulated through 1,2-diacylglycerol activation of protein kinase C and arachidonic acid may directly stimulate exocytosis by interacting with the membrane of secretory granules. This model is proposed to serve as a guide to further investigation into the molecular events that couple TRH-receptor interaction at the cell surface to the transduction and amplification of intracellular signals that lead to stimulation of TSH secretion.

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REFERENCES

1. Gershengorn MC. Endocrinology 102: 1122, 1978.
2. Gershengorn MC, Cohen M, and Hoffstein ST. Endocrinology 103: 648, 1978.
3. Gershengorn MC. In JH Oppenheimer and HH Samuels (eds), Molecular Basis of Thyroid Hormone Action, Academic Press, New York and London, 1983, p 387.
4. Furth J, Moy P, Hershman JM, et al. Arch Pathol 96: 217, 1973.
5. Rasmussen H and Barrett PQ. Physiol Rev 64: 938, 1984.

6. Douglas WW. Ciba Found Symp 54: 61, 1978.
7. Berridge MJ. Biochem J 220: 345, 1984.
8. Nishizuka Y. Nature (Lond) 308: 693, 1984.
9. Brenner-Gati L and Gershengorn MC. Endocrinology, in press.
10. Geras EJ and Gershengorn MC. Am J Physiol 242 (Endocrinol Metab 5): E109, 1982.
11. Geras E, Rebecchi MJ, and Gershengorn MC. Endocrinology 110: 901, 1982.
12. Gershengorn MC and Thaw C. Am J Physiol 243 (Endocrinol Metab 6): E298, 1982.
13. Gershengorn MC, Geras E, Rebecchi MJ, et al. J Biol Chem 256: 12445, 1981.
14. Kolesnick RN, Musacchio I, Thaw C, et al. Endocrinology 114: 671, 1984.
15. Gershengorn MC, Geras E, Purello VS, et al. J Biol Chem 259: 10675, 1984.
16. Gershengorn MC. Mol Cell Biochem 45: 163, 1982.
17. Koenig RJ, Senator D, and Larsen PR. Biochem Biophys Res Commun 125: 353, 1984.

THYROID HORMONES AND THE DEVELOPING BRAIN

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There are three main situations in which an association has been noted between anomalies of thyroid function and mental retardation: (A) Severe endemic goiter associated since the XVIth century with the birth of deaf-mute and imbecile inhabitants, known as cretins (1). This association was recognized before it was even known that goiter is an enlarged thyroid. (B) Congenital hypothyroidism associated with severe mental retardation since Curling described two cases in the mid-XIXth century (2). (C) Maternal hypothyroxinemia, with or without clinical hypothyroidism, associated since the studies of Man et al. (3) with decreased mean I.Q. of the progeny.

The possibility that in utero or neonatal exposure to hypothyroid conditions also affects brain development has been reviewed by Hollingsworth (4) and will not be discussed here.

The severity of the brain damage is greatest for situation A, and least for situation C. The first could be recognized by laymen; the last required the development of quantitative tests to measure intellectual performance, but this does not mean that our understanding of the etiology of the brain damage parallels its severity. Indeed, our understanding of cretinism is still far from clear, despite the fact that it still affects millions. Fortunately, however, though we do not understand the underlying mechanisms, it is possible to prevent the causes, or the consequences, of all three situations. The birth of cretins can be eradicated by adequate iodine prophylaxis of the mother before, or very early in pregnancy. Though we cannot yet prevent congenital hypothyroidism, mass screening programs for the prevention of the ensuing mental retardation by early treatment with thyroid hormone are being very successful everywhere. These two points will be dealt with extensively in the present Congress by others (5,6). Adequate treatment of hypothyroxinemic mothers throughout pregnancy prevents adverse effects on the I.Q. of the progeny (3). There is no scientific reason at present for brain damage to continue to occur because of iodine deficiency, congenital hypothyroidism, or maternal hypothyroxinemia.

Some experimental models which have been used to clarify problems related to conditions A-C will be briefly discussed here, not in the chronological order in which these associations were recognized, but in the order in which experimental models were established and used to investigate the underlying mechanisms.

CONGENITAL HYPOTHYROIDISM

Typical clinical findings regarding the mental development of babies with congenital hypothyroidism have indicated that the earlier the hypothyroid condition supervenes and the longer the condition is left untreated, the more profound the ensuing irreversible damage. On the contrary, the earlier an adequate treatment with thyroid hormone is instituted, the greater the likelihood of ensuring a normal I.Q. Onset of treatment after three months of age reduces drastically the probability that mental retardation will be prevented (7) in babies with prenatal hypothyroidism. However, in order to prevent other less neuropsychological sequelae, treatment has to be instituted before one month. On the other hand, if hypothyroidism supervenes after birth, the probability increases that a normal I.Q. will be attained even when treatment is delayed for a few months. Thus, it would appear from such information that lack of fetal thyroid hormones would already have a damaging effect on phase(s) of brain development occurring after midgestation (when the fetal thyroid becomes active), and that the thyroid hormone deficiency has to continue and affect brain development for more than the first three months after birth for brain damage to result in a low I.Q. Fig. 1 tries to summarize known events regarding fetal thyroid function (upper panel) and brain maturation (lower panel) in the human. The most active phase of neuroblast multiplication occurs at about 18-20 weeks of gestation, antedating the onset of active T_4 secretion by the fetal thyroid. Thus, lack of fetal thyroid function is not expected to affect neuroblast multiplication markedly, but it cannot be excluded that it might still affect neuroblasts undergoing mitosis during the late third trimester, or in brain structures, such as the cerebellum, where neuroblast multiplication is retarded with respect to that of the forebrain, and mostly postnatal. At the end of gestation and during the first months after birth, the brain growth spurt takes place. This reflects rapid multiplication of the glial

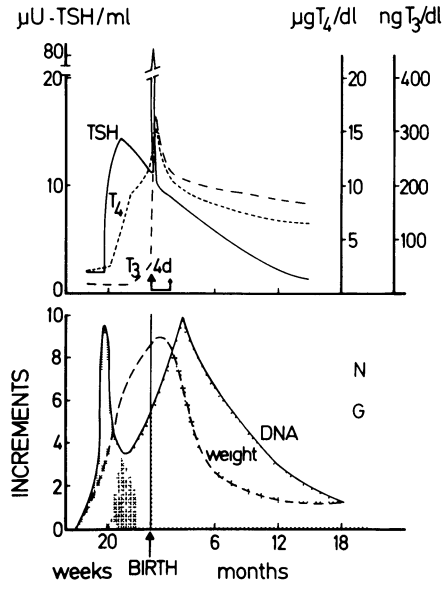

Fig. 1. Schematic representation of the timing of the changes in human fetal and neonatal TSH, T_4, and T_3 levels (upper panel) and of main events during the brain growth spurt (lower panel). The fetal thyroid starts secreting hormone at the beginning of the 4th month of gestation, but is fully active at about the 20th week (mid-gestation) as assessed from the sharp rise in serum T_4, consequent to the previous surge in TSH (8). The developmental events in the brain are shown as increments in DNA or weight over 5-week periods, and are taken from Dobbing (9). The shaded areas, N and G, represent the phases of peak neuroblast and glial cell replication. The diagram represents mainly events in the forebrain, timing of cerebellar events being retarded, with most of the growth spurt occurring after birth. Reprinted from Morreale de Escobar et al. (14) by courtesy of Marcel Dekker, Inc.

cell population, and increased deposition of proteins and lipids, as the neurons and glial cells differentiate. In addition, there is rapid growth of axonal and dendritic processes, which permit the establishment of neuronal circuits and connections. Some of these processes (myelination, for instance) may continue well into the second and third year after birth. All of them might be affected by a lack of fetal thyroid hormones persisting for more than three months after birth.

Since the pioneering work of Eayrs (10), the rat thyroidectomized at birth, or within the first two weeks after birth, has been used to obtain a model for the understanding of human congenital hypothyroidism. In this species, the phase of active neuroblast multiplication is taking place during the last days preceding birth with the brain growth spurt peaking at about the 2nd-3rd postnatal week. Plasma T_4 is low at birth and increases postnatally. Thus, it was hoped that identification of some event in brain maturation which would be affected by thyroidectomy at birth would give us an insight into the "physical basis within the brain of higher mental functions" (9). Unfortunately, it turned out that "everything appears to go wrong in the brain of animals rendered prematurely hypothyroid" (11). Many excellent overviews covering hundreds of studies on different morphological, biochemical, electrophysiological, and behavioral aspects of the problem have appeared, some being quoted in reviews from our group (12-14). It is in general believed that thyroid hormones are needed for normal differentiation of brain cells; in their absence, normal brain maturation would not take place. An excess of thyroid hormone during a critical period would have the opposite effect, by signaling the cells to stop mitosis prematurely and initiate differentiation. The end result would also be an abnormal brain. However, we cannot yet give an account of the primary event(s) of brain development in which the thyroid hormones are involved directly.

As already proposed by Eayrs, the connectivity of cortical neurons might ultimately be altered. On the assumption that neurons of the cerebral cortex

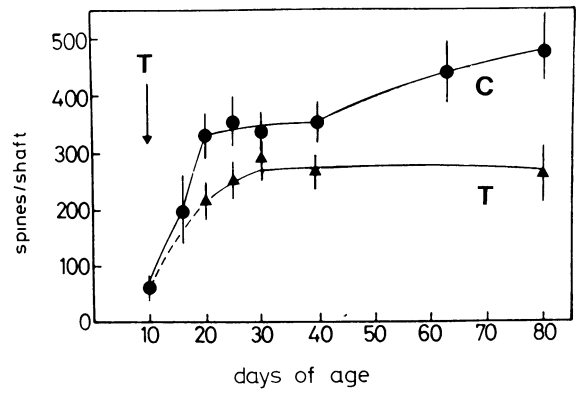

Fig. 2. Effects of thyroidectomy (T) at 10 days of age on the number of spines per apical shaft of pyramidal neurons from the rat visual cortex. The number of spines was decreased by T from the earliest date studied (20 days of age) as compared to that of age-paired controls (C). Data are from Ruiz-Marcos et al. (15).

are necessary for the establishment of the circuitry needed for *higher* mental functions, we have investigated the effects of early thyroidectomy (T) on the connectivity of pyramidal cells. This might be assessed indirectly by measuring the number of dendritic spines of the apical shaft, and the distribution of their density along the shaft. Both of these variables change during brain development, following a pattern which is essentially the same for several species, the rat and man included (13). We decided to study first the effects of T at 10 days of age (T10), a moment when the differentiation of pyramidal cells is starting, and to choose those pyramidal cells with the cell body deep in layer V, as their shaft transverses the entire cortex. Both the number and distribution of apical shaft spines were markedly affected (15) by T10. In normal rats, the number of spines increases from 10 to 80 days of age, but this increase was blunted markedly in T10 rats (Fig. 2): spine number no longer increased after 25 days of age, and remained at about 60% of the control (C) value thereafter. Spine distribution was markedly deranged (Fig. 3). Such changes were also found in pyramids from the auditory cortex (16). Considering the role of spines as connecting apparatus (13) and that synaptic contacts from afferents are needed for spines to appear, it may be concluded that connectivity of the pyramidal neurons is decreased by T10, and that this might be due to a decrease in specific afferents making contact with the pyramidal cell.

When T_4 was injected into T10 rats at a dose of 1.5 µg/100 g body weight (BW) per day, starting at 12, 15, 20, 30, or 40 days of age and continuing until 60 days of age, it was found that the hormone had no ameliorating effect on spine number or distribution when onset of treatment was delayed to 20 days of age, or later, despite normalization of other thyroid hormone-dependent developmental processes, such as body growth (17). Onset of T_4 treatment at 12 days of age had a clear-cut ameliorating affect, with

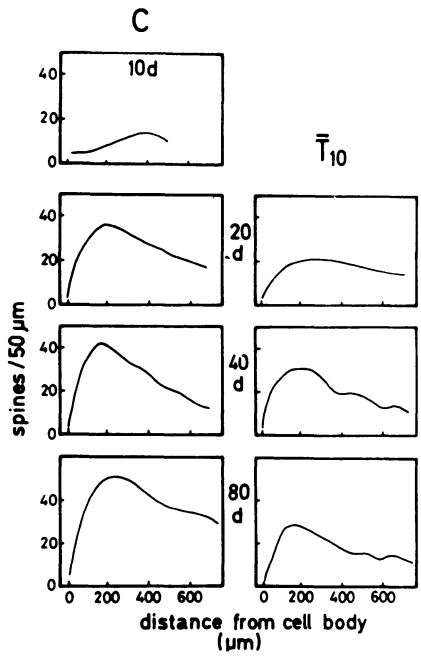

Fig. 3. Effects of thyroidectomy (T) at 10 days of age on the distribution of apical spines along the shaft. The number of spines in each 50 µm segment is plotted against the distance from the cell body. The left-hand panels show the normal evolution of the distribution in C rats; the right-hand panels the corresponding distribution in T rats at 20, 40, and 80 days of age. Data are from Ruiz-Marcos et al. (15).

onset at 15 days of age having an intermediate effect. The lack of effectiveness of T_4 started after 20 days of age was not due to the shorter treatment period, as illustrated in Fig. 4, where spine distribution is compared for T10 rats treated with T_4 for about 30 days, starting at 12 (left-hand panel), or at 30 (right-hand panel) days of age. Thus, these findings suggest that alterations in neuronal connectivity could be related to permanent defects in behavior (and to mental retardation), which can only be prevented by very early treatment with thyroid hormone. There is at present no evidence that early T_4 treatment actually reverses the effects of T10, as data suggest that it merely prevents further deterioration of the measured variables, but this important point remains to be clarified.

We carried out a control experiment in rats thyroidectomized at 40 and 120 days of age, predicting that no alterations would be detected in cortical neurons, as it was then believed that the adult rat brain is insensitive to thyroid hormone. However, we found (18) that the number of spines decreased, both when compared to that of age-paired C rats and to the number found before thyroidectomy. A decrease may already be shown by 10 days after T40 (Cartagena et al., unpublished). Fig. 5 illustrates the effects of T40 on spine distribution. There is an important difference between juvenile- or adult-onset hypothyroidism and neonatal thyroidectomy. The variables we measured were permanently altered by T10 unless almost immediate treatment with T_4 was instituted, whereas in the case of T40, they were practically reversed to normality with 1.75 µg T_4/100 g BW per day despite a 30 day delay in onset of treatment (19). These results are consistent with the clinical observation that defects of mental function in cases of "acquired" hypothyroidism are reversed with treatment, in contrast to those of congenital hypothyroidism left untreated for several months after birth.

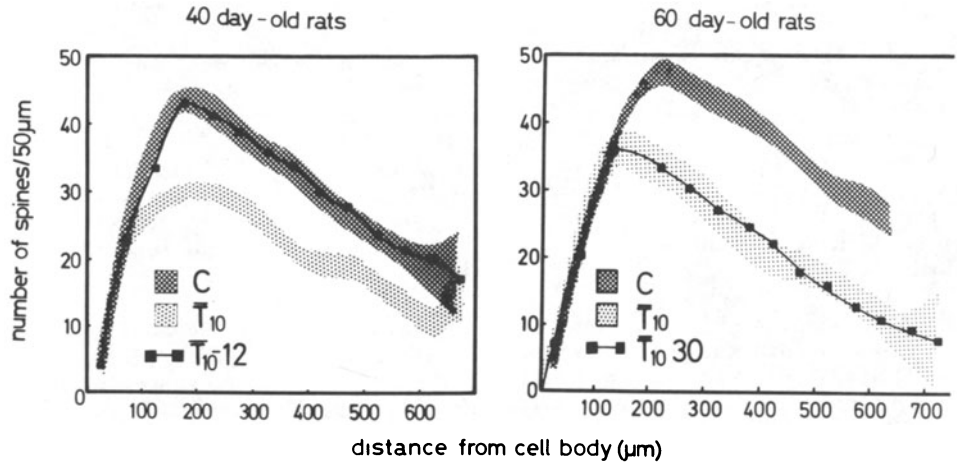

Fig. 4. Effect of treatment of rats T at 10 days of age with 1.5 µg T_4/100 g BW/day, starting at 12 (left-hand panel) or 30 (right-hand panel) days of age and continuing for about 30 days. Shaded areas represent the 95% confidence intervals for spine distribution of C and T rats given placebo; the black squares the data for the T_4-treated rats. As may be seen, spine distribution fell within C confidence intervals when T_4 treatment was started at 12 days of age, but fell within the confidence intervals of placebo-treated T rats if onset of treatment was delayed until 30 days of age. Reprinted from Morreale de Escobar et al. (14) by courtesy of Marcel Dekker, Inc. Data from Ruiz-Marcos et al. (17).

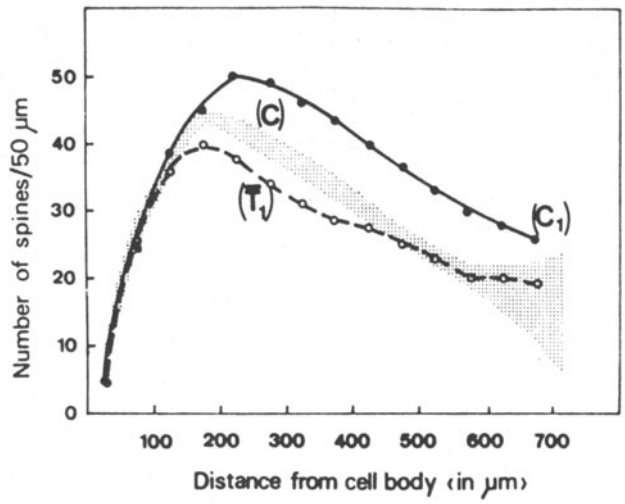

Fig. 5. Effects of thyroidectomy (T) performed at 40 days of age, on the distribution of spines along the shaft of pyramidal cells. The shaded area (C) corresponds to the 95% confidence intervals for normal rats at 40 days of age (C), the full circles (C1) to normal rats at 120 days of age, and the open circles (T1) to rats T at 40 days of age, studied at 120 days of age. Data are from Ruiz-Marcos et al. (18).

The underlying mechanisms resulting in permanent decrease of the measured variables after T10, and a reversible decrease after T40, are still not understood. Some possibilities are discussed elsewhere (13).

Recently, other investigators have described rapid biochemical responses of the adult brain to thyroidectomy (20,21), and maintenance of cerebral T_3 homeostasis (22), findings which all support an important role of thyroid hormones in brain function throughout life, and not only during brain development.

Although many changes occur in the brain after both neonatal- or adult-onset hypothyroidism, this does not necessarily mean that thyroid hormone exerts direct effects on brain cells. They could, for instance, be mediated through growth hormone, other growth-promoting factors, etc. However, it is now known that both neurons and glial cells contain nuclear receptors for T_3, which are more abundant in the neurons (23). Receptor numbers in glial cell lines are increased by *in vitro* incubation with short chain fatty acids known to affect proliferation and differentiation of mammalian cells (24). Using cells cultured in thyroid hormone-free serum, direct effects of T_3 added *in vitro* in physiological concentrations have been shown both in glial cells (25) and neurons (26). Thus, thyroid hormones do have direct effects on brain cells, though we do not know which would be the primary event(s) leading to many varied biological end-points of thyroid hormone action in the brain.

Are any of these findings relevant to our understanding of endemic cretinism? The clinical characteristics of the endemic cretin, especially those of the neurological type, which is the one most frequently found in

most severe goiter endemias, differs in important respects from those of untreated congenital hypothyroidism (27). The neurological lesions most frequently found (28), apart from mental deficiency of a characteristic type, are severe impairment of hearing and speech, spasticity, particularly involving the proximal lower extremities, extrapyramidal disorders of rigidity and bradykinesia, and, less frequently, strabismus. Thyroid function is usually comparable to that of other non-cretin inhabitants of the same area, and there are no clear signs that neurological cretins were as hypothyroid during the prenatal and perinatal period, as endemic cretins of the myxedematous type, or congenital hypothyroid babies. The fact that primary organ formation within the CNS is normal would indicate that the timing of the insult should be placed after the first three months of gestation, and the cochlear damage further localizes the period of injury to the second trimester of pregnancy, at about 14-18 weeks. This does not preclude that insults leading to other CNS abnormalities and mental retardation might occur later in pregnancy, but it already indicates that timing of the damage is earlier than that which leads to the defects described in congenital hypothyroidism. Though it was initially proposed (29) that it was the lack of iodine itself, as trace element, which was responsible for the early damage, maternal hypothyroxinemia has received increasing attention (30), as many of the iodine deficiency disorders (increased abortions, stillbirths and perinatal mortality, congenital defects, neuromotor impairment of non-cretin offspring) (1) are similar to those described in hypothyroxinemic women without iodine deficiency (3).

Thus, considering that the experimental model used for congenital hypothyroidism is not adequate to investigate the problem of endemic cretinism, it appeared appropriate to first investigate the effects of maternal hypothyroxinemia on the CNS of the developing rat embryo.

MATERNAL HYPOTHYROXINEMIA

The clinical and experimental observations indicating that maternal hypothyroidism decreases reproductive competence and the development of the offspring, intellectual competence included, have been summarized elsewhere (31); our experimental results in rats, and those of others in sheep, being described in detail elsewhere in this Congress (6,32). In brief, adult female rats were thyroidectomized (T) and later mated. the concepta were obtained at 11, 14, 16, 17, 20, and 21 days of gestation (dg), and compared to those from normal (C) age-paired rats. Their development was assessed by their BW and the organ weights of 20 and 21-day-old fetuses. The concentrations of both T_4 and T_3 were determined by RIA in embryotrophoblasts (ET), embryos (E), and placentas (P), and in 20 and 21-day-old fetal organs. The analytical procedure involves extensive extraction in chloroform; methanol, back-extraction into aqueous phase, and purification of this phase through BioRad AG 1X2 Columns. The final eluates used for RIA were free of interfering substances.

It was found that maternal T resulted in a marked decrease in the number of viable embryos and in their individual BW. Individual placental weights were hardly affected. Onset of fetal thyroid secretion (at 17.5 dg in the rat) did not result in complete and immediate catch-up, and fetuses at term were still smaller than C. Cerebral weights were lower, DNA concentrations increased, and protein/DNA ratios decreased.

Before onset of fetal thyroid function, T_4 and T_3 could be clearly detected in 9-12-day-old ET, and 13-14-day-old E and P from C mothers. Concentrations of both iodothyronines were decreased by maternal T to undetectable levels. However, even after 17.5 dg, the total extrathyroidal concentrations of both iodothyronines were reduced as compared to fetuses from C

mothers (Figs. 5 and 6). This decrease in total body T_4 and T_3 concentrations did not affect all organs to the same degree, the iodothyronine concentrations in the brain being normal in 20-day-old fetuses, although still decreased in other organs and tissues. In 21-day-old fetuses, cerebral concentrations were actually increased in parallel to the increase in cerebral DNA concentration.

Thus, present results confirm that T_4 and T_3 are found in developing embryonic tissues early in pregnancy (33) and that they are of maternal origin. Maternal T results in thyroid hormone deficiency of the developing embryo before onset of fetal thyroid function, a situation not immediately corrected by its initiation. The possibility exists that early thyroid hormone deficiency affects early brain development and that some adverse effects might have become permanent by the time fetal thyroid function overcomes the early hypothyroid situation. In this connection, it is of interest that Bernal et al. (34) have found nuclear receptors for T_3 in brain from 14-day-old rat fetuses and in 13-day-old whole rat embryos. Both the hormone and its nuclear receptor were also found in human fetal brain as early as 10 weeks of gestation and in a 7-week-old whole human embryo (34). Our findings do not necessarily exclude that the adverse effects of maternal hypothyroxinemia on the offspring are due to the hypothyroid condition of the mother, making maintenance of pregnancy, transfer of nutrients, etc., difficult, but they do raise the possibility that the thyroid hormone deficiency of the embryonic tissues themselves contributes to their delayed development.

IODINE DEFICIENCY AND CRETINISM

In areas of severe iodine deficiency, inhabitants may have very low circulating T_4, and high TSH levels, T_3 usually remaining the same, but do not usually present overt signs of hypothyroidism. This situation may be reproduced by feeding rats a low iodine diet (LID). Although it was assumed that euthyroidism is maintained at the expense of the normal circulating T_3 levels, we have previously shown for the rat on LID that the concentrations of T_3 and/or biological end-points of thyroid hormone action are lower in the anterior pituitary, liver, and brain than those for LID+I rats (35,36),

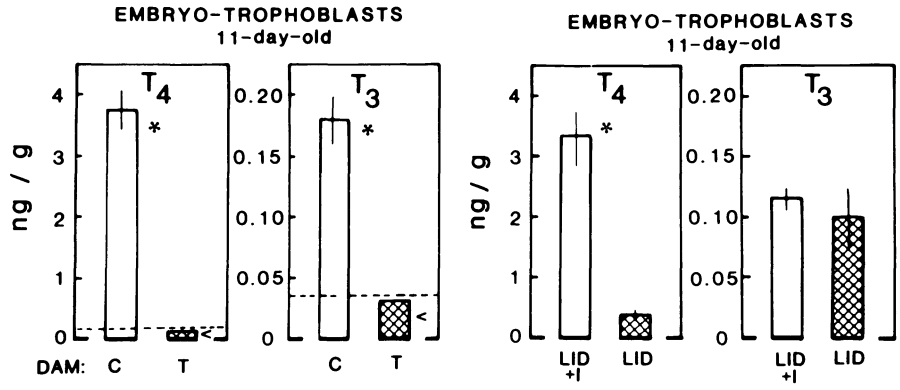

Fig. 6. Concentrations of T_4 and T_3, measured by RIA, in 11-day-old embryo-trophoblasts obtained from control (C) and thyroidectomized (T) dams, and from dams on a low iodine diet (LID) or the same diet supplemented with KI (LID+I). Data are from ref. 31, 32, and 37.

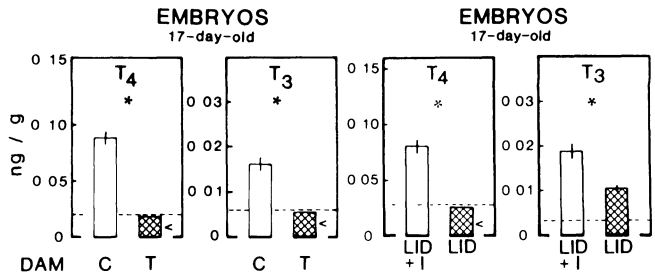

Fig. 7. Concentrations of T_4 and T_3 in 17-day-old embryos from the same groups of dams indicated in the legend to Fig. 6.

despite normal plasma T_3 levels. How would the low circulating T_4 and normal T_3, levels affect embryonic tissues developing in LID rats?

Adult female rats were fed an LID containing 0.02-0.06 µg I/g, or the same LID supplemented with KI (7-10 µg/rat/day). the LID and LID+I rats were mated, and 11-day-old ET, 17-day-old E and P obtained. At 21 dg, P were obtained, as well as fetal blood, brain, liver, and the rest of the fetus (trachea + thyroid exluded, termed carcass). More detailed results are reported elsewhere in this Congress (37). LID feeding resulted in a marked decrease of maternal T_4 concentrations in plasma, liver, and brain. T_3 concentrations in maternal plasma from LID dams fluctuated around those of the LID+I dams. However, as previously found (35,36), the concentrations of T_3 in the liver and brain were always lower than expected from the plasma T_3 levels alone. Figs. 6 and 7 show T_4 and T_3 concentrations in embryonic tissues obtained before onset of fetal thyroid activity, from LID and LID+I dams, and compares them with those from T and C dams (31,32). T_4 in 11-day-old ET from LID dams was very low, but T_3 concentration was not altered, whereas both were low in ET from T dams. In 17-day-old E from LID dams, T_4 concentration was markedly decreased and T_3 was also lower, although not as low as after maternal T. Although not shown, the same was found for 17 and

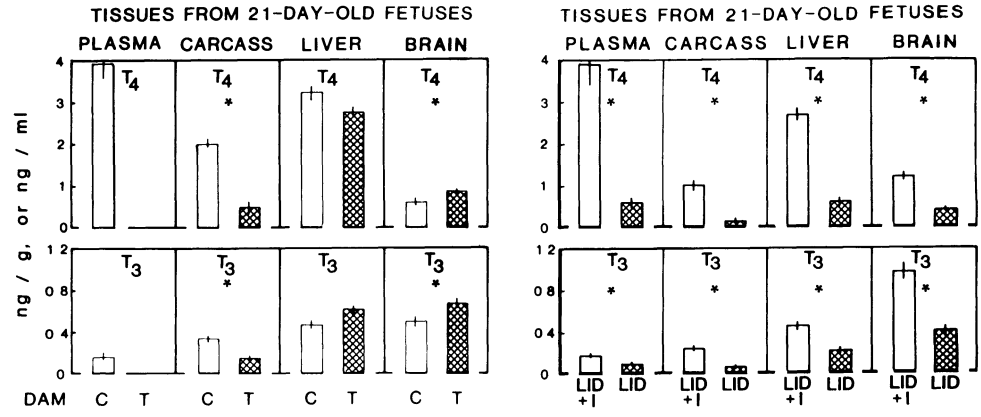

Fig. 8. Concentrations of T_4 and T_3 in blood and tissues from 21-day-old fetuses obtained from the same groups of dams indicated in the legend to Fig. 6. See text for the meaning of "carcass".

21-day-old P. Thus, before onset of fetal thyroid secretion, embryonic tissues from LID dams were markedly deficient in T_4 and increasingly *deficient* in T_3. Once fetal thyroid secretion had started, a fetal deficiency in T_4 and T_3 persisted, as shown for 21-day-old fetal plasma, brain, liver, and carcass in Fig. 8. Thus, although the T_3 deficiency of embryonic tissues from LID dams is initially less severe than for T dams, it persists until term in all organs studied.

The number of viable embryos and their individual BW were reduced in LID dams, but not to the same degree as in T dams. The brain weight, both absolute and relative to BW, and the DNA and protein contents were decreased in 21-day-old fetuses from LID dams. Their thyroids were visibly enlarged (about 3-5 times).

Thus, it may be concluded at present that the adult iodine-deficient rat is somewhat hypothyroid (at least as respects pituitary, liver, and brain), and has a decreased reproductive competence, as regards the number of viable embryos and their development. The embryonic tissues developing in such mothers are thyroid hormone-deficient both before and after onset of fetal thyroid function, at least until term. It does not appear unreasonable to assume that if the severity of the iodine deprivation were greater than that obtained with the diet we have used, maternal serum T_3 might also decrease, and the embryonic tissues might be almost as T_3-deficient as those from T dams early in pregnancy.

Might these findings have any bearing on the congenital defects, cretinism included, described for inhabitants of severely iodine-deficient areas? As indicated above, in such areas there is an increased reproductive failure, poor motor coordination of offspring having been related to the low maternal hypothyroxinemia of severely iodine-deficient women (30). The accompanying deficiency in thyroid hormones of embryonic tissues early in gestation, which would persist after onset of fetal thyroid function because of the low availability of iodine, might be playing an important role. Bernal et al. (34) have found nuclear receptors for T_3 in human and rat fetuses well before the thyroid becomes active. Iodothyronines were found in the human brain at 10 weeks of gestation, and in a whole 7-week-old embryo. It does not seem unreasonable that the simultaneous presence of the hormone and its nuclear receptor might lead to a biological effect earlier in development than previously believed. Thus, embryos from severely iodine-deficient mothers might be thyroid hormone-deficient and hypothyroid even before the stage of brain development when the damage leading to neurological cretinism is supposed to occur (28).

Fig. 9 attempts to summarize some of the concepts briefly discussed here, using information available from Fig. 1 as basis. It should be borne in mind that too little information is available at present to permit them to be little more than purely hypothetical. Thus, the upper panels show thyroid hormones available to the fetus and newborn, using the plasma T_4 data from Fig. 1, but superimposing on it the possible T_4 of maternal origin. The relative proportions of the maternal T_4 versus the fetal T_4 are not known at present. As regards the lower panels, the lowering of the brain DNA and weight curves which might result from the insult should not be taken quantitatively; the insult might not affect total DNA appreciably, for instance, but might have affected proliferation of selected cohorts of brain cells, leaving irreversible functional defects. The graphs also do not illustrate possible effects on differentiation, which have been included in the weight curve. They simply point out the likely duration of the insult which might be attributed to fetal hypothyroidism and to maternal hypothyroxinemia. In the first case (B) the insult is not likely to affect acquisition of neurons, except those underoing mitosis late in gestation or after birth, but might affect acquisition of glial cells, and the differentiation of both neurons

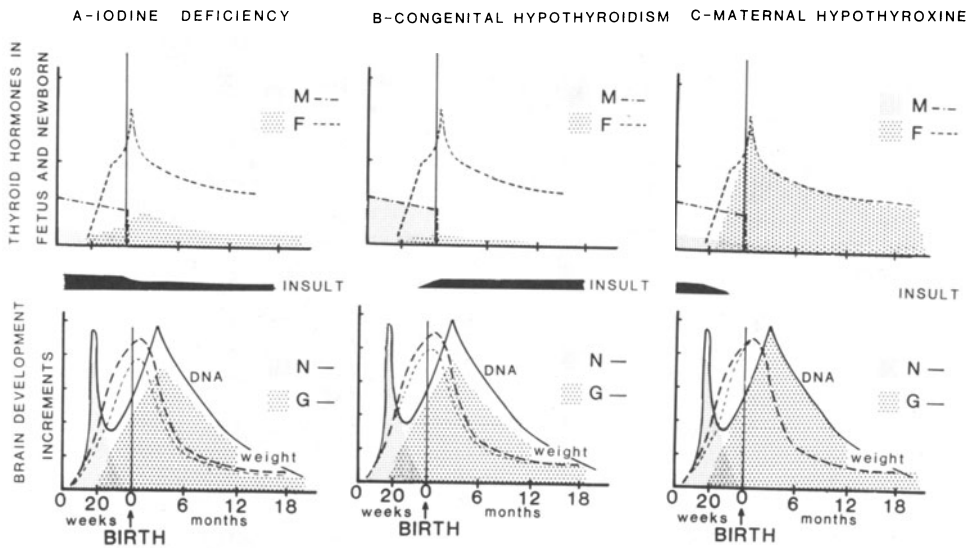

Fig. 9. Schematic representation of possible timing of events relating changes in thyroid hormone contents and cerebral development events in the case of human congenital hypothyroidism (B), maternal hypothyroxinemia (C), and maternal iodine deficiency (A). The illustration is based on Fig. 1 for the normal pattern of events. The shaded areas, superimposed on the normal pattern, indicate the thyroid hormone contents and cerebral maturational events in the three situations (A-C); the black areas indicate the duration and likely intensity of the insult to the developing brain.

and glial cells. In the second case (C), the insult would be present earlier in gestation, but onset of fetal thyroid function would overcome it. Thus, it might well affect neuronal cell acquisition and differentiation. Despite onset of fetal thyroid function, some permanent defect might remain. In the case of iodine deficiency (A), both the maternal T_4 supply and fetal thyroid function are affected. Thyroid function of the newborn might improve if more iodine becomes available through milk than through the placenta. However, the period of insult would extend from early in gestation throughout pregnancy and after birth. Thus, it might affect both neuronal and glial cell acquisition and their differentiation and lead to more severe damage than either maternal hypothyroxinemia or fetal hypothyroidism alone.

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REFERENCES

1. Koenig MP. In BS Hetzel and RM Smith (eds), Fetal Brain Disorders-Recent Approaches to the Problem of Mental Deficiency, Elsevier North Holland Biomedical Press, Amsterdam, 1981, p 229.
2. Klein R. In GN Burrow and JH Dussault (eds), Neonatal Thyroid Screening, Raven Press, New York, 1980, p 51.
3. Man EB and Serunian SA. Am J Obstet Gynecol 125: 949, 1976.
4. Hollingsworth DR and Mabry CC. In DA Fisher and GN Burrow (eds), Perinatal Thyroid Physiology and Disease, Raven Press, New York, 1975, p 163.
5. Irie M. AOTA Prize Award, Ninth International Thyroid Congress, 1985.
6. Hetzel BS. AOTA Prize Award, Ninth International Thyroid Congress, 1985.
7. Wolter R, Noel P, DeCock P, et al. Acta Pediat Scand 277: 41, 1980.
8. Fisher DA and Klein AH. New Engl J Med 304: 702, 1981.
9. Dobbing J. Am J Dis Child 120: 411, 1970.
10. Eayrs JT. In RP Michael (ed), Endocrinology and Human Behaviour, Oxford University Press, London, 1968, p 239.
11. Hamburgh M, Mendoza LA, Bennet I, et al. In GD Grave (ed), Thyroid Hormones and Brain Development, Raven Press, New York, 1977, p 40.
12. Morreale de Escobar G and Escobar del Rey F. In GN Burrow and JH Dussault (eds), Neonatal Thyroid Screening, Raven Press, New York, 1980, p 25.
13. Ruiz-Marcos A, Sanchez-Toscano F, Escobar del Rey F, et al. In BS Hetzel and RM Smith (eds), Fetal Brain Disorders - Recent Approaches to the Problem of Mental Deficiency, Elsevier North Holland Biomedical Press, Amsterdam, 1981, p 205.
14. Morreale de Escobar G, Ruiz-Marcos A, and Escobar del Rey F. In JH Dussault and P Walker (eds), Congenital Hypothyroidism, Marcel Dekker, New York, 1983, p 85.
15. Ruiz-Marcos A, Sanchez-Toscano F, Escobar del Rey F, et al. Brain Res 162: 315, 1979.
16. Ruiz-Marcos A, Sala J, Sanchez-Toscano F, et al. Develop Brain Res 9: 205, 1983.
17. Ruiz-Marcos A, Sanchez-Toscano F, Obregon MJ, et al. Brain Res 239: 559, 1982.
18. Ruiz-Marcos A, Sanchez-Toscano F, Escobar del Rey F, et al. Brain Res 185: 91, 1980.
19. Cartagena P and Ruiz-Marcos A. ETA Meeting, Rotterdam, Ann d'Endocrinologie 45: 29 (abstract), 1984.
20. Dembri A, Michel O, Belkhiria M, et al. ETA Meeting, Madrid, Ann d'Endocrinologie 44: 139 (abstract), 1983.
21. Leonard JL, Kaplan MM, Visser TJ, et al. Science 214: 571, 1981.
22. Dratman MB, Crutchfield FL, Gordon JT, et al. Am J Physiol 245 (Endocrinol Metab 8): E185, 1983.
23. Haidar MA, Dube S, and Sarkar PK. Biochem Biophys Res Comm 112: 221, 1983.
24. Ortiz-Caro J, Montiel F, Pascual A, et al. Ninth International Thyroid Congress, Sao Paulo, 71A, 1985.
25. Bhat NR, Rao GS, and Peringer RA. J Biol Chem 256: 1167, 1981.
26. Puymirat J, Barret A, Picart R, et al. Neuroscience 10: 801, 1983.
27. Hetzel BS and Querido A. In JB Stanbury and BS Hetzel (eds), Endemic Goiter and Endemic Cretinism, John Wiley and Sons, New York, 1980, p 461.
28. DeLong GR. International Symposium on Iodine Nutrition, Thyroxine and Brain Development, New Delhi, 1985.
29. Pharoah POD, Ellis SM, Ekins RP et al. Clin Endocrinol 5: 159, 1976.
30. Pharoah POD, Connolly K, and Hetzel BS. Develop Med Child Neurol 23: 76, 1981.

31. Morreale de Escobar G, Pastor R, Obregon MJ, et al. Endocrinology 117, Nov, 1985.
32. Morreale de Escobar G, Pastor R, Obregon MJ, et al. Ninth International Thyroid Congress, Sao Paulo, 62A, 1985.
33. Obregon MJ, Mallol J, Pastor R, et al. Endocrinology 114: 305, 1984.
34. Bernal J, Perez-Castillo A, Pans T, et al. In L Labrie and L Proux (eds), Endocrinology, International Congress Series 655, Excerpta Medica, Amsterdam, 1984, p 977.
35. Santisteban P, Obregon MJ, Rodriguez-Pena A, et al. Endocrinology 110: 1780, 1982.
36. Obregon MJ, Santisteban P, Rodriguez-Pena A, et al. Endocrinology 115: 614, 1984.
37. Escobar del Rey F, Pastor R, Mallol J, et al. Ninth International Thyroid Congress, Sao Paulo, 232A, 1985.

IODINE-SUFFICIENT GOITER AND AUTOIMMUNE THYROIDITIS: THE KENTUCKY AND COLOMBIAN EXPERIENCE

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Thyroid disorders constitute today a significant public health problem in Colombia, S.A. and in the United States (1,2). Goiter continues to occur in some areas despite adequate iodine supplementation. For instance, a continued high prevalence of goiter in many localities of western Colombia, as well as in the coal-rich Appalachian area of eastern Kentucky, has been reported (1,2). The incidence of lymphocytic autoimmune thyroiditis (AT) has has steadily increased in the U.S. during the past five decades and it has been attributed to iodine prophylaxis and excessive iodine intake (2). In western Colombia, S.A., where iodine prophylaxis was instituted thirty years ago, a similar trend has been documented (3). However, lymphocytic infiltration was of high degree in most of the U.S. glands, while it was minimal in 70% of the Colombian cases. Thus, recent studies were designed to reassess and gain insight into the problem of goiter and AT in areas of eastern Kentucky and western Colombia, where goiter persists despite iodine supplementation. Nearby localities where goiter has not been a problem were similarly investigated.

EPIDEMIOLOGICAL STUDIES

Studies were conducted in 1983-1984 in a randomized representative sample of 1,321 schoolchildren, equal numbers of each sex, between the ages of 9 and 15 years living in Bourbon, Breathitt, and Owsley Counties of Kentucky, U.S.A. and in the municipal localities of La Cumbre, La Habana, Vijes, Darien, and Alaska in western Colombia, S.A. Goiter size was graded using a modification of the W.H.O. criteria (Grades 1b and 2) (4) and measured by the surface outline method (5). The coefficient of variation among observers was 10%. Spot urine samples were collected from 50% of the children examined to determine iodine (6) and thiocyanate (7) excretions, and blood samples from 35% to measure free T_4, Total T_4, T_3, and TSH by RIA and anti-thyroglobulin (TgAB) and antithyroid microsomal (TmAB) antibodies by hemagglutination techniques. Fine-needle aspiration cytologies were obtained from 50 of the Colombian children with goiter and their immediate goitrous family (8). Data was statistically analyzed by one-way analysis of variance, the Student-Newman-Keuls multiple range test, and the Chi-square after adjustment for percentage with and without goiter.

Results show (Fig. 1) that goiter, lymphocytic autoimmune thyroiditis (AT), and subclinical hypothyroidism (SH) are intimately related in Kentucky

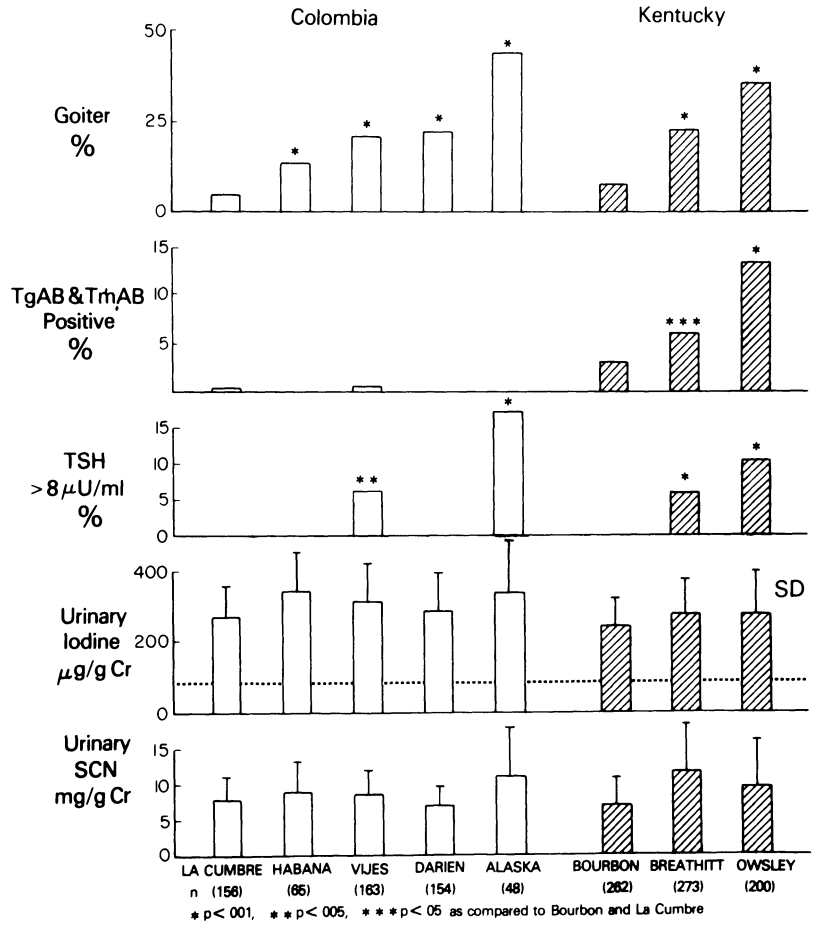

Fig. 1. Goiter among school children 9-15 years of age from Kentucky (USA) and Colombia (SA) in 1983-1984.

children. Seventy percent (70%) of positive antibodies are found in children with the largest goiters (Grade 2), and only 6% in non-goitrous individuals. There was equal distribution of goiters by sex among children with negative antithyroid antibodies, but three out of four with positive ABs were females. By contrast, a low incidence of AT is documented in the Colombian goitrous children using immunological tests and fine-needle aspiration cytologies in children with Grade 2 goiters and those with SH. Urinary iodine, SCN, and other hormonal (FT_4, T_4, T_3U, FTI, and T_3-RIA) measurements were comparable between the American and Colombian groups.

Results clearly demonstrate that these goiters are not due to iodine deficiency and that iodine supplementation is not the primary environmental cause of AT. Other region-specific environmental (organic and microbial water pollutants) and immunogenetic factors (HLA-DR5) (9) may be responsible for the significantly different prevalence rates in goiter, AT, and SH (Fig. 1) among nearby localities, as well as between the North American and and Colombian populations.

ENVIRONMENTAL GOITROGENESIS

Epidemiological evidence from western Colombia indicates that environmental factors other than iodine deficiency are responsible for the persistence of goiter (1). A relationship between drinking water and goiter prevalence rates has been documented (10-12). These studies indicate that goitrogenic organic compounds contaminate the water supply, constituting the main factor underlying the endemia. Investigation in 37 localities of western Colombia showed a significant correlation between the distribution of rock types (sedimentary, metamorphic, and igneous) and goiter prevalence (13,14), supporting the hypothesis that sedimentary rocks rich in organic matter (coals, shales, cherts, etc.), are the source of water-borne goitrogens.

Because the correlation ($p<.0005$) between goiter prevalence and rock types accounted for 57% of the variation in goiter prevalence, bacterial contamination of water supplies was investigated as a potential factor involved in the remaining 43% (15). The overall concentration of bacteria in the pipeline system was significantly associated with increased goiter prevalence, and *K. pneumoniae* in the water source associated with decreased prevalence. A model fitted with the geological and bacteriological variables accounted for 80% of the variability in goiter prevalence. These findings may be explained on the basis of bacterial synthesis and degradation of the organic pollutants that cause goiter, and/or production of gram (-) bacterial antibodies (e.g., *E. coli*) with growth promoting effects on the thyroid (16,17).

We have gained knowledge on the physical state of organic goitrogens in water. Physico-chemical analyses (1,18) from the goitrogenic Well of Candelaria Town in western Colombia (1,10-12) indicate that the active compounds form dissociable complexes and/or they are part of larger organic molecules, possibly humic substances (HS). This notion is strongly reinforced by results of elemental analysis and infrared spectrometry.

Recently, over 30 organic compounds, including resorcinol, were identified (19) by GC/MS in activated carbon extracts from the Candelaria Well water with antithyroid activity. However, only four compounds, not including resorcinol, were found in the extracts from the non-goitrogenic Well water. Besides aliphatic hydrocarbons and dibutyl and dioctyl phthalates, also found in earlier studies (10,11), halogenated hydrocarbons, fatty acids, phenol, resorcinol and its derivative cyclopentanediol, were identified in the goitrogenic Well water extract. In the earlier studies, sulfur-bearing organic compounds, possibily aliphatic disulfides, with potent antithyroid activity were also found (10,11). Studies in 1984 (Table 1) indicated the presence of a substituted resorcinol (2,4-dihydroxyacetophenone), as well as phthalate esters in the drinking water from the coal-rich Appalachian area of eastern Kentucky.

Humic substances (HS), high molecular weight complex polymeric compounds, are the principal organic components of soils and waters (20,21). HS are also present in coals, shales, and possibly other carbonaceous sedimentary rocks. Resorcinol and other parent antithyroid phenolic and phenolic-carboxylic compounds (21,22) (phloroglucinol, pyrogallol, 5-methylresorcinol (orcinol), 3,4- and 3,5-dihydroxybenzoic acids (DHBA), and the ortho (*o*)- and meta (*m*)-phthalic acids) are monimeric byproducts of reduction, oxidation, and microbial degradation of HS. As much as 8% of shale bitumen is constituted by phenols which are also the major organic pollutants in aqueous effluents from coal-conversion processes (21). Resorcinol and other antithyroid phenolic pollutants comprise as much as 4 g/l in the aqueous effluent from a bench-scale coal liquefaction unit (23). Coal-conversion waste waters also contain thiocyanate and S^{2-} (24,25), again known to possess antithyroid properties.

Table 1. Tentative Identifications Resulting from GC/MS Analysis of Organics Separated from Water Samples by Purge/Trap Method and by Adsorption on XAD or Activated Carbon and Extracted with Organic Solvents

Compounds	Kentucky	Colombia
Phenolics		
Resorcinol (1,3-dihydroxybenzene)* and Subst.		
Resorcinol (2,4-dihydroxyacetophenone)*	+	+
Phenol*	+	?
Polycyclic Aromatic Hydrocarbons (PAH)		
Naphthalenes*	+	+
Methoxyanthracene*	+	ND
Halogenated Aliphatic Hydrocarbons		
Bromoform	+	ND
Others	+	+
Halogenated Aromatic Hydrocarbons		
Dichlorobenzene	+	ND
Phthalates	+	+
Sulfurated Organics		
Thiophenes	+	+
Aliphatic disulfides	ND	+
Higher mol. wt. S cpd.	ND	+
Other Organics		
Aliphatic hydrocarbons*	+	+
Fatty acids	+	+

*Coal and shale derived; + - Identified; ND - Non-detected

Our results (22), are in agreement with those of others demonstrating in vitro and in vivo the antithyroid and goitrogenic effects of resorcinol and other parent phenolic and phenolic carboxylic compounds (21). We have demonstrated also significant enhancement of the goitrogenic activity of resorcinol in the Gunn rat (26) with UDP-glucuronyl transferase deficiency. This defect results in decreased conjugation and excretion of resorcinol with glucuronic acid. Cytoplasmic melanin-like granules in the thyroid gland are also characteristic of the Gunn rat strain (27). Microscopic examination of thyroid follicular cells obtained by fine-needle aspiration from the Colombian children in 1984, disclosed that 11 (24%) goiters were also filled with brownish granules, which by Masson-Fontana Ammoniacal Silver stain contained melanin-like material. These granules may represent peroxidase catalyzed cross-coupling of dihydroxyphenols (resorcinol, dihydroxybenzoic acids) with tyrosine. Formation of dark-colored nitrogenous polymers by this type of reaction, has been observed in soils and microbial culture (28). Colombian goiter may then be comparable to that of Gunn rats in pathogenesis.

We have also examined for antithyroid effects substituted resorcinols present in high concentrations in aqueous effluents from coal liquefaction plants (23) and found potent antithyroid activities of these pollutants (29), which may enter community water supplies, constituting a potential environmental goitrogenic factor.

Similarly, we have investigated the antithyroid effects of some bioflavonoids, C_6-C_3-C_6 polyphenols, widely distributed in nature. Results indicate that they possess intrinsic (30,31) antithyroid activity. Cyanidin, a naturally-occurring bioflavonoid used as model subunit of flavonoid-type HS, yields by reductive degradation the antithyroid monomers resorcinol, phloroglucinol, and orcinol (21,22). Decaying organic matter (plants and animals) rich in these materials, becomes the substrate of flavonoid-type HS during the process of fossilization (humification and carbonification). Besides, bioflavonoids in high concentrations are present in various staple foods of the Third World (millet, shorghum, beans, ground nut) (30,32). Thus, bioflavonoid structures might be the link for phenolic goitrogens in foodstuffs (millet) and those in rocks, soils, and water.

The ubiquitous phthalate esters have been frequently identified as water pollutants (21). Dibutyl and dioctyl phthalates have consistently been present in the goitrogenic well water supplying the endemic goiter district of Candelaria Town (10,11,19,33). Phthalate esters were also found recently in water supplies of Kentucky and Colombia. Most commonly, they result from industrial pollution or artificial contamination, but phthalates are also reported to occur naturally in shale, crude oil, petroleum, plants, and as fungal metabolites (21). Although phthalates and phthalic acids do not possess intrinsic goitrogenic activity (22), they undergo biodegradation by gram-negative bacteria with production of intermediate metabolites, such as dihydroxybenzoic acids (DHBAs) (21), known to possess antithyroid properties. Thus, phthalates, with bacterial intermediation, may become a source of environmental goitrogens.

ENVIRONMENTAL "TRIGGERS" OF AUTOIMMUNE THYROIDITIS

Except for an increased prevalence of primary hypothyroidism with elevated titers of antithyroid microsomal ABs (TmAB) which was documented among workers in a plant that manufactured polybrominated biphenyls (PBBs) (34), no association thus far has been investigated in humans between environmental pollutants and AT. However, AT has been observed after administration of the carcinogens methylcholanthrene (MCA) and dimethylbenzanthracene (DMBA) (polycyclic aromatic hydrocarbons) (PAH) and of carbon tetrachloride to the BUF inbred strain of rats (9,35). Similarly, injection of mouse thyroglobulin with bacterial lipopolysaccharide or with complete Freund's adjuvant containing mycobacteria, induce autoimmune thyroiditis in "good-responder" mice, whereas "poor-responder" strains develop little pathologic response. "Good" and "poor" responders differ in their H-2 haplotype (9). A relationship between loci in the major histocompatibility complex (MHC) and susceptibility to autoimmune thyroid disease has also been demonstrated in man. For instance, the histocompatibility (HLA)-DR5 antigen is seen with increased frequency in patients with goitrous thyroiditis, whereas atrophic thyroiditis is associated with the HLA-DR3 (9).

Our studies in 1984 demonstrated the presence of bromoform, as well as other halogenated aliphatic and aromatic hydrocarbons, and the PAH, anthracene, in drinking water from the coal-rich Appalachian area of eastern Kentucky (Table 1). Thus, it appears that resorcinol and other antithyroid compounds, as well as organic potential "triggers" of AT, possibly derived from HS and sedimentary rocks, may play with bacterial intermediation, an important role in the causation of goiter and AT in eastern Kentucky and

western Colombia. Furthermore, we have constructed some family pedigrees of children with goiter from Owsley County and Colombia. These pedigrees clearly indicate the familial nature of iodine-sufficient goiters and suggest that AT may be under separate genetic control. Thus, the question arises as to whether the same organic pollutants that cause goiter operate in genetically predisposed individuals to trigger the pathogenic mechanism leading to AT and/or they simply act as "promoters", sensitizing the thyroid to the "trigger" action of other organic or microbial pollutants.

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REFERENCES

1. Gaitan E. Ecology of Disease 2: 295, 1983.
2. Matovinovic J and Trowbridge FL. In JB Stanbury and B Hetzel (eds), Endemic Goiter and Endemic Cretinism, John Wiley and Sons, Inc., New York, 1980, pp 31-67.
3. Duque E, Cuello C, and Correa P. Patologia (Mexico) 14: 81, 1976.
4. Querido A, Delange F, Dunn JT, et al. In FT Dunn and GA Medeiros-Neto (eds), Endemic Goiter and Cretinism, Sc. Pub. 292 Pan American Health Organization, Washington, DC, 1974, pp 267-272.
5. MacLennan R and Gaitan E. In JT Dunn and GA Medeiros-Neto (eds), Endemic Goiter and Cretinism, Sc. Pub. 292, Washington, DC, 1974, p 195.
6. Acland JA. Biochem J 66: 177, 1957.
7. Aldridge WN. Analyst (London) 70: 474, 1945.
8. Van Herde AJ, et al. Ann Intern Med 96: 221, 1982.
9. Weetman AP and McGregor AM. Endocrine Reviews 5: 309, 1984.
10. Gaitan E, Island DP, and Liddle GW. Trans Assoc Amer Physns 82: 141, 1969.
11. Gaitan E. World Rev Nutr Diet 17: 53, 1973.
12. Gaitan E, Cooksey RC, Matthews D, et al. J Clin Endocrinol Metab 56: 767, 1983.
13. Meyer JD, Gaitan E, Merino H, et al. Int J Epidemiol 7: 25, 1978.
14. Gaitan E, Merino H, Rodriguez G, et al. Bull Wld Hlth Org 56: 957, 1980.
15. Gaitan E, Medina, DeRouen TA, et al. J Clin Endocrinol Metab 51: 1957, 1980.
16. Weiss M, Ingbar SH, Winblod S, et al. Science 219: 1331, 1983.
17. Drexhage HA, Bottazzo GH, Doniach D, et al. Lancet 2: 287, 1980.
18. Gaitan E. In F Delange and R Ahluwalia (eds), Cassava Toxicity and Thyroid: Research and Public Health Issues, International Development Research Centre-IDRC, IDRC-207e, Ottawa, 1983, pp 27-34.
19. Jolley RL, Gaitan E, Lee NE, et al. Am Chem Soc Environ Chem 23: 179, 1983.
20. Choudhry GG. Tox Env Chem 4: 209-259, 1981.

21. Gaitan E. In AB Tarcher (ed), Principles and Practice of Environmental Medicine, Plenum Publ. Corp., New York and London, 1986.
22. Cooksey RC, Gaitan E, Lindsay RH, et al. Organic Geochemistry 8: 77, 1985.
23. Pitt WW, Jolley RL, and Jones G. Environment International 2: 167, 1979.
24. Jahnig CE and Bertrand RR. Chem Eng Prog 72: 51, 1976.
25. Klibanov AM, Tu TM, and Scott KP. Science 221: 159, 1983.
26. Gaitan E, Cooksey RC, and Legan JS. Proc. 7th International Congress of Endocrinology, Quebec, No. 823, July, 1984, p 672.
27. Gomba S, et al. Virchows Archiv (Cell Pathol) 20: 41, 1976.
28. Bollag JM. In RF Christman and ET Gjessing (eds), Aquatic and Terrestrial Humic Materials, Ann Arbor Science Publ., Ann Arbor, MI, 1983, pp 127-141.
29. Gaitan E, et al. Proc Am Thyroid Assoc, T-22, September, 1982.
30. Gaitan E, Cooksey RC, Legan J, et al. In G Medeiros-Neto and E Gaitan (eds), Frontiers in Thyroidology, Plenum Publ. Corp., New York and London, 1986.
31. Gaitan E, Cooksey RC, and Lindsay RH. In JT Dunn, E Pretell, F Viteri, et al. (eds), Endemic Goiter and Cretinism, Sc Publ Pan Am Health Organization, Washington, DC, 1985.
32. Hulse JH (ed). International Development Research Centre, Ottawa, Canada, IDRC-145e, 1980.
33. Gaitan E, Jolley RL, Lee NE, et al. American Chemistry Society, Environmental Chem 23: 175, 1983.
34. Bahn AK, et al. N Engl J Med 302: 31, 1980.
35. Biggazi PE and Rose NR. Prog Allergy 19: 245, 1975.

NEONATAL HYPOTHYROID SCREENING: NATIONWIDE EXPERIENCE IN JAPAN

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Our initial motivation for this study was, of course, to identify the patients with congenital hypothyroidism and treat them as early as possible. Klein et al. (1) have shown that mental retardation can be prevented if the patient is treated at an early stage. Clinical diagnosis is often difficult except in severe cases with quite typical signs and symptoms, and this is often too late for effective treatment to prevent mental retardation. A screening test is, therefore, thought to be necessary.

In 1973, Dussault et al. (2) in Canada reported that T_4 concentration can be measured using blood filter paper disc, which is commonly employed for the screening of inborn errors of metabolism, and they were the first to screen by this method (3). We successfully measured TSH by radioimmunoassay (RIA) using dried heel blood disc on filter paper, which then was used for the pilot study on the screening of primary hypothyroidism. We selected TSH determination because blood TSH shows marked elevation in patients with primary hypothyroidism, both in cases with low or low normal T_4 concentrations, enabling detection not only of severe, but also of mild or subclinical hypothyroidism. The other reason for this selection was that primary hypothyroidism, especially cases with thyroid aplasia, is the most important disorder compared to secondary or tertiary hypothyroidism as the cause of mental retardation.

In 1975, the Research Project Team on the Screening of Congenital Hypothyroidism was started by the Ministry of Health and Welfare in our country, and after the Team detected approximately 40 cases, the screening of congenital hypothyroidism was included as a part of the Multiple Screening Program of the government in 1979. This program now includes six disorders: phenylketonuria, maple syrup urine disease, homocystinuria, histidinemia, galactosemia, and congenital hypothyroidsm.

I will briefly describe the selection of TSH or T_4 as the primary screening method, since I have presented a detailed paper at this meeting (5). To attempt to solve this problem, three institutes (Tokyo and Chiba, Kanagawa, and Sapporo) measured both TSH and T_4 from the same blood disc samples taken mostly at the 5th-7th day after birth, and compared results. Table 1 shows the number of newborns screened and types of hypothyroidism detected. The total number screened by both TSH and T_4 measurement was more than 734,000 and total cases detected were 115. This group consisted

Table 1. Types and Numbers of Hypothyroidism Detected by Both TSH and T_4 Screening

Items/Institutes	I	II	III	Total
Number of newborns screened	278,378	337,531	118,401	734,310
Primary hypothyroidism	32	43	19	94
Transient primary hypothyroidism	3	12	3	18
Secondary hypothyroidism	0	3	0	3
Total	35	58	22	115

of 94 cases with primary hypothyroidism, 18 with transient primary hypothyroidism, and only three with secondary or tertiary hypothyroidism. Table 2 summarizes the comparison of determination of TSH and T_4 for the screening of primary congenital hypothyroidism. Sixty-four cases were detected by high TSH and low T_4, but it is noteworthy that 30 cases were detected by high TSH and normal T_4, and no case was found by normal TSH and low T_4. From these and other results, we believe that primary TSH screening is preferable to primary T_4 for the detection of congenital hypothyroidism, and that secondary or tertiary hypothyroidism are extremely rare at the neonatal period. Thus, we use primary heel blood TSH measurement routinely and enzyme immunoassay of neonatal TSH is also being employed in our country (6).

Table 3 is the summary of the nationwide neonatal hypothyroid screening in Japan, which shows the statistics of the Ministry of Health and Welfare, starting April, 1979, to March, 1985. Total number of the screened subjects is close to 7,430,000 and patients detected were 932. If we add

Table 2. Comparison of TSH and T_4 Determination for Screening of Primary Congenital Hypothyroidism

Determinations/ Institutes	I	II	III	Total (n=94)
High TSH Low T_4	23	27	14	64
High TSH Normal T_4	9	16	5	30
Normal TSH Low T_4	0	0	0	0
Total	32	43	19	94

Table 3. Summary of Neonatal Hypothyroid Screening in Japan (April, 1979 - March, 1985)*

Year	No. of births	No. of subjects screened	Screening rate	No. of patients	Incidence
1979, April	1,633,434	335,795	20.6%	41	1/8,200
1980, April	1,555,076	1,206,905	77.6%	151	1/8,000
1981, April	1,520,293	1,441,472	94.8%	185	1/7,800
1982, April	1,519,436	1,489,600	98.0%	177	1/8,400
1983, April	1,508,058	1,487,573	98.6%	181	1/8,200
1984, April	1,472,196	1,467,728	99.7%	197	1/7,500
Total	9,208,593	7,429,073	---	932	1/8,000

*Statistics by Ministry of Health and Welfare.

the patients detected during the period of pilot study to those detected after April, 1985, the total number exceeds 1,000. The incidence in Japan is then about 1:8000. The screening rate increased every year, and the latest percentage reached as high as 99.7%.

Apart from the statistics of the government, the Research Project Team on Congenital Hypothyroidism (Chief: Dr. H. Nakajima and Dr. M. Irie) is

Table 4. Cases of Congenital Hypothyroidism Detected by the Screening

	Congenital hypothyroidism	Transient hypothyroidism	Transient hyperthyro-tropinemia	Under follow-up (no final diagnosis)
By Mar. 1977	2			
1978	7	1	1	
1979	21	2	6	
1980	46	6	0	1
1981	110	18	16	4
1982	116	17	15	12
1983	101	13	17	14
1984	98	34	22	3
Total	501	91	77	34

Table 5. Types of Congenital Hypothyroidism Detected by Screening

	Male	Female	Ratio	Total (%)
Thyroid aplasia or hypoplasia	28	59	1 : 2.1	87 (27.0)
Ectopic thyroid	48	132	1 : 2.8	180 (55.9)
Dyshormonogenesis	21	32	1 : 1.5	53 (16.5)
Secondary hypothyroidism	1*	0	---	1 (0.3)
Total	98	223		321
Not determined				180

*Due to hamartoma of pituitary gland.

sending questionnaires to the specialized hospitals and collecting the clinical and laboratory data every year. Most of the past results have already been reported (7-12). I would like to show the most recent data accumulated by the Research Project Team.

Table 4 shows the types and numbers of congenital hypothyroidism detected every year beginning in 1977 to the present time. The types of hypothyroidism, detected by TSH measurement, were primary hypothyroidism, transient primary congenital hypothyroidism, and transient hyperthyrotropinemia. The case numbers of each category are shown in Table 4. Among the cases of congenital hypothyroidism, etiology of the disease was defined as shown in Table 5. Thyroid aplasia or hypoplasia was present in 27%, ectopic thyroid in 56%, and dyshormonogenesis in 16.5% of the 321 cases examined. Only one case of secondary hypothyroidism was recorded at that time. This patient died eventually with a hamartoma in the pituitary region found at autopsy. The other two cases mentioned before were diagnosed recently.

Fig. 1 shows some of the characteristics of the patients detected by the screening. Sex ratio revealed that there are twice as many female patients as males, accounting for 68% of total patients. The gestational age, birth weight, and birth height are not essentially different from the normal control newborns. Fig. 2 shows some radiological and laboratory data at the time of examination by the pediatrician. The absence of distal femur epiphysis is known to be characteristic of typical congenital hypothyroidism, but this was present in 72% of the total 434 patients examined. Serum TSH was high in all patients, average being more than 200 μU/ml, but serum T_4 and T_3 values were variable, with average values for T_4 of 4.5 μg/dl, and for T_3 of 122 μg/dl. Antimicrosomal antibody and antithyroblobulin antibody were positive in 9.8% and 5.1%, respectively, which are definitely higher than in the general population. This finding suggests a causative role of autoimmunity for this disorder, which should be studied further. Fig. 3 shows the family history on thyroid disorders of the cases detected. This was high and positive in 8.6%, including hyperthyroidism, hypothyroidism, and various other thyroid disorders. The 10 cases who had congenital hypothyroidism were mostly due to dyshormonogenesis. Complications of other disorders (Table 6) were present in 11.6%, which is unexpectedly high; among those, congenital heart disease, Down's syndrome, and miscellaneous anomalies were the main clinical complications. The reasons for this should also be studied further.

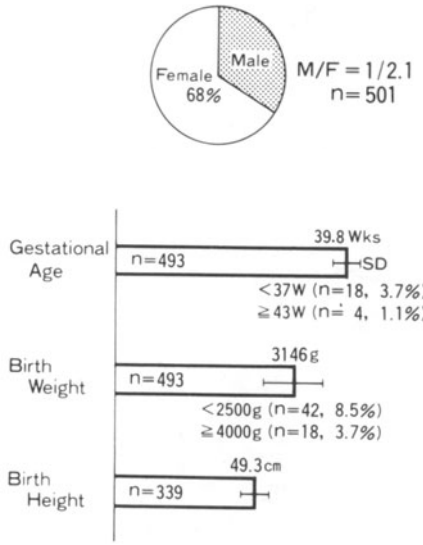

Fig. 1. Summarized data of congenital hypothyroidism detected by the screening.

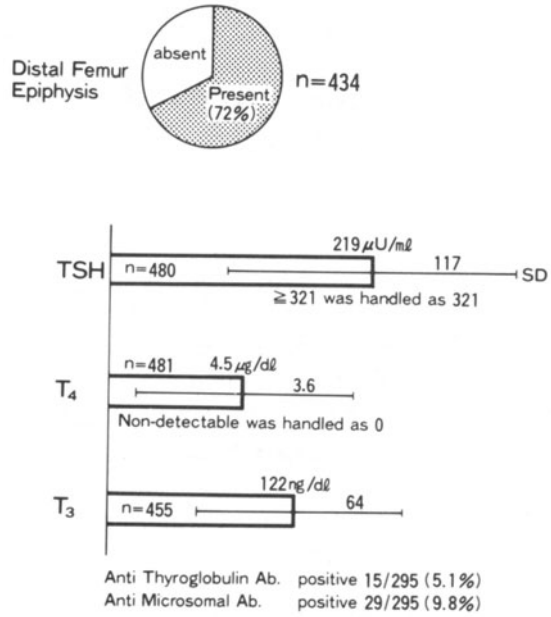

Fig. 2. Summarized data of congenital hypothyroidism.

We have found 77 cases of transient primary hypothyroidism, and Fig. 4 shows the known etiology of this condition. Fetography and dyshormonogenesis were the main causes, but seven cases had very low birth weight. Premature infants are known to have low T_4 and some of them high TSH (13). In cases of premature infants, we should then be cautious in the interpretation of the screening values of TSH and T_4 at the time of birth. For these infants, we decided recently to do a second screening at least one month after birth. Among seven premature subjects who showed high TSH and low T_4 in our series, only one case was eventually proven to be primary hypothyroidism. Positive TSH binding inhibitor immunoglobulin (TBII) as a cause

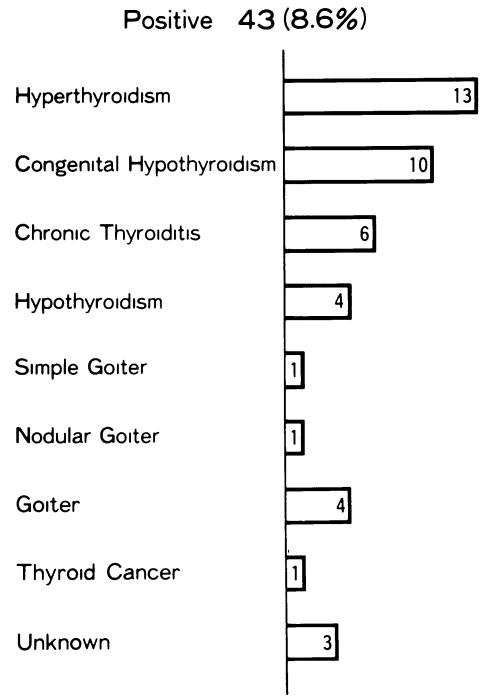

Fig. 3. Family history of thyroid disorders in patients with congenital hypothyroidism.

Table 6. Complications Associated with Congenital Hypothyroidism

Present 58 (11.6%)	
Congenital heart diseases	16
Minor anomaly	14
Down's syndrome	10
Cerebral anomaly	7
Dislocation of hip joint	4
Inguinal hernia	3
Rectal atresia	2
Cornelia de Lange syndrome	1
45X/47XXX	1
Protein-losing gastroenteropathy	1

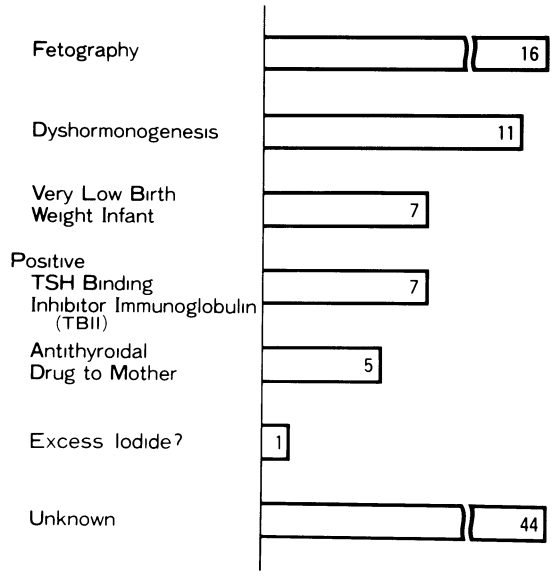

Fig. 4. Causes of transient hypothyroidism (n=91; 46 male, 45 female).

of transient primary hypothyroidism was first reported in Japan by Matsuura and co-workers (14), with the collaboration of the Kyoto University group, and this has now been demonstrated in seven cases of our series. As to TBG deficiency or decrease, we are finding more patients in Japan. During this study concomitantly to TSH and T_4 measurement (Table 7), the Sapporo City Institute of Public Health measured TBG by radioimmunoassay using the blood filter paper discs. They found 352 cases with TBG less than 14.9 µg/ml among 118,401 cases, the ratio being 1:336. If we take the cases with TBG less than 4.9 µg/ml, the number is 128, the ratio being 1:925. Regarding the sex ratio, the group of TBG less than 4.9 µg/ml consists of all males, and that of TBG 5.0-14.9 µg/ml consists of about 50% males.

Table 7. Distribution of TBG Deficiency and Decrease (Institute III*)

Values of TBG	Number	Ratio
0 - 4.9 µg/ml	128	(1 : 925)
5.0 - 9.9 µg/m	63	(1 : 1879)
10.0 - 14.9 µg/ml	161	(1 : 735)
Total 0 - 14.9	352	(1 : 336)

*Total number screened: 118,401; normal newborns: 140; mean ± SD: 25.5 ± 2.67 µg/ml; range: 17.6 ± 31.3 µg/ml.

Finally, I will describe briefly the treatment and follow-up study. The standard method of treatment is the administration of L-T_4, 5-10 μg/kg initially, and adjustment of the dose within four weeks by clinical and laboratory findings in the patients. We now have one to six years' data on DQ or IQ in treated patients with congenital hypothyroidism detected by our screening. As shown in Table 8, the results are excellent in both mental and physical development, and no neurological and psychological disturbances have been noted up to now. Some patients with DQ or IQ below 70 were complicated with either Down's syndrome or Cornelia de Lange syndrome. The overall distribution of DQ and IQ is quite favorable when compared to the general population. Before we started the screening and treatment, we were not certain of the influence of the lack of thyroid hormone in utero for brain development, but it appears that this may not be a problem.

In summary, we have initiated the screening of congenital hypothyroidism with TSH measurement of the blood filter paper disc, and it is now proceeding on a governmental basis as part of a multiple neonatal screening program in Japan. We have screened more than 7.4 million infants and detected approximately 1,000 patients, the incidence being about 1:8,000 in our country. The comparison of primary TSH and T_4 measurement was made in three major institutes in more than 734,000 subjects, and it was concluded that primary TSH determination is preferable to primary T_4 for the screening of congenital hypothyroidism if one has to choose between these measurements. Secondary or tertiary hypothyroidism was extremely rare in neonates in our study. Besides, most of them are also diagnosed clinically, so the screening should lean towards the diagnosis of primary hypothyroidism. Some of the clinical features and laboratory and radiological findings described here are from data of the Research Project Team on Congenital Hypothyroidism of the Ministry of Health and Welfare in Japan. The follow-up study of DQ and IQ during these six years have showed quite satisfactory results.

Table 8. Follow-up Data of DQ or IQ in Treated Patients with Congenital Hypothyroidism Detected by Screening

Age at examination	Mean ± SD (n)	≧90	89-85	84-80	79-70	70>
1Y DQ	106 ± 13 (192)	180	5	2	3	2
2Y DQ	110 ± 19 (166)	146	6	7	2	5
3Y DQ	105 ± 18 (91)	81	3	1	4	2
4Y DQ or IQ	105 ± 14 (40)	36	1	3	0	0
5Y DQ or IQ	113 ± 15 (11)	10	0	1	0	0
6Y IQ	112 ± 16 (8)	7	0	1	0	0
The last DQ or IQ at this study (%)	107 ± 17 (311)	277 (89.1)	8 (2.6)	13 (4.2)	6 (1.9)	7 (2.3)

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REFERENCES

1. Klein AH, Meltzer S, and Kenny FM. J Pediatr 81: 912, 1972.
2. Dussault JH and Laberge C. Union Med Can 102: 2062, 1973.
3. Dussault JH, Coulombe P, Laberge C, et al. J Pediatr 86: 670, 1975.
4. Irie M, Enomoto K, and Naruse H. Lancet ii: 1233, 1975.
5. Irie M, Naruse H, Nakajima H, et al. Presented at the Ninth International Thyroid Congress, Sao Paulo, Brazil, 1985.
6. In H Naruse, M Irie, and A Tsuji (eds), Neonatal Hypothyroid Screening by Enzyme Immunoassay, Boshi-Aiiku-kai, Okado Publishing Co., Ltd., Tokyo, 1982.
7. Irie M and Naruse H. In H Bickel, R Guthrie, and G Hammersen (eds), Neonatal Screening for Inborn Errors of Metabolism, Springer-Verlag, Berlin-Heidelberg-New York, 1980, p 247.
8. Irie M. In GN Burrow and JH Dussault (eds), Neonatal Thyroid Screening, Raven Press, New York, 1980, p 139.
9. Irie M, Nakajima H, Inomata H, et al. In H Naruse and M Irie (eds), Neonatal Screening, Excerpta Medica, International Congress Series 606, Amsterdam-Oxford-Princeton, 1983, p 130.
10. Irie M, Nakajima H, and Inomata H. In Abstracts, Seventh International Congress of Endocrinology, Excerpta Medica, International Congress Series 652, Amsterdam-Oxford-Princeton, 1984, p 856.
11. Irie M, Nakajima H, Inomata H, et al. In M Lee, CS Koh, and BY Cho (eds), Current Problems in Thyroid Disease, Korean Thyroid Society, Seoul, Korea, 1985, p 225.
12. Inomata H, Nakajima H, Naruse H, et al. In Abstracts, Seventh International Congress of Endocrinology, Excerpta Medica, International Congress Series 652, Amsterdam-Oxford-Princeton, 1984, p 855.
13. Delange F, Dalhem A, Bourdoux P, et al. J Pediatr 105: 462, 1984.
14. Matsuura N, Yamada Y, Nohara Y, et al. New Engl J Med 303: 738, 1980.

IODINE DEFICIENCY, THE THYROID, AND THE BRAIN

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The establishment of the essential link between iodine deficiency, thyroid function, and brain development has emerged over the past 20 years from a fascinating combination of clinical, epidemiological, and experimental studies.

The central human phenomenon that focuses this relationship is the condition of endemic cretinism, a condition well known in Alpine Europe in the Middle Ages, which was mentioned in Diderot's Encyclopedia in 1754 and was of such importance as to warrant a special Commission set up by the King of Sardinia (who was also King of Savoy) in 1848 (1). It was also reported by McCarrison (2) from the N.W. Frontier Region of India.

However, endemic cretinism was virtually forgotten in the first half of the twentieth century after McCarrison's description until it was "rediscovered" in New Guinea by McCullagh (3) in the East - Papua New Guinea, and Choufoer, Querido, et al. (4) in the West - in what is now Irian Jaya, a province of Indonesia. In the rugged Highlands of this very large island, these investigators were astounded to find not only very high rates of goiter prevalence, but a great abundance of mental defectives who were usually suffering from deaf-mutism and sometimes spastic diplegia.

McCarrison (2) in his classical study of 203 cretins distinguished two types: One a "nervous" cretin with mental deficiency, deaf-mutism, ataxia, and spasticity without hypothyroidism, which he clearly distinguished from a hypothyroid type which he considered characteristic of Europe. Over the last 20 years the nervous or neurological cretin has been recognized to be the most common type in all parts of the world - Alpine Europe, Latin America, Papua New Guinea, Indonesia, China, and the Himalayas with the exception of Zaire in Africa where the hypothyroid cretin predominates, probably due to the high cassava (goitrogen) intake (5). Only the hypothyroid form of cretinism is responsive to thyroid hormones.

The important observation that raised great doubt as to the importance of iodine deficiency was the apparent "spontaneous" disappearance of cretinism from the Alpine region. This was noted by Wespi (6) in Switzerland where deaf-mutism disappeared in cantons where both iodized salt was and was not used. Costa (7) provided further data with his observations in the Piedmont, formerly part of Savoy, the same area which was the subject of the original Sardinian Commission in 1848 - cretins were still to be seen

but they were in older age groups in spite of the lack of an iodized salt program.

The relation of iodine deficiency to the thyroid and brain development has become my personal special interest. It has provided me with a series of three adventures which I will review.

My first adventure was in the Western Highlands of Papua New Guinea, where we were able to demonstrate the prevention of endemic cretinism in a controlled trial with iodized oil injections. This epidemiological study established the relationship of iodine to brain development but raised questions about the mechanisms involved. It did indicate that the spontaneous disappearance of cretins from Alpine Europe was probably due to an increase in iodine intake due to greater availability of foodstuffs from non-iodine deficient areas.

This led to my second adventure which was concerned with animal models and a systematic investigation of the mechanism of the effect of iodine deficiency on fetal brain development. This finally proved the effect at the experimental level and established the role of both maternal and fetal thyroid function.

My third adventure has been concerned with the implication of this work for international public health. The recognition of the gap between our knowledge and its application to the millions suffering from iodine deficiency has led to the formation of the International Council for the Control of Iodine Deficiency Disorders (ICCIDD).

PAPUA NEW GUINEA

My first adventure was in Papua New Guinea over the period 1964-1972. When this small developing country became the responsibility of the Australian Government after World War II, an active Department of Public Health developed with strong leadership from the late Dr. John Gunther and Dr. Roy Scragg. Research collaboration with Australian investigators was encouraged. So it was that with a thyroid laboratory and excellent staff, and a long-standing interest in the thyroid hormones and thyrotoxicosis, I decided to explore the problem of endemic goiter in Papua New Guinea in collaboration with the Department of Public Health.

In due course, we established the presence of severe iodine deficiency in Papua New Guinea and were able to demonstrate the value of the injection of iodized oil in its correction (8). The effect of a single injection was shown to last up to 4 1/2 years. The effect of the iodized oil in reducing existing goiter with rise in thyroxine iodine and fall in TSH was also shown (8). This effect became well known to the villagers who presented themselves in large numbers at health centers and hospitals, requesting the injections. This fact continues to be a very useful aid in acceptance of an iodized oil program.

We, like our predecessors in Papua New Guinea, were confronted with the remarkable phenomenon of the neurological cretins. We reported a series of 254 cases that usually showed some degree of deaf-mutism (70%), followed by neurological signs of an upper motor neurone lesion in less than half (9). In view of the doubt about the relation of iodine deficiency to cretinism, we decided to set up a controlled trial with the iodized oil, although we were far from sure as to whether we would be able to successfully complete it.

The study was carried out with the support and collaboration of the Public Health Department which accepted that the study was ethically justified. Iodized oil or saline injections were given to alternate families in the Jimi River District in the Western Highlands at the time of the first census (1966). Each child born subsequently was examined for evidence of motor retardation, as assessed by the usual milestones of sitting, standing, or walking, and for evidence of deafness. Examination was carried out without knowledge as to whether the mother had received the iodized oil injection or saline (10).

Infants presenting with a full syndrome of hearing and speech abnormalities, together with abnormalities of motor development with or without squint, were classified as suffering from endemic cretinism. By these criteria, there were seven cretins born to women who had received iodized oil out of a total of 687 children. In six of these seven cases, conception had occurred prior to the iodized oil injections.

In the untreated group, there were 25 endemic cretins out of a total of 688 children born since the trial began. In five of these 25, conception had occurred prior to saline being given.

It was concluded that an injection of iodized oil given prior to pregnancy could prevent the occurrence of the neurological syndrome of endemic cretinism in the infant. The occurrence of the syndrome in those who were pregnant at the time of oil injection indicated that the damage probably occurred during the first half of pregnancy, possibly in the first trimester.

The value of iodized oil injection in the prevention of endemic cretinism was subsequently confirmed in Zaire and in South America (11).

Subsequent observations in Papua New Guinea revealed the existence of a coordination defect in otherwise apparently normal children subjected to iodine deficiency in pregnancy, in contrast to the group whose mothers had received an iodized oil injection (12). This observation has also been made in Indonesia, and indicates a wider spectrum of neurological effects of iodine deficiency in pregnancy.

ANIMAL MODELS

My second adventure (1976-1985) has been the establishment of animal models, first in the sheep and then in the marmoset. This followed my move to the CSIRO Division of Human Nutrition - the National Nutrition Research Institute in Australia, where I was fortunate to have a group of colleagues (Brian Potter, Gordon Jones, Graham McIntosh, and others) who had long experience of trace element work in sheep. Arising from the New Guinea work, we decided to try to investigate the effect of severe iodine deficiency in pregnancy on the development of the fetal brain in the lamb. We chose this animal because of its availability and because we knew that surgical procedures would be possible in which to investigate the role of the maternal and fetal thyroid.

Iodine Deficiency in the Sheep

Severe iodine deficiency was produced in sheep (13) with a low-iodine diet of crushed maize and pelleted pea pollard (8-15 µg iodine/kg) which provided 5-8 µg of iodine per day. After a period of five months, although body weights were maintained, iodine deficiency was evident with the appearance of goiter, low plasma T_4 and T_3 values, elevated TSH levels, and low daily urinary excretion of iodine. Control animals received the same diet

but were supplemented with an iodized oil injection (1 ml = 400 mg iodine). The ewes were mated with normal fertile rams, dates of conception established, and fetuses delivered at 56, 70, 98, and 140 days' gestation by hysterectomy (13).

Goiter was evident from 70 days in the iodine-deficient fetuses and thyroid histology revealed evidence of hyperplasia from 56 days' gestation. The increase in thyroid weight was associated with a reduction in fetal thyroid iodine content, reduced plasma T_4 values, and increased plasma TSH.

The iodine-deficient fetuses at 140 days were grossly different in physical appearance to the control fetuses (13). There was reduced weight, absence of wool growth, goiter, varying degress of subluxation of the foot joints, and deformation of the skull. There was also delayed bone maturation as indicated by delayed appearance of epiphyses in the limbs.

There was a lowered brain weight and brain DNA as early as 70 days, indicating a reduction in cell number probably due to slowed neuroblast multiplication which normally occurs from 40-80 days in the sheep (13). Delayed maturation of the cerebellum was shown by reduced migration of cells from the external granular layer to the internal granular layer and increased density of Purkinje cells. In the cerebral hemispheres, the cells were more densely packed in the motor and visual areas (13).

The effects of severe iodine deficiency on fetal brain development in the sheep were more severe but similar to those of fetal thyroidectomy carried out at 50-60 days or at 98 days (14). Maternal thyroidectomy carried out some six weeks before pregnancy had a significant effect on fetal brain development in mid-gestation (14). The combination of maternal thyroidectomy and fetal thyroidectomy at 98 days produced more severe effects than that of iodine deficiency (14,15).

The findings following maternal, fetal, and combined thyroidectomy suggest that the effect of iodine deficiency on fetal brain development is mediated by the combination of reduced maternal and fetal thyroid secretion and not by a direct effect of iodine (14). The effect of reduced maternal secretion occurs in the first half of pregnancy and the effect of reduced fetal secretion in the latter half of pregnancy. The conclusion is consistent with recent evidence in the rat of the passage of maternal thyroxine across the placental barrier early in pregnancy (16,17). It also explains the significant relationship found between the level of maternal T_4 and the extent of the coordination defect in the Papua New Guinea trial (18).

THE KNOWLEDGE APPLICATION GAP - TOWARD PREVENTION AND ERADICATION

The new knowledge of the effects of iodine deficiency on brain development required a modification of our traditional thinking of "goiter" as the major effect of iodine deficiency. It was apparent that the effects extended through fetal life to the neonate and child, constituting a spectrum ranging from stillbirths, cretinism, neonatal hypothyroidism, and juvenile hypothyroidism, to impaired hearing and brain development and function, as well as goiter at all ages. This led to my introduction of the term "Iodine Deficiency Disorders" (IDD) to refer to all the effects of iodine deficiency on human growth and development which can be prevented by correction of iodine deficiency (19). This latter feature is very important in distinguishing IDD from other forms of goiter or mental deficiency which are not due to iodine deficiency.

The lack of awareness of these broad effects of iodine deficiency on growth and development, particularly brain development, has been a major

factor in the continued inertia surrounding the development of iodization programs. This inertia is worldwide. Effective IDD control programs are an exception rather than the rule. In Latin America, Argentina, Brazil, Guatemala and Colombia have achieved success; in Africa, Zaire; and in Asia, only Indonesia and China are making some progress, apart from Papua New Guinea (19). In SE Asia, there are 300 million people at risk (20). In China, approximately one third of the population (310,000,000) is at risk with only half the population adequately covered so far, in spite of energetic efforts dating mainly from as recently as 1978 (21).

A series of international meetings over the past decade has called for the eradication of goiter and cretinism and the iodine deficiency disorders. These include the World Food Council (1974), the General Assembly of the United Nations (1978), the International Nutrition Congress (Rio de Janiero, 1978), the Regional WHO/UNICEF Committee for Southeast Asia (1981, 1982), the Asia and Oceania Thyroid Congress (Tokyo, 1982), the 4th Asian Congress of Nutrition (Bangkok, 1983), and the PAHO/WHO Meeting (Lima, 1983).

The 4th Asian Congress of Nutrition in Bangkok included an international Symposium on the Control of Iodine Deficiency in Asia. This symposium (22) agreed on a series of resolutions including adopting the term IDD, noting the importance of effects on the brain and the limitations imposed by iodine deficiency on social life and development. They pointed to the feasibility of eradication using iodized salt and iodized oil, the need for monitoring and reappraisal of existing programs, and the opportunity to make a major input to Health for All by the Year 2000.

In May 1984, I received an invitation through the Australian Government to prepare a Report on a Global Strategy for the eradication of IDD. This invitation came from the Administrative Coordinating Committee of the United Nations Agencies Standing Committee on Nutrition (ACC/SCN). This body, which meets each year, coordinates international nutrition activities for the UN Agencies (there are about 14 of them with such an interest including particularly WHO, FAO, and UNICEF). It also includes representatives of National Governments (called "bilaterals") with an interest in international nutrition. This report was duly prepared with the assistance of a grant from the Australian Development Assistance Bureau (ADAB). It was accepted as a background document by the ACC/SCN at its 1985 meeting in Nairobi. At that time, a subcommittee was appointed to prepare a short statement for submission to the Secretary-General of the United Nations for consideration and, if approved, transmission to the UN Agencies for the development of a 10 year plan of action. This Subcommittee duly met at PAHO Washington in May under the Chairmanship of Dr. E. de Maeyer (WHO) and included J. B. Stanbury, C. H. Thilly, and myself.

In my report, I recommended the establishment of an International Consultative Group to provide expertise for the international agencies and national governments with major IDD problems as a necessary step to develop more active IDD control programs. The ACC/SCN approved this step at the Nairobi meeting in February, 1985. It followed earlier initiatives which established international consultative groups for Vitamin A (INAVC) and for nutritional anemia (INACG) eight to ten years ago.

Subsequently, in Delhi in March, 1985, I convened a meeting of a group of interested experts at the time of the WHO/UNICEF Intercountry Workshop which decided to establish the International Council for Control of Iodine Deficiency Disorders (ICCIDD). Dr. John B. Stanbury was elected Chairman, Professor V. Ramalingaswami, Vice-Chairman, Dr. J. T. Dunn, Secretary, and I was appointed Executive Director. The original group of 12 has been designated the Founding Board. It has now been extended by invitation to

a total of about 30 members who have substantial research or practical interest in IDD. A further preliminary meeting has just been held in Sao Paulo (1st September). The formation of the ICCIDD was announced at the IDD Satellite Meeting and I am pleased to take the opportunity of a further announcement to the Ninth International Thyroid Congress.

The ICCIDD will be formally inaugurated at a meeting in Kathmandu, Nepal, March 23-27, 1986. By that time, a draft constitution will be drawn up and a strategy and work plan will be submitted.

Initial support for ICCIDD has been generously provided by UNICEF. The New York office has agreed to an initial grant of $150,000 a year for two years. ADAB in Australia has also provided funds for the Secretariat which is being established in Adelaide at the CSIRO Division of Human Nutrition.

The ICCIDD will act as an expert resource on all aspects of IDD and control programs. Its primary function is to bridge the great knowledge application gap which is so obvious in this field. Its prime purpose is to help the international agencies and national governments with significant IDD problems to develop effective programs.

It aims to establish a multidisciplinary global network of 200 members who will act as a resource on all aspects, including communication, planning and economics, as well as technical expertise on iodinated salt and iodinated oil, and thyroid pathology, physiology, and biochemistry. New technology will be fostered. Adequate monitoring and evaluation procedures will be introduced. To this end, ICCIDD plans to introduce a global monitoring system for IDD, with the establishment of a global network of laboratories providing determinations of T_4 and TSH on cord bloods from remote areas. These data will be provided to national governments and international agencies to provide evidence indicating the urgency of the IDD problem in relation to the prevention of mental deficiency. This system will also help to provide quality control for new laboratories, as well as monitoring of IDD control programs.

Reginal IDD Coordinators will be appointed to be responsible for general surveillance of the countries with major IDD problems in the various WHO Regions. This will include the gathering of prevalence data and program development. The Regional Coordinator will also be able to develop a Regional multidisciplinary team to be available for consultation in the Region.

Consultations with national governments and international agencies are being arranged. Further funding from international agencies and bilateral countries will be sought at a modest level for the ICCIDD itself. Its function is to assist the development of IDD control programs by national governments but not to actually run these programs.

It is hoped that the ICCIDD will be an important development in relation to the ultimate objective of eradication of IDD. It will provide scientists with an active interest in IDD with a framework for operation, so that initiatives produce a greater return than is the case at present.

The technology for eradication of IDD is available and feasible; however, much more than technology is involved. That is why the ICCIDD is necessary to secure the proper application and use of science and technology for the benefit of the very large populations living in iodine-deficient areas who are at risk of IDD.

This, then, is the end of my third adventure, the outcome of research on the relation of iodine, thyroid, and brain into a great global public health program, which could be a triumph for our generation in the field of international health and nutrition. It would be great if at the next ITC in 1990, substantial progress towards global eradication could be reported.

ACKNOWLEDGMENTS

My adventures in this field have been shared with many friends and colleagues over the last 20 years. I cannot mention them all but I thank all of them for their stimulation, comradeship and support. I especially thank my senior friends and colleagues, John Stanbury, Andries Querido and V. Ramalingaswami, my colleagues Graham Clugston and Rolf Carriere in Delhi, my collaborators in CSIRO led by Brian Potter, and my original group at the Queen Elizabeth Hospital, notably Ian Buttfield and Brain Good, and Peter Pharoah in New Guinea who shared with me the laying of the foundation of this work.

REFERENCES

1. Konig MP. In BS Hetzel and RM Smith (eds), Fetal Brain Disorders, Elsevier Biomedical, Amsterdam, 1981, p 229.
2. McCarrison R. Lancet 2: 1275, 1908.
3. McCullagh SF. Med J Aust 1: 769, 1963.
4. Choufoer JC, Van Rhijn M, and Querido A. J Clin Endocrinol 25: 385, 1965.
5. Pharoah POD, Delange F, Fierro-Benitez R, et al. In JB Stanbury and BS Hetzel (eds), Endemic Goiter and Endemic Cretinism, Wiley, New York, 1980, p 395.
6. Wespi HJ. Schweiz Md Wochenschr 75: 625, 1945.
7. Costa A, Cottino F, Mortara M, et al. Panminerva Med 6: 250, 1964.
8. Buttfield IH and Hetzel BS. Bull WHO 36: 243, 1967.
9. Buttfield IH and Hetzel BS. Aust Ann Med 18: 217, 1969.
10. Pharoah POD, Buttfield IH, and Hetzel BS. Lancet 1: 308, 1971.
11. Hetzel BS, Thilly CH, Fierro-Beintez R, et al. In JB Stanbury and BS Hetzel (eds), Endemic Goiter and Endemic Cretinism, Wiley, New York, 1980, p 513.
12. Connolly KC, Pharoah POD, and Hetzel BS. Lancet 2: 1149, 1979.
13. Potter BJ, Mano MT, Belling GB, et al. Neuropathol Appl Neurobiol 8: 303, 1982.
14. Hetzel BS and Potter BJ. In IE Dreosti and RM Smith (eds), Neurobiology of the Trace Elements, Humana Press, New Jersey, 1983, p 45.
15. McIntosh GH, Potter BJ, Mano MT, et al. Neuropathol Appl Neurobiol 9: 215, 1983.
16. Obregon MJ, Mallol J, Pastor R, et al. Endocrinol 114: 305, 1984.
17. Woods RJ, Sinha AK, and Ekins, RP. Clin Sci 67: 359, 1984.
18. Pharoah POD, Connolly KJ, Ekins RP, et al. Cl Endo 21: 265, 1984.
19. Hetzel BS. Lancet 2: 1126, 1983.
20. WHO/SEARO. A Regional Strategy for the Control of Iodine Deficiency Disorders in Countries of the SE Asian Region. WHO/SEARO, Delhi, 1985.
21. Tai M, Tizhang L, Uybin R, et al. Food Nutr Bull 4: 13, 1982.
22. Control of Iodine Deficiency in Asia. Lancet 2: 1244, 1983.

AN OVERVIEW OF THE NEUROENDOCRINE CONTROL OF TSH AND PRL, TSH BIOSYNTHESIS, PITUITARY DEIODINASE, AND TSH-PRODUCING TUMORS

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NEUROENDOCRINE CONTROL OF TSH AND PRL: ROLE OF DOPAMINE

The association of hypothyroidism and hyperprolactinemia has been known for many years and suggested that the abnormally elevated PRL levels in primary hypothyroidism might result from a decreased hypothalamic dopamine (DA) secretion rather than increased TRH stimulation, reflecting a general action of thyroid hormones on hypothalamic DA activity (1,2). The same mechanism is probably present in hypothalamic hypothyroidism defined by exaggerated and/or delayed TSH response to TRH, with reduced biological activity as suggested by the decreased T_3 response to endogenous TSH released, probably dependent on alterations on its carbohydrate moiety, since it was demonstrated that TRH (decreased in central hypothyroidism) regulates TSH glycosylation (3). Furthermore, patients with hypothalamic hypothyroidism have an excess of β-TSH (4), reflecting an alteration in the combining properties of TSH subunits which may result from the abnormal glycosylation (3). As can be seen in Fig. 1, the greatly increased PRL levels were corrected by thyroid replacement. Besides, a decrease in hypothalamic and/or pituitary DA content or activity would be associated with decreased response to DA receptor blocking drugs such as chlorpromazine, metoclopramide, and domperidone. In effect, in patients with primary hypothyroidism and slight increase in basal PRL, we have shown a significantly lower increment in serum PRL after chlorpromazine when compared to normals (unpublished data). Scanlon and co-workers demonstrated that after the administration of metoclopramide (1) or domperidone (5), there was a significant release of TSH in euthyroid and subclinical hypothyroid patients, but not in those with overt hypothyroidism who lack DA inhibition of TSH release but may show marked DA inhibition of PRL secretion. It is then possible that thyroid hormones regulate hypothalamic DA either by enhancing DA secretion or by modulating DA receptors on the thyrotroph, the last possibility being the most likely. Therefore, thyroid hormones act directly on the thyrotrophs, inhibiting TSH secretion and stimulating hypothalamic DA secretion which acts as an intermediate step in the inhibition of TSH release (2). The stimulation of hypothalamic DA by thyroid hormones also inhibits PRL secretion by lactotrophs (2). Thus, it appears that thyroid hormones modulate the DA inhibitory tonus on the thyrotroph.

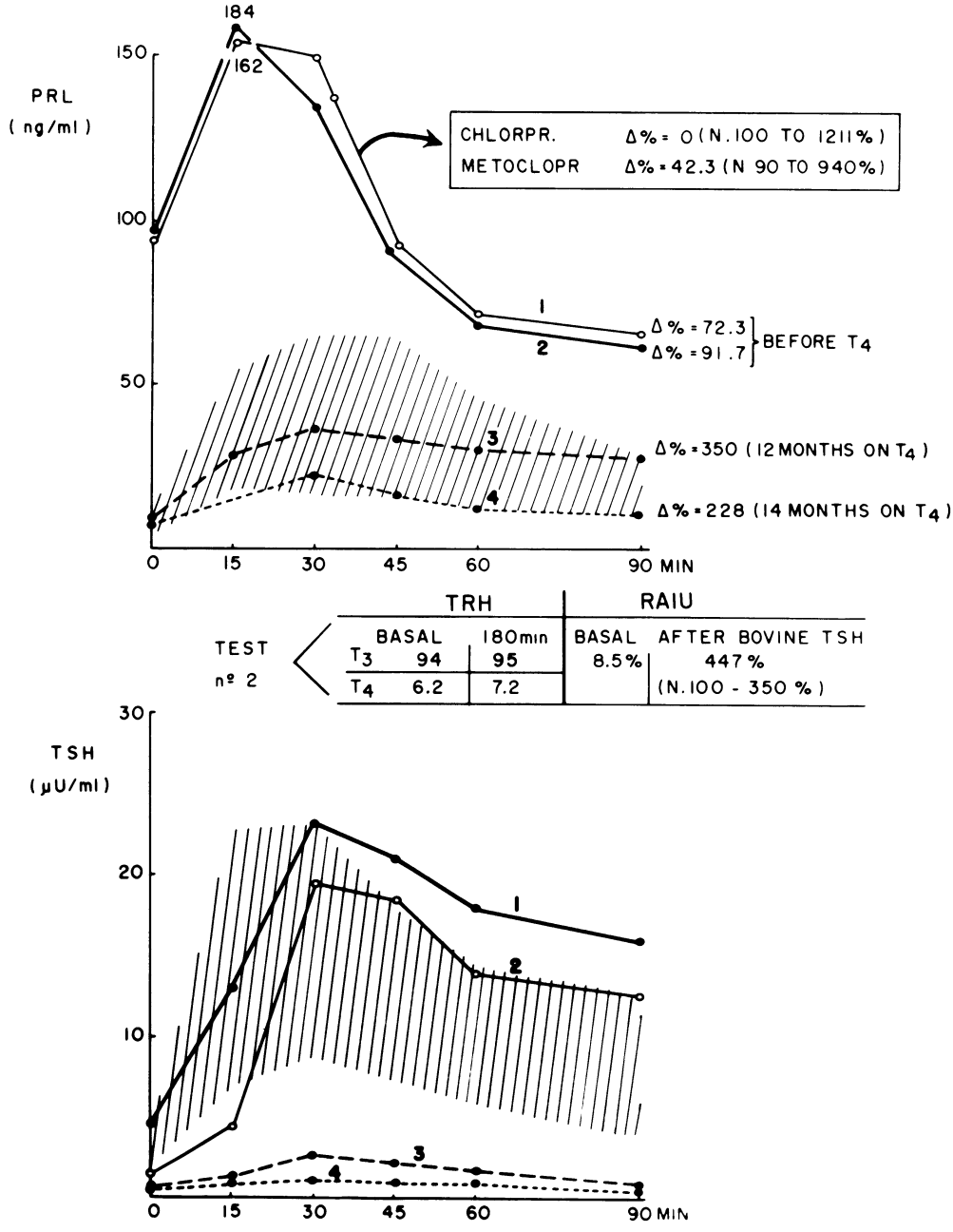

Fig. 1. TSH and PRL responses to TRH stimulation in a patient with idiopathic hypopituitarism, hypothalamic hypothyroidism, and hyperprolactinemia. (Hatched lines indicate mean ± SD.) A.J.M. - 13 year old male.

TSH BIOSYNTHESIS

The α and β-subunits are independently synthesized in precursor forms by the translation of separated mRNA molecules. These subunits are then processed to glycosylated forms, followed by their combination to form the biologically active TSH molecule prior to secretion from the thyrotroph (6) (Fig. 2). Recombinant DNA techniques have been utilized by Kourides and co-workers to clone DNA molecules complementary to α and β-TSH-mRNA (6). They demonstrated that in the mouse, the genes for α and β-TSH reside in different chromosomes. RIAs have indicated that human plasma and pituitary ratios of TSH/β-TSH are greater than one, suggesting that the limiting factor in the production of complete TSH is β-subunit biosynthesis.

As for human DNA, it was shown that there is only one gene for the α-subunits of all four glycoprotein hormones and two β-TSH genes, at least in the mouse. Kourides et al. (6) demonstrated that the negative feedback of thyroid hormones on the pituitary of mice was predominant on β-TSH-mRNA levels by inhibition of β-TSH transcription. Finally, in mouse pituitary TSH tumor, there was found a relationship between α and β-TSH-mRNA levels and correspondent protein values, suggesting transcriptional control of α and β-TSH levels (6).

ROLE OF THE PITUITARY DEIODINASE

It has been demonstrated by Silva et al. (7) that, in the rat, serum T_3 is the most important source of nuclear T_3 to the peripheral tissues (liver and kidney). However, half to two-thirds of nuclear T_3 is derived from intracellular T_4 to T_3 conversion in the anterior pituitary, cerebral cortex, and cerebellum. The enzyme that converts T_4 to T_3 (5'-iodothyronine deiodinase) was classified as Type I or PTU-sensitive (found in the kidney and liver) to distinguish it from Type II or PTU-insensitive 5'-iodothyronine deiodinase found in the CNS which has a Km for T_4 much lower

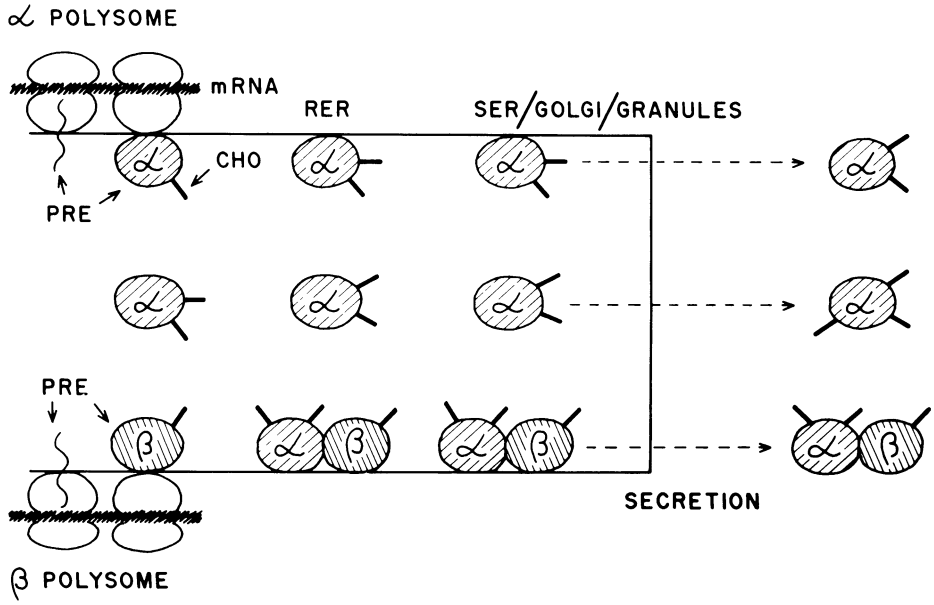

Fig. 2. Schematic representation of TSH biosynthesis.

than that of the Type I enzyme. The physiological characteristics of the 5'-iodothyronine deiodinase clearly indicate that the two different enzymatic pathways of T_3 generation are a mechanism regulating the thyroid status of peripheral tissues independently of the CNS, providing another level of regulation of tissular T_3, typically demonstrated in conditions of reduced T_4 when the brain is protected by an increase in its capacity to convert T_4 to T_3. The clinical implication of decreased 5'-iodothyronine deiodinase is seen in the "euthyroid sick patient" with low T_4 and T_3 levels and normal TSH, accumulation of rT_3 (reflection of the impaired Type I T_4 5'-deiodinase), and maintenance of the Type II enzyme activity. The peripheral reduction in the rate of T_4 to T_3 conversion, thus decreasing the availability of T_3, can be seen as of physiological advantage in such condition.

TSH-PRODUCING TUMORS

TSH-producing tumors which induce hyperthyroidism have the following characteristics (8-10): a) Autonomy-unresponsive (α-TSH and TSH) to TRH and T_3 and/or T_4. b) Rapid growth and invasion with frequent recurrences. c) Increase in α-TSH subunit relative to TSH but undetectable β-TSH: Molar α-TSH/TSH ratio > 1. d) Heterogenous forms of TSH with different bioactivities, as found in serum and pituitaries of mice with TSH tumors by Weintraub and co-workers (11). These tumors can be classified as: a) Not associated with hypersecretion of other hormones. b) Associated with overproduction of other hormones: PRL and/or GH, representing a relatively common tumor frequently associated with acromegaly, less frequently with hyperprolactinemia, and even less frequently with hyperthyroidism (12). The reason for the clinical silence of some of the hormones detected in tumor tissue is that either they are not secreted in excess and/or there is secretion of abnormal hormones, the last possibility being more frequent regarding TSH (12). All data suggest a common cytogenesis of these tumors.

REFERENCES

1. Scanlon MF, Weightman DR, Shale DJ, et al. Clin Endocrinol 10: 7, 1979.
2. Feek CM, Sawers JSA, Brown NS, et al. J Clin Endocrinol Metab 51: 585, 1980.
3. Beck-Peccoz P, Amr S, Menezes-Ferreira M, et al. New Engl J Med 312: 1085, 1985.
4. Faglia G, Beck-Peccoz P, Ballabio N, et al. J Clin Endocrinol Metab 56: 908, 1983.
5. Pourmand M, Rodriguez-Arnao MD, Weightman DR, et al. Clin Endocrinol 12: 211, 1980.
6. Kourides IA, Gurr JA, and Wolf O. Rec Prog Horm Res 40: 79, 1984.
7. Larsen PR, Silva JE, and Kaplan MM. Endocrine Rev 2: 87, 1981.
8. Gershengorn MC. In B Weintraub, Inappropriate Secretion of Thyroid-stimulating Hormone. Ann Int Med 95: 340, 1981.
9. Kourides IA. In B Weintraub, Inappropriate Secretion of Thyroid-stimuating Hormone. Ann Int Med 95: 344, 1981.
10. Hill SA, Falko JM, Wilson CB, et al. J Neurosurg 57: 515, 1982.
11. Pekonen F, Carayon P, Amr S, et al. Horm Metab Res 13: 617, 1981.
12. Horvath E, Kovacs K, Scheithauer BW, et al. Ultrastruct Path 5: 171, 1983.

THE NEUROREGULATION OF TSH AND PROLACTIN SECRETION

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INTRODUCTION

It is well known that the secretion of the anterior pituitary (AP) hormones, thyrotropin (TSH) and prolactin (PRL), is under tonic stimulatory and inhibitory control, respectively, by the hypothalamus. It is now clear also that the hypothalamic control of AP hormone secretion is mediated by the release into the portal blood vessels of a variety of peptidic and non-peptidic substances. Data gathered during the last few years have demonstrated that TRH and the central neurotransmitter dopamine (DA) have important actions on TSH and PRL release from the AP. Furthermore, somatostatin and a variety of other central neurotransmitters such as noradrenaline have effects which will be considered briefly.

BASIC PHYSIOLOGY AND CONTROL MECHANISMS

The Role of TRH

TRH is secreted by hypothalamic neurons into the hypophyseal portal system which originates at the median eminence. From here, the peptide is transported to the anterior pituitary gland. There is now no doubt that the dominant stimulatory effect exerted by the hypothalamus on TSH synthesis and released is mediated by TRH since: 1) TRH stimulates TSH release *in vivo*, *in vitro*, and after infusion into portal blood vessels in a physiological dose-range (1). 2) Passive immunization with anti-TRH antiserum leads to a decline in basal TSH levels in euthyroid and hypothyroid rats. In addition, sheep in which antibodies to TRH are raised show a decline in thyroid function (2). 3) TRH immunoreactivity can be detected in portal blood vessels (3). 4) Specific, high affinity, low capacity TRH receptors are present on thyrotroph membranes (4).

In contrast to the well-established role of TRH in the control of TSH, a biphasic pattern of TSH release is seen after prolonged intravenous infusion of TRH in man. The early phase may well reflect the release of a readily releasable pool of stored TSH within the thyrotrophs, whereas the later phase would be due to release of newly synthesized TSH produced under the influence of increased TRH drive (5).

This dominant stimulatory effect exerted by the hypothalamus via TRH on TSH synthesis and release is counterbalanced by the direct pituitary inhibition of TSH by thyroid hormones. In this process, the local intrapituitary conversion of T_4 to T_3 is most important, whereas other tissues have a greater capacity to take up circulating T_3. In addition to their direct inhibitory effects on TSH synthesis and secretion, thyroid hormones modulate the number of TRH receptors on the thyrotroph. In vitro studies have demonstrated a twofold increase in TRH binding to anterior pituitary membranes from hypothyroid animals, which can be reduced by thyroid hormone replacement. In contrast, it has been shown that estrogens have an opposite effect, acting to increase the number of TRH receptors. This latter process could explain in part the greater TSH responses to TRH observed in females than in males (5).

Whether thyroid hormones have any direct hypothalamic action on TRH synthesis and release remains controversial, although there is some evidence to suggest a negative feedback inhibitory role. After microinjections of nanomolar concentrations of T_3 into the hypothalami of hypothyroid monkeys, there is a rapid reduction in TSH levels (6). It remains to be demonstrated, however, whether this effect is mediated by actions on TRH or on other hypothalamic regulators of TSH release.

In contrast to the well-established role of TRH in the control of TSH secretion, it is still disputed whether TRH is a physiological prolactin-releasing factor (7). Factors supporting such a role are: a) the minimal effective threshold dose of TRH is the same for both TSH and PRL (5), b) in rats, the PRL response to suckling is accompanied by TSH release and by an increase of TRH in portal blood (2,5), and c) in sheep immunized against TRH, the PRL response to heat exposure is reduced (2).

However, against a physiological PRL-releasing role for TRH are the following: a) in man, there is no elevation in TSH levels following physiological stimuli to PRL release such as suckling and stress (5,7), b) the circadian rhythms of TSH and PRL are dissociated (5), and c) injection of TRH antibodies in rats has no consistent effect on PRL concentrations in serum (2).

In summary, it is clear that TRH plays a major role in the neuroregulation of TSH, as well as in the PRL response to suckling in the rat. However, it still remains to be established whether TRH plays an important role in the control of PRL secretion in humans.

The Role of DA

The role of DA as a physiological inhibitor of TSH and PRL secretion is now clearly established (5,7) and the evidence is summarized: 1) DA inhibits TSH and PRL release in vivo and in vitro, 2) endogenous DA antagonism leads to a rapid increase in PRL and TSH levels, 3) DA is present in portal blood at concentrations which inhibit PRL and TSH in vivo, and 4) specific high affinity DA receptors are present on anterior pituitary cells.

Furthermore, there is a striking parallelism between the inhibition of TSH and PRL by DA and DA agonist and antagonist drugs and the displacement of the specific binding of ^{3}H-DHE to rat AP cells in vitro. There is also good animal evidence that PRL and TSH may regulate their own secretion via an increase in hypothalamic dopaminergic activity and an increase in the number of DA receptors on the thyrotroph respectively (Fig. 1). Such evidence can be summarized as follows. 1) PRL administration to rats causes: a) reduced PRL levels in intact but not in pituitary transplanted rats, b) increased DA turnover in the median eminence, c) a selective increase in the

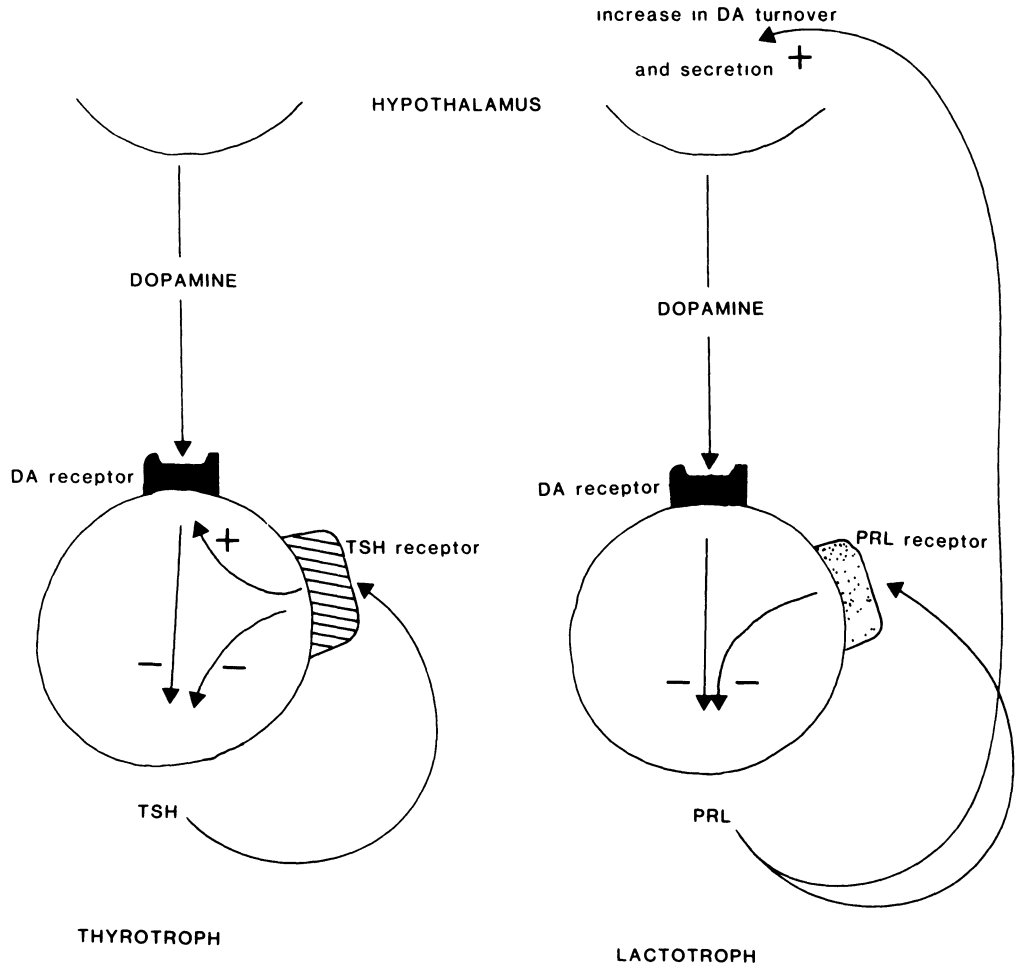

Fig. 1. Hypothetical model of PRL and TSH modulation of DA activity. According to this model, TSH (left) will influence the DA inhibition of TSH secretion by an increase in the number of DA receptors in g the thyrotroph. In contrast, PRL (right) acts mainly by increasin DA release by the TIDA neurons, resulting in increased PRL inhibition. The possibility of a direct PRL negative auto feedback is also considered.

activity of tubero-infundibular but not striatal DA neurons. 2) intraventricular PRL injections reduce PRL and TSH release. 3) DA levels in hypophyseal portal blood are increased in rats with ectopically transplanted PRL-secreting adenomas. 4) Recent in vitro data have shown that administration of TSH increases the number of DA receptors and the Bmax of the inhibition of TSH but not PRL secretion by DA (8).

In addition, other peripheral factors such as thyroid hormones and estrogens may influence the inhibition of PRL and TSH secretion by DA. Recent data from cultured rat AP cells from both euthyroid and hypothyroid animals suggests that thyroid hormones have opposite actions with regard to the DA

control of PRL and TSH; a slight facilitation of DA inhibition of PRL secretion and a powerful antagonism of the DA control of TSH (9). It is likely that these actions of thyroid hormones are mediated through modulation of the number of DA receptors on lactotrophs and thyrotrophs respectively.

With regard to the effects of estrogens on the control of PRL and TSH secretion by DA, there is also considerable debate (7,10). In vitro data from rat AP cells have demonstrated that estrogen treatment impairs PRL responsiveness to DA, and it has been shown also that DA receptors on the AP decrease markedly on the morning of proestrus which coincides with the preovulatory PRL surge. However, in the hypogonadal monkey and in humans, estrogen administration increases the inhibitory effect of exogenously administered DA on PRL and TSH secretion (10). The explanation for these apparently contradictory findings is unclear. It is possible that this could simply be a reflection of differences in the duration and dosage of administered estrogen. In support of this, it has been recently demonstrated that administration of very low doses of estradiol to rats leads to an increase in pituitary dopamine receptor binding, while higher doses have the opposite effect (11).

Other Central Factors Involved in the Control of PRL and TSH Release

Although DA is the dominant physiological mediator of the hypothalamic inhibition of PRL release, there is increasing evidence for the involvement of other neurotransmitter pathways in the inhibition of PRL release. GABA may be an inhibitory neurotransmitter, and possibly has a direct action on the pituitary gland mediated by specific GABA receptors. This area is somewhat controversial, since pharmacological concentrations of GABA are necessary to inhibit PRL release, and hypophyseal stalk plasma from diestrous rats contains low levels of GABA, similar to those found in the peripheral circulation (7). Histaminergic pathways have been implicated also in PRL inhibition, since administration of the H_2-receptor blocking drug, cimetidine, causes an acute rise in plasma PRL concentrations. This effect is mediated by central H_2-receptors rather than via a peripheral action on the pituitary lactotroph DA receptor. Similarly, an inhibitory role for the TRH metabolite, his-pro-diketopiperazine (DKP), has been suggested. However, conflicting data have been reported concerning the actions of DKP on PRL secretion in vitro (7). Recently, we have investigated the effect of administration of a 200 µg i.v. bolus of DKP on TSH and PRL secretion in both normal subjects and patients with microprolactinomas and primary hypothyroidism. We were unable to detect any effect of DKP on basal or stimulated PRL and TSH levels (12). More recently, it has been reported that Sauvagine, a peptide which is similar in structure to CRF, exerts a powerful inhibitory action on PRL secretion in vitro. More importantly, the inhibition was long-lasting and PRL levels remained suppressed several hours after Sauvagine administration (13). This latter finding clearly merits further investigation. Finally, it has been proposed recently that 6AP (part of the precursor protein of GnRH) may be the long sought peptidic prolactin-inhibiting factor. However, further data are needed before this can be established (14).

In addition to thyroid hormones and DA, somatostatin may also be a physiological inhibitor of TSH release (5). Administration of somatostatin antisera to cultured anterior pituitary cells causes elevation of both growth hormone (GH) and TSH levels in the medium, and when administered to intact rats enhances the TSH response to both cold stress and TRH, as well as elevating basal GH and TSH levels. There is no such evidence in man. Somatostatin infusion does, however, lower the elevated basal plasma TSH levels in patients with primary thyroid failure, suppresses the TSH response to TRH, and abolishes the nocturnal elevation in basal TSH levels. Other neuroregulators such as neurotensin, cholecystokinin, and acetyl-choline have also been shown to influence the release of TSH from the anterior pituitary, but the significance of these actions is unclear at present (5).

In addition to TRH, there are other factors that may stimulate PRL and TSH secretion by the AP gland. With regard to PRL, there are a large number of molecules which have been reported to stimulate synthesis and/or secretion in vitro. These include: VIP, β-endorphin, leu-enkephalin, bombesin, acetyl-choline, angiotensin II, vasopressin, substance P, secretin, PHI, and cholecystokinin (4). However, it still remains to be established whether they can stimulate PRL secretion when spontaneous PRL secretion is under tonic dopaminergic inhibition (as in the in vivo setting). Nevertheless, there is now clear evidence indicating that VIP may well be a prolactin-releasing factor (4) since: a) VIP is present in hypophyseal stalk plasma, b) low concentrations of VIP stimulate PRL release in vivo and in vitro, and c) VIP receptors are found on pituitary membrane preparations.

With regard to TSH, it is established that alpha-noradrenergic pathways play a stimulatory role in the intact animal. We have reported recently that this stimulatory action could be mediated, at least in part, direct-at pituitry level via α_1 adrenergic receptors (15). Moreover, this in vitro stimulatory effect can be demonstrated at agonist concentrations similar to to those found in hypophyseal portal blood. Other factors reported to have a stimulatory action on TSH release include melatonin and opioids but, again, the significance is unclear at present (5).

Alterations in TSH Regulation

These may be manifest either as alterations in basal plasma concentrations of TSH or alterations in the pattern and degree of the TSH response to TSH or DA blocking drugs. The most clear-cut alterations in TSH control occur in primary thyroid diseases (5,16). Basal TSH and responses to TRH are suppressed in primary hyperthyroidism from any cause. It should be remembered that in severe primary hypothyroidism the plasma concentrations of TSH may be lower than anticipated and the TSH responses to both TRH and DA antagonists may also be low (5,16).

Until recently, more reliance has been placed on the TSH response to dynamic testing unless the basal TSH is clearly elevated, since most current TSH radioimmunoassays are unable to distinguish between low and normal basal TSH levels. Even here, however, in so-called "TSH toxicosis" the absence of a TSH response to TRH in the presence of elevated TSH levels points to the presence of a TSH-secreting pituitary adenoma. In most other pituitary disease states (with the exception of hyperprolactinemia), the TSH response to TRH is usually normal or reduced and a flat TSH response is strongly suggestive of a pituitary lesion. It should be emphasized that factors other than tumor size may also determine the degree of TSH responsiveness to TRH.

In hypothalamic disease of any etiology, the TSH response to TRH may be suppressed or show a delayed and, not infrequently, exaggerated pattern, peak TSH levels being achieved at 60 minutes following TRH administration (5,16). However, these patterns of TSH response to TRH are not limited to patients with hypothalamic disease, but may also be observed in patients with pituitary stalk lesions. Alterations in basal TSH levels and responses to TRH and DA antagonist can also occur in a variety of neuropsychiatric (p.c. anorexia nervosa, unipolar depression) and endocrine/metabolic disorders such as chronic renal failure, starvation, Cushing's syndrome, etc. However, there are considerable overlaps and, although such alterations in the TSH response may be helpful, diagnostically they provide little information as to etiology, site of disease, or precise neuropeptide/neurotransmitter imbalances (5,16).

Alterations in PRL Regulation

Hyperprolactinemia is a common clinical hypothalamic-pituitary disorder and it is now clear that prolactinomas are the most common anterior pituitary

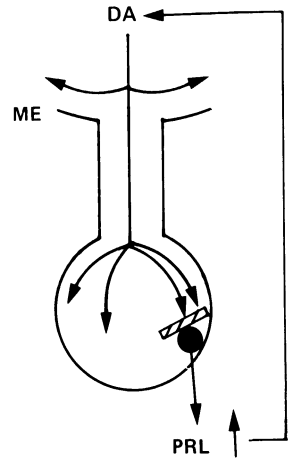

Fig. 2. Schematic representation of possible DA-mediated interactions between TSH and PRL in patients with autonomously functioning microprolactinomas. PRL stimulates increased DA release into hypophyseal portal blood leading to increased dopaminergic inhibition of TSH release. However, the increased DA concentrations are unable to control PRL release by the adenomatous cells, possibly because of reduced sensitivity to DA, or because of defective delivery of DA caused by dissociation of the adenoma from the hypophyseal portal blood supply.

tumors. The diagnosis of prolactinoma can be particularly difficult in the case of microadenomas because of the difficulty of interpreting minor radiological changes and because basal PRL levels can be near normal. Although a reduced or absent PRL response to TRH or DA antagonist correlates well with the presence of a prolactinoma, they are also observed in stalk-compression hyperprolactinemia (17) and the presence of considerable PRL responses to these agents by no means excludes a diagnosis of adenomatous hyperprolactinemia (17). Probably the best approach is to investigate the TSH response to DA antagonism (5), which is usually clearly exaggerated in the absence of stalk-compression. This exaggerated response probably reflects the increase in hypothalamic DA turnover caused by the positive feedback actions of high PRL levels (Fig. 2).

REFERENCES

1. Porter JC, Vale W, Burgus R, et al. Endocrinology 89: 1054, 1971.
2. Fraser HM and McNeilly AS. In EC Griffiths and GW Bennett (eds), Thyrotropin Releasing Hormone, Raven Press, New York, 1983, p 179.
3. Sheward WJ, Harmer AJ, Fraser HM, et al. Endocrinology 113: 1865, 1983.
4. De Lean A, Ferland C, Drouin J, et al. Endocrinology 100: 1496, 1977.
5. Peters JR, Foord SM, Dieguez C, et al. Endocrinol Metab 12: 669, 1983.
6. Belchetz PE, Gredley G, Bird D, et al. J Endocrinol 76: 439, 1977.
7. Leong DA, Frawley LS, and Neill JD. Ann Rev Physiol 45: 109, 1983.
8. Foord SM, Peters JR, Dieguez C, et al. Endocrinology 1985 (in press).
9. Foord SM, Peters JR, Dieguez C, et al. Endocrinology 115: 407, 1984.
10. Valcavi R, Harris P, Foord SM, et al. Clin Endocrinol 1985 (in press).
11. Falardeau P and Di Paolo T. 67th Annual Meeting of the Endocrine Society, Baltimore, USA. Abstract 459, 1985.
12. Peters JR, Foord SM, Dieguez C, et al. Clin Endocrinol 1985 (in press).
13. Motta M. First Meeting of the British Neuroendocrine Group, Oxford, UK, 1985.
14. Nikolics K, Mason A, Stonyl E, et al. Nature 316: 511, 1985.
15. Peters JR, Foord SM, Dieguez C, et al. Endocrinology 113: 133, 1983.

16. Scanlon MF, Peters JR, Foord SM, et al. In EC Griffiths and GW Bennett (eds), Thyrotropin Releasing Hormone, Raven Press, New York, 1983, p 303.
17. Scanlon MF, Peters JR, Salvador J, et al. Clin Endocrinol 1985 (in press).

TSH BIOSYNTHESIS

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The pituitary glycoprotein hormones, thyroid-stimulating hormone (TSH), luteinizing hormone (LH), and follicle-stimulating hormone (PSH), and the placental hormone chorionic gonadotropin (CG) constitute a family of hormones, each of which consists of two dissimilar, non-covalently bound subunits, α and β (1). In a particular species, these hormones share a common α subunit, whereas each β subunit is unique and confers biologic specificity to the complete dimeric hormone. Each subunit is glycosylated, the α and β subunits of TSH having two and one oligosaccharide side chains, respectively, which are N-linked to the protein through asparagine residues (1). Glycosylation is required for subunit combination and protection from intracellular degradation, but not for secretion (2,3). Both subunits are required for biologic activity (1).

In the absence of cultured cells which produce either complete TSH or its free α and β subunits, we and other workers have used mouse TSH-secreting pituitary tumors to study TSH biosynthesis (4-14). These tumors, first described by Jacob Furth (15), arise spontaneously in hypothyroid LAF_1 mice. They are transplantable into the flanks of these mice and, thus, can provide large amounts of tissue for the isolation of TSH protein and mRNA. These tumors do not secrete the related gonadotropins LH and FSH, nor pituitary growth hormone and prolactin (4,15). Moreover, these tumors are benign and show qualitatively normal responses to physiological regulators of TSH production such as thyroid hormones (T_3 and T_4) and TRH (4-16). However, with repeated passage in the animal, production of complete TSH and TSH-β subunit decreases such that, ultimately, only free α subunit is synthesized (16,17). These α-secreting tumors will also grow in euthyroid animals and are no longer hormonally regulated. Unfortunately, it has not proved possible to maintain thyrotropic tumors in culture.

The multi-step pathway by which thyroid-stimulating hormone and the gonadotropins must be synthesized contains many potential points for the regulation of subunit biosynthesis. For example, control may occur at the level of α or β subunit gene transcription, nuclear mRNA precursor processing or stability, cytoplasmic mRNA stability, mRNA translation, protein processing and glycosylation, subunit combination, or secretion from the cell. Despite this complexity, many insights into the mechanism of glycoprotein hormone biosynthesis at the molecular level have now been gained, particularly by the application of recombinant DNA techniques.

The secretion, and in some cases the synthesis, of TSH is regulated by TRH, glucocorticoids, thyroid hormones, somatostatin, and dopaminergic agents. TRH causes the rapid stimulatio of TSH and subunit secretion from the pituitary. Although the exact mechanism of TRH action on TSH secretion is unknown, it has been shown that TRH treatment of thyrotropic tumor cells results in changes in calcium flux, phosphatidylinositol breakdown, and an increase in diacylglycerol levels (18,19). By analogy with similar results from more extensive studies of the action of TRH on prolactin secretion, TRH may also stimulate TSH secretion both via calcium-dependent protein kinases and the activation of protein kinase C by diacylglycerol (20-22). TRH also appears to stimulate the glycosylation of secreted α and TSH-β subunits in the hypothyroid and normal pituitary (23,24). Thyroid hormones inhibit TSH and subunit secretion (4-14), and they also inhibit subunit synthesis (5-13), predominantly at the level of TSH-β gene transcription (11, 12). Glucocorticoids reduce plasma levels of TSH and its α and β subunits, but these effects are not mediated by changes in α and TSH-β mRNA levels (25). Both somatostatin (26) and dopamine (27) inhibit the TRH-induced release of TSH and its subunits from pituitary cells.

Elucidation of the post-translational events involved in α and TSH-β subunit glycosylation, carbohydrate processing, and subunit combination has been primarily undertaken by Weintraub and coworkers employing pulse-chase labeling studies in tumor minces and isolated cells (2,3,14,23,24, 28,29). During the synthesis of glycoproteins, preformed core oligosaccharides ($glucose_3$-$mannose_9$-N-$acetylglucosamine_2$) are transferred <u>en bloc</u> from dolichol intermediates to asparagine residues in the rough endoplasmic reticulum (ER) (30). Complex carbohydrate side chains are then formed by removal of glucose and mannose residues and subsequent addition of residues such as galactose, N-acetylglucosamine, and sialic acid (30). The enzyme endoglycosidase H has been used to characterize the carbohydrate side chains attached to immunoprecipitated α and TSH-β subunits since it will cleave the high-mannose core, but not the complex, oligosaccharide side chains (31,32). This work has shown that the α and TSH-β subunits are co-translationally glycosylated with the addition of a single, endoglycosidase H-sensitive, oligosaccharide moiety in the rough ER. This is followed by the addition of a second high mannose side chain to the α subunit, also in the rough ER. Combination of these core glycosylated subunits begins in the rough ER, but occurs predominantly in the smooth ER and Golgi compartments of the cell. Processing of carbohydrate side chains to their complex forms takes place mainly in these latter compartments. Carbohydrate processing appears not to be a requirement for subunit combination since endoglycosidase H-sensitive and resistant forms of both the α and TSH-β subunits are found in intact TSH (14). However, core glycosylation is required for the α subunit to combine effectively with TSH-β; it also prevents both intracellular aggregation and degradation of the TSH subunits (2,3). Interestingly, it has been found that free TSH-β subunits migrate through the processing pathway at a faster rate than do TSH or the free α subunit and that the rate-limiting steps in oligosaccharide processing of free α subunits differ from those of complete TSH and free TSH-β (28,29). In related work, Strickland and Pierce (31) have reported that core glycosylation is required for correct folding of the α subunit during cell-free biosynthesis and, hence, for effective combination with a β subunit. Additionally, various investigators had shown that the secreted free α subunit had a slightly higher molecular weight and carbohydrate content than α forms combined with β, either intra- or extracellularly (3,32-35). Parsons, Bloomfield, and Pierce (36) then reported that the free α subunit derived from bovine pituitaries is glycosylated at an additional site with O-linked oligosaccharide. This α subunit with a third carbohydrate side chain cannot combine with a β subunit (36).

It has recently been shown that modification of α and β carbohydrate structure may be important in regulation (23,24). Hypothyroidism, for

example, caused a specific stimulation of carbohydrate synthesis of both combined TSH subunits (23). Acute TRH treatment of hypothyroid pituitaries increased the relative glycosylation of both combined α and TSH-β subunits (24). These hormonally induced changes in carbohydrate structure may be of regulatory significance, since TSH glycosylation is a determinant of TSH biological activity and clearance rate (3).

Mouse TSH-secreting tumors characteristically vary widely in their production of TSH. Moreover, they usually synthesize in excess of the α subunit protein relative to TSH-β (4,5,7,10,37), as does the normal pituitary (38). This suggested that the α and TSH-β subunits might be synthesized from separate mRNAs and that α and TSH-β biosynthesis might not be concordantly regulated. We and others have shown independent biosynthesis of α and TSH-β pre-subunits by in vitro translation (39-42). Messenger RNA was extracted from TSH-secreting tumors, poly (A)-enriched and fractionated by sucrose density gradient centrifugation. Individual mRNA fractions were translated in reticulocyte lysate or wheat germ cell-free translation systems. Immunoprecipitation of [^{35}S]-methionine-labeled α and TSH-β protein showed that α and TSH-β synthesis was directed by separate mRNAs and not by a common mRNA precursor (40-42). The mature α and TSH-β subunits were also shown to be formed from pre-subunits which were cleaved of a signal peptide and glycosylated by translation in the presence of microsomal membranes. Our experiments consistently showed excess α to β synthesis in these in vitro systems, using different TSH-secreting pituitary tumor mRNAs (40-42).

We then separated the α and TSH-β mRNAs utilizing sucrose gradient centrifugation and urea/polyacrylamide gel electrophoresis (42). Separate bands eluted from the gel contained mRNA that directed the synthesis of either α or TSH-β subunit, but not both, when translated in a cell-free system. Subsequently, it was shown that α and β subunits of all the glycoprotein hormones are synthesized utilizing separate mRNAs (43-45). α mRNA is modestly larger than TSH-β mRNA, about 850 vs 750 nucleotides in length (46).

To facilitate the study of the structure of TSH genes and the regulation of their expression, we synthesized and cloned DNAs complementary to the mRNAs encoding the presubunits of α and TSH-β (46). The α and TSH-β colonies in our cDNA library were identified by hybridization-selection and cell-free translation. Colonies were first screened in groups of seven and later individually by fixing the plasmid DNA to nitrocellulose filters and hybridizing them with mouse tumor poly(A) mRNA; the specifically hybridized mRNA was eluted and translated in the reticulocyte lysate translation system supplemented with microsomal membranes. The protein product was identified by polyacrylamide gel electrophoresis and immunoprecipitation. The complementary DNAs for mouse (47,48), rat (49), cow (50,51), and human (52) α subunits have not been sequenced, as have complementary DNAs for the mouse (46), rat (53), and cow (54) TSH-β subunits. The deduced amino acid sequences show that each pre-α subunit consists of 96 amino acids plus a 24 amino acid signal sequence, whereas the pre-TSH-β subunits each contain 118 amino acids and a 20 amino acid signal sequence. The nucleotide sequence homology in the coding sequences among the various α subunits is more than 75% in the coding sequences and among the TSH-β subunits is more than 80%.

These cDNA probes have been used to isolate and characterize the genes for several α and TSH-β subunits from genomic libraries. We have isolated gene fragments of mouse α (55), mouse TSH-β (55), and human TSH-β . Other investigators have studied the structure of the bovine (56) and human (57, 58) α genes. The single human and bovine α genes each contain three introns which interrupt the exon sequences in precisely the same locations. That the bovine α gene is 16.5 kb long, whereas the human α gene is 9.4 kb long,

is due solely to the longer 5'-intron of the bovine gene. We have so far determined that there is a sigble mouse α gene which is at least 4 kb long and includes two introns which interrupt the coding region of the mouse α gene in similar positions to the 3'-ward introns in the human α gene. The human and mouse TSH-β genes are currently being characterized in our laboratory. The single mouse TSH-β gene is ∿ 5 kb long with 3 introns; the human TSH-β gene is also single with at least 2 introns, the 3'-ward of which is in exactly the same position as that in the mouse TSH-β gene. The 3'-ward intron of the mouse and human TSH-β genes interrupts these gene sequences in a similar position as in the LH-β and CG-β genes (59-61). However, the TSH-β genes differ in the position and size of their remaining introns from the LH-β and CG-β genes.

We have localized the genes for the mouse α and TSH-β subunits to specific chromosomes using mouse-hamster somatic cell hybrids whose chromosome composition was determined by karotype and isoenzyme analysis (62). Chromosomes were assigned by hybridization of α and TSH-β cDNA probes with Southern blots of chromosomal DNA digested with BamHI. The mouse α gene was found to reside on chromosome 4 and the TSH-β gene on chromosome 3. In addition, mouse LH-β was assigned to mouse chromosome 7 (62,63).

Moreover, we have recently determined the chromosomal location of the gene for human TSH-β (64). A fragment of the human TSH-β gene was used as a hybridization probe to analyze Southern blots of DNA from rodent-human somatic cell hybrids. Analysis of the segregation of the EcoRI fragment containing human TSH-β sequences allowed the assignment of the TSH-β gene to human chromosome 1. A subregional assignment of the gene to 1p22 was achieved using a set of hybrids containing partially overlapping segments of this chromosome. We also have independently confirmed the assignment by Naylor et al. (63) of the α subunit gene to human chromosome 6. In the human, the LH-β/CG-β gene cluster has been assigned to chromosome 19. Thus, it appears that the genes for the subunits of the glycoprotein hormones may all be found on different chromosomes, with the exception of the LH-β/CG-β gene cluster in which CG-β has probably only recently evolved by gene duplication from LH-β, in primates.

Negative feedback by thyroid hormones is the major regulator of pituitary TSH synthesis and secretion, with the effect on secretion being more rapid (3-14). Mouse thyrotropic tumors almost always show an excess of free α subunit over TSH-β, as in fact do the normal mouse and human pituitary (37,38). In hypothyroidism, however, the ratio of free α to TSH-β becomes more balanced (37,38). As further evidence for the independent regulation of α and TSH-β gene expression, the TSH-β content of tumors and of the pituitaries of hypothyroid mice is invariably decreased much more markedly and rapidly than α content, by thyroid hormone treatment. In studying the mechanism of this discordant regulation of α and TSH-β subunit biosynthesis by thyroid hormones, we (10) and others (6,7) have shown unbalanced changes in α and TSH-β mRNA levels. Thus, T_3 treatment (20 μg/100 g body weight) or tumor-bearing mice for either 4 or 10 days reduced the tumor TSH-β protein content to 29% and 10% of control, respectively, with a similar decrease in tumor TSH content. However, there was no significant change in tumor α content at either time point. Hybridization of tumor poly(A) mRNA with labeled α and TSH-β cDNA probes showed that tumor TSH-β mRNA content was reduced to less than 10% of control at both 4 and 10 days, whereas there was no change in α mRNA content at 4 or 10 days. Similar changes were seen when translatable α and TSH-β mRNA levels were assayed in the reticulocyte lysate system (10). Chin et al. (13) have reported similar discordant effects on tumor α and TSH-β mRNAs as early as 1 and 4 hours after T_4 treatment. In addition, we and others have found that thyroid hormones cause a more marked and rapid effect on TSH-β than on α mRNA levels in the pituitaries of non-tumor-bearing hypothyroid mice (6,7,25). In our experiments

on the effects of thyroid hormone on hypothyroid pituitary α and TSH-β mRNA, we used lower doses of T_3 (0.5 to 2.0 μg/100 g body weight) and a shorter treatment time (1, 2, or 3 days); we again noted faster and more potent suppression of TSH-β than α mRNA. We also demonstrated minor stimulatory effects of TRH on α and TSH-β mRNA, but no effect of dexamethasone or bromocriptine (25).

We have also used the "run off" in vitro transcription assay to determine whether thyroid hormones exert discordant effects on α and TSH-β mRNA levels at the level of α and TSH-β gene transcription (11). In this

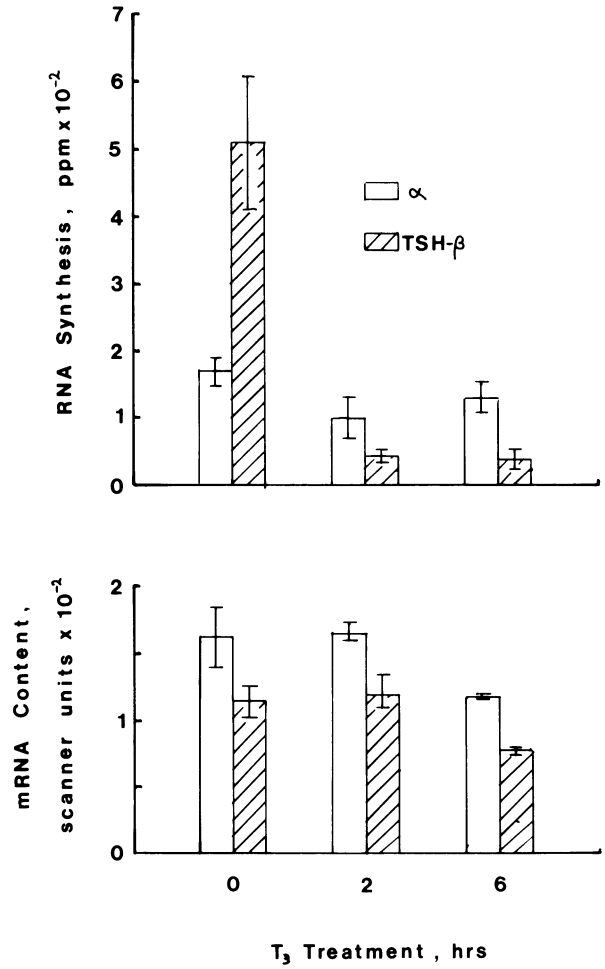

Fig. 1. Effects of T_3 treatment on α and TSH-β gene transcription and mRNA content in tumor IAK 109D. Mice were treated with T_3 (5 μg/100 g body weight) for the indicated times. α and TSH-β RNA synthesis was determined by an in vitro transcription assay. α and TSH-β mRNA content was determined by dot-blot hybridization and scaning densitometry. Each value is the mean ± SD for two tumors.

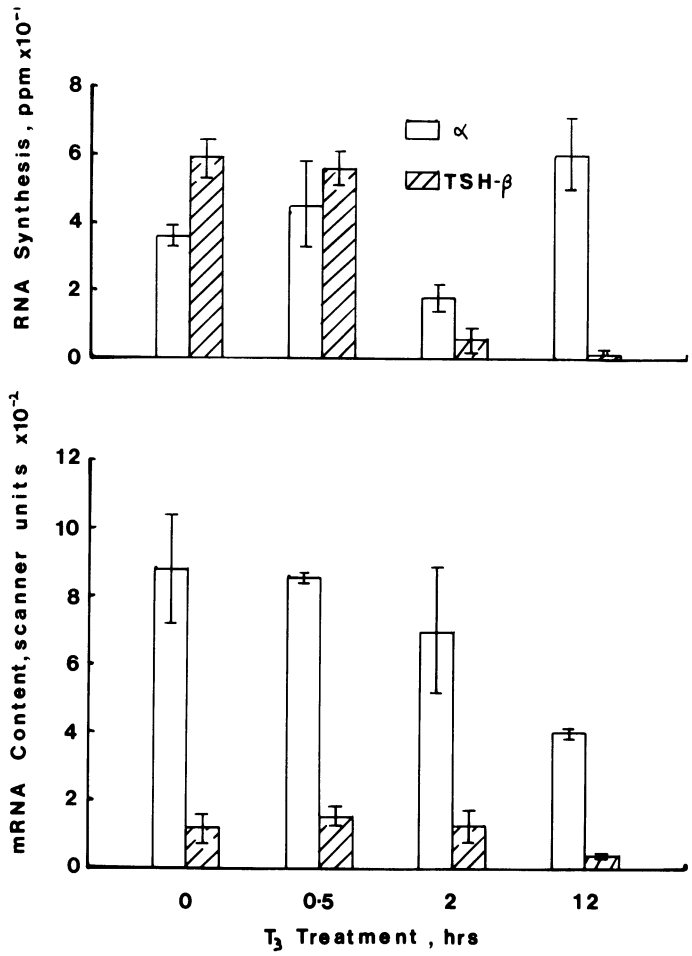

Fig. 2. Effect of T_3 treatment on α and TSH-β gene transcription and mRNA content in tumor 109F. Methods are as described for Fig. 1.

assay, isolated nuclei are allowed to synthesize RNA in vitro in the presence of [αP^{32}]-UTP; the labeled RNA is isolated and then hybridized to filter-bound plasmid DNA probes to measure specific RNA synthesis. There is no re-initiation in vitro, and the assay measures the number of RNA polymerase molecules on the gene at the time of isolation of the nuclei. Using this assay we found that T_3 treatment decreased tumor TSH-β gene transcription to less than 10% of control levels after both 2 and 6 hours. In contrast, α gene transcription was only slightly reduced at 2 hours and had returned to control levels at 6 hours. Although the tumor contained approximately equal amounts of α and TSH-β mRNA, the rate of TSH-β gene transcription was approximately three times greater than the rate of α gene transcription (Fig. 1). Similarly, in a second tumor in which α mRNA content was about sixfold greater than TSH-β mRNA, the rate of TSH-β gene transcription was still 50% greater than the rate of α gene transcription (Fig. 2). These findings suggest that there is a considerable difference in stability between α and TSH-β mRNAs or mRNA precursors. Although we do not yet know whether thyroid hormones influence mRNA stability, these findings suggest that mRNA turnover rates are important in the regulation of α and TSH-β subunit biosynthesis (11). In similar studies, Shupnik et al.

(12) have found a more rapid and marked effect of thyroid hormone on TSH-β gene transcription than on α gene transcription, with a significant decrease in TSH-β gene transcription as early as 30 minutes after T_4 administration. The rapidity of the thyroid hormone effect is consistent with inhibition of gene transcription by direct interaction of the nuclear thyroid hormone receptor complex with control regions of the α and TSH-β genes. Shupnik et al. (12) noted that the transcription rates of α and TSH-β mRNA were equivalent. This finding could be due to the fact that they used different tumors for their studies.

In conclusion, TSH biosynthesis is primarily affected by thyroid hormones; regulation of the subunits is discordant and mainly exerted on TSH-β. Thyroid hormones decrease α and TSH-β mRNA levels by inhibiting α and TSH-β gene transcription. Other levels of regulation, such as RNA stability, glycosylation, and secretion, appear to modulate TSH biosynthesis more subtly.

REFERENCES

1. Pierce JG and Parsons TF. Ann Rev Biochem 50: 465, 1981.
2. Weintraub BD, Stannard BS, Linnekin D, et al. J Biol Chem 255: 5715, 1980.
3. Weintraub BD, Stannard, BS, and Meyers L. Endocrinology 112: 1331, 1983.
4. Blackman MR, Gershengorn MC, and Weintraub BD. Endocrinology 102: 499, 1978.
5. Marshall Jr MC, Williams D, and Weintraub BD. Endocrinology 108: 908, 1980.
6. Ross DS, Downing MF, Chin WW, et al. Endocrinology 112: 2050, 1983.
7. Ross DS, Downing MF, Chin WW, et al. Endocrinology 112: 187, 1983.
8. Cacicedo L, Pohl SL, and Reichlin S. Endocrinology 108: 1012, 1981.
9. Gershengorn MC. Endocrinology 102: 1122, 1978.
10. Gurr JA and Kourides IA. J Biol Chem 258: 10208, 1983.
11. Gurr JA and Kourides IA. DNA (in press), 1985.
12. Shupnik MA, Chin WW, Habener JF, et al. J Biol Chem 260: 2900, 1985.
13. Chin WW, Shupnik MA, Ross DS, et al. Endocrinology 116: 873, 1985.
14. Magner J and Weintraub BD. J Biol Chem 257: 6709, 1982.
15. Furth J, Moy P, Hershman J, et al. Arch Pathol 96: 217, 1973.
16. Ross DS, Kieffer, JD, Shupnik MA, et al. Mol Cell Endocrinol 39: 161, 1985.
17. Ridgway EC, Kieffer JD, Ross DS, et al. Endocrinology 113: 1587, 1985.
18. Gershengorn MC. Mol Cell Biochem 45: 163, 1982.
19. Kolesnick RN, Mussachio I, Thaw C, et al. Endocrinology 114: 671, 1984.
20. Kolesnick RN and Gershengorn MC. J Biol Chem 260: 5217, 1985.
21. Martin TFJ and Kowalchyk JA. Endocrinology 115: 1517, 1984.
22. Martin TFJ and Kowalchyk JA. Endocrinology 115: 1527, 1984.
23. Taylor T and Weinbraub BD. Endocrinology 116: 1968, 1985.
24. Taylor T and Weintraub BD. Endocrinology 116: 1535, 1985.
25. Gurr JA, Wagner CR, Athanasian E, et al. Hormone Metab Res (in press), 1985.
26. Ridgway EC, Klibanski A, Martorana MA, et al. Endocrinology 112: 1937, 1983.
27. Cooper DS, Klibanski A, and Ridgway EC. Clin Endocrinol 18: 265, 1983.
28. Magner JA, Ronin C, and Weintraub BD. Endocrinology 115: 1019, 1984.
29. Ronin C, Stannard BS, Rosenbloom IL, et al. Biochemistry 23: 4503, 1984.
30. Hubbard SC and Ivatt RJ. Annu Rev Biochem 50: 555, 1981.

31. Strickland TW and Pierce JG. J Biol Chem 258: 5927, 1983.
32. Fein HG, Rosen SW, and Weintraub BD. J Clin Endocrinol Metab 50: 1111, 1980.
33. Kourides IA, Hoffman BJ, and Landon MB. J Clin Endocrinol Metab 49: 700, 1979.
34. Ruddon RW, Bryan AH, Hanson CA, et al. J Biol Chem 256: 5189, 1981.
35. Cox GS. Biochem Biophys Res Commun 98: 942, 1981.
36. Parsons TF, Bloomfield GA, and Pierce JG. J Biol Chem 258: 240, 1983.
37. Gurr JA and Kourides IA. Endocrinology 115: 830, 1984.
38. Kourides IA, Landon MB, Hoffman BJ, et al. Clin Endocrinol 12: 407, 1980.
39. Chin WW, Habener JF, Kieffer JD, et al. J Biol Chem 253: 7985, 1978.
40. Kourides IA and Weintraub BD. Proc Natl Acad Sci USA 76: 298, 1979.
41. Kourides IA, Vamvakopoulos NC, and Maniatis GM. Biol Chem 254: 11106, 1979.
42. Vamvakopoulos NC and Kourides IA. Proc Natl Acad Sci USA 76: 3809, 1979.
43. Daniels-McQueen S, McWilliams S, Birken S, et al. J Biol Chem 253: 7109, 1978.
44. Godine JE, Chin WW, and Habener JF. J Biol Chem 255: 8780, 1980.
45. Godine JE, Chin WW, and Habener JF. J Biol Chem 256: 2475, 1981.
46. Gurr JA, Catterall JF, and Kourides IA. Proc Natl Acad Sci USA 80: 2122, 1983.
47. Schorr-Toshav NL, Gurr JA, Catterall JF, et al. Endocrinology 112: 1434, 1983.
48. Chin WW, Kronenberg HM, Dee PC, et al. Proc Natl Acad Sci USA 78: 5329, 1981.
49. Godine JE, Chin WW, and Habener JF. J Biol Chem 257: 8368, 1982.
50. Nilson JH, Thomason AR, Cserbak MT, et al. J Biol Chem 258: 4679, 1983.
51. Erwin CR, Croyle ML, Donelson JE, et al. Biochemistry 22: 4856, 1983.
52. Fiddes JC and Goodman HM. Nature 281: 351, 1979.
53. Croyle ML and Maurer RA. DNA 3: 231, 1984.
54. Maurer RA, Croyle ML, and Donelson JE. J Biol Chem 259: 5024, 1984.
55. Kourides IA, Gurr JA, and Wolf O. Rec Prog Horm Res 40: 79, 1984.
56. Goodwin RG, Moneman CL, Rottman FM, et al. Nucleic Acids Res 11: 6873, 1983.
57. Fiddes JC and Goodman HM. J Mol Appl Genetics 1: 3, 1981.
58. Boothby M, Ruddon RW, Anderson C, et al. J Biol Chem 256: 5121, 1981.
59. Talmadge K, Vamvakopoulos NC, and Fiddes JC. Nature 307: 37, 1984.
60. Jameson L, Chin WW, Hollenberg AN, et al. J Biol Chem 259: 15474, 1984.
61. Virgin JB, Silver BJ, Thomasen AR, et al. J Biol Chem 260: 7072, 1985.
62. Kourides IA, Barker PE, Gurr JA, et al. Proc Natl Acad Sci USA 81: 517, 1984.
63. Naylor SL, Chin WW, Goodman HM, et al. Somatic Cell Genet 9: 757, 1983.
64. Dracopoli NC, Rettig WJ, Old LJ, et al. Science (submitted).

ROLE OF THE PITUITARY IODOTHYRONINE 5'DEIODINASE ACTIVITY IN THE NEGATIVE FEEDBACK BY THYROID HORMONE UPON TSH RELEASE

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It is widely accepted that triiodothyronine (T_3) accounts for nearly all of the thyromimetic potency of thyroidal secretion. The nuclear thyroid hormone receptors bind T_3 with > 10-fold higher affinity than thyroxine (T_4), and the intracellular concentration of T_4 is not enough to occupy more than 10% of these receptors--a substantial fraction of which may actually be non-specifically bound. About a decade ago, this and the relatively rapid exchange of plasma T_3 with the receptor, led to the concept that the thyroid status of the tissues was closely reflected by the plasma concentration of T_3 (1).

There was, however, one conspicuous exception. In a number of conditions, experimental as well as clinical, TSH was elevated in spite of normal plasma T_3 levels. For example, in experimental iodine deficiency, serum TSH rises promptly after placing the animals on the iodine-deficient diet, yet the plasma T_3 remains normal (2), unless the deficiency is extreme and prolonged. Patients in early stages of thyroid gland insufficiency exhibit elevated serum TSH levels with T_3 well within normal limits. In both patients and iodine-deficient rats, tissue hypothyroidism is barely evident or simply inapparent, consistent with the concept that the availability of T_3 for most tissues depended largely on plasma T_3 (3). Only the pituitary gland is evidently hypothyroid, as reflected by the elevation in TSH (2). The reduced plasma T_4 in these conditions appeared as the driving force in the elevation of TSH.

These findings could be explained in a number of ways: 1) the suppression of TSH could be mediated by mechanisms different from the other effects of thyroid hormone, i.e., other than via the nuclear receptors, with T_4 being as active as, or more active than, T_3; 2) there could be previously unrecognized nuclear T_4 receptors in the pituitary; and 3) there could be intrapituitary T_3 generation and plasma T_4 could be a rate-limiting factor. Two pieces of evidence favored either the first or the second explanation. One was the failure to demonstrate T_3 generation in pituitary homogenates by Galton (4) and, the other, the observation by Larsen and Frumess that propylthiouracil (PTU) did not prevent the acute T_4-mediated inhibition of TSH release (5).

The first experiments performed to explore this seemingly unique effect of T_4 on the thyrotrophs addressed the question as to whether or not the T_3-induced TSH release inhibition was mediated by the nuclear receptors. The

results of these experiments showed that the acute inhibition of TSH release after a single injection of T_3 to hypothyroid rats correlates chronologically and quantitatively with the T_3 specifically bound to the nuclear receptors (6), and that the inhibition after various doses of T_3 is linearly related to nuclear occupancy (7). Along with the earlier observations that the inhibition of protein synthesis by cycloheximide or actinomycin D prevented the suppressive effects of T_3 upon TSH, these results suggested that this effect of T_3 was not different from other effects of the hormone, but the T_4 inhibition of TSH release could still be mediated by other mechanisms.

Although cytoplasmic pituitary proteins can bind T_3 and T_4 in a saturable fashion (8), because of their low affinity for T_4, these binding proteins do not appear to be receptors; so, other than the weak binding of T_4 to the nuclear T_3 receptors, no binding sites qualifying as receptors for T_4 appeared to be present in the pituitary. When we examined the nuclei of the pituitary cells for T_4 binding after injecting tracer amounts of radioactive T_4 into euthyroid rats, we only found radioactive T_3 bound to these nuclei (9). Most importantly, the radioactively labeled T_3 found in the nuclei was specifically bound to the nuclear receptors and could not be accounted for by the minute amounts of radioactive plasma T_3 present under those experimental conditions (6,7). In those and subsequent experiments, the nuclear to plasma ratio of ^{125}I-T_3 [T_4] shortly after the injection of ^{125}I-T_4 and ^{131}I-T_3 exceeded the nuclear to plasma ratio of ^{131}I-T_3 [T_3] by a factor of 2-3, indicating rapid and active intrapituitary T_4 to T_3 conversion (6,7,9). When TSH suppression after T_4 was examined in hypothyroid rats, the time course and the extent of the suppression could be closely related to the T_3 [T_4] found specifically bound to the nuclear receptors (6,7). Only in the pituitary gland, but not in liver or kidney, did the simultaneous administration of T_3 and T_4 result in significantly larger amounts of T_3 than after the same doses of T_3 alone (7), and the simultaneous administration of submaximal doses of T_4 and T_3 resulted in additive TSH suppression without higher levels of plasma T_3 than after the T_3 alone (7). Radioisotopic kinetic analyses with pulse injections of ^{125}I-T_4 and ^{131}I-T_3 (9), and subsequently by constant infusion by van Doorn et al. (10) or by isotopic equilibrium by Obregon et al. (11), indicate that in euthyroid rats the T_3 nuclear receptors are about 80% saturated, and that about half of this T_3 derives from local (intrapituitary) production. That at physiological doses of T_4 all, or nearly all, of the suppressive effect of T_4 on TSH was due to the T_3 generated in the pituitary was demonstrated by the observation that iopanoic acid, a competitive inhibitor of T_4 to T_3 conversion, prevented the effect of T_4 on TSH, but not that of T_3 (12). Although basically indirect, all of this evidence suggested that the apparently unique effect of T_4 on TSH release was due to intrapituitary T_3 generation with subsequent binding of the T_3 [T_4] to the nuclear receptors, and to the fact that the latter constituted about 50% of the nuclear T_3.

Thus, pituitary 5'deiodination of T_4 seems to play a crucial role in determining the physiological characteristics of the feedback of thyroid hormones on TSH secretion. I will now discuss briefly some characteristics of the pituitary 5'deiodinase activity. Before the experiments described above, Larsen and Frumess had found that PTU did not prevent the acute inhibition of TSH by a single replacement dose of T_4, and that in thyroidectomized rats maintained on T_4, the concomitant administration of PTU reduced plasma T_3 levels by 60-70%, prevented the normalization of the thyroid hormone-dependent hepatic alpha-glycerophosphate dehydrogenase, but barely affected the suppression of TSH by the replacement with T_4 (5). In light of the experiments described above, these results strongly suggest that pituitary T_4 5'deiodination is PTU-sensitive. In vivo studies demonstrated that the quantity of T_3 specifically bound to the pituitary nuclei after T_4 was not affected by pretreatment with PTU (7). Experiments with pituitary

fragments also showed that nuclear ^{125}I-T_3 derived from labeled T_4 placed in the incubation medium was resistant to PTU inhibition and, at variance with liver-catalyzed T_4 to T_3 conversion, this reaction in the pituitary fragments was not affected by fasting (13). Furthermore, all of the locally generated ^{125}I-T_3 [T_4] after *in vivo* injection of ^{125}I-T_4 was PTU-insensitive while, in the same animals, PTU decreased serum and liver ^{125}I-T_3 [T_4] (14). Studies with pituitary homogenates demonstrated that T_4 5'deiodination in the pituitary was highly dependent on thiol reducing agents like dithiothreitol, and also confirmed that T_4 to T_3 conversion was not affected by as much as 1 mM PTU (15). Similar observations in central nervous tissue, both *in vivo* and *in vitro* (14), and the partial inhibition of reverse T_3 (rT_3) 5'deiodination by PTU, led to the conclusion that in these tissues there were two separate enzymatic pathways that could catalyze rT_3 5'deiodination (16). One of these enzymes was indistinguishable from the well-characterized 5'deiodinase in liver and kidney (termed by our group Type I 5'deiodinase or 5'D-I), and the other was different in a number of features: preferred T_4 over T_3 as substrate, had a markedly lower Km for T_4 and T_3 than the renal or hepatic enzymes, exhibited sequential type of kinetics, was insensitive to PTU, and responded markedly, rapidly, and in opposite direction to hypo and hyperthyroidism when compared with 5'D-I (17). This is the Type II 5'deiodinase, 5'D-II. It is the pituitary 5'D-II that accounts for all the T_3 locally generated *in vivo* in this gland, and for all the other results described above. This enzyme is a key element in determining the pituitary responsiveness to feedback by T_4.

Before analyzing the physiological implications of the peculiarities of the interactions of the pituitary with T_4 and T_3, I would like to examine some of the evidence indicating that a similar mechanism operates also in humans. For obvious reasons, this evidence is largely indirect and is summarized in Table 1.

Whereas the observation of hypothyroxinemia with normal T_3 and elevated TSH is familiar to all clinicians, the other evidences deserve some comment. Moderate iodine deficiency prevents the normal elevation in total serum T_4 seen in pregnancy, resulting in a reduction of serum free T_4 concentration. This situation is corrected by the administration of 300 µg of iodine daily which is accompanied by a normalization of free T_4 concentration, a modest but highly significant reduction in TSH, but no change in total or free serum T_3 concentration. These findings suggest that the modest increase in TSH maintains the level of T_3 in the face of reduced T_4, and that the latter drives the elevation of TSH. When the iodine supply is

Table 1. Evidence Suggesting that Plasma T_4, Probably Through T_4 to T_3 Conversion, is at Least as Important as Plasma T_3 in the Feedback on TSH Secretion in Humans

1)	Elevation of serum TSH in hypothyroxinemia with normal serum T_3 in a number of conditions such as iodine deficiency, failing thyroid syndrome, etc.
2)	Pharmacological blockade of T_4 to T_3 conversion is followed by TSH elevation unaccounted for by reduced serum T_3 (18,19,20).
3)	Cases of TSH-induced hyperthyroidism where TSH can be suppressed with exogenous T_3 but not T_4 (21).
4)	Some cases of nonthyroidal illness where the normal serum TSH can be, in part, explained by elevated levels of free T_4.

normalized, the secretion of TSH decreases and with it that of T_3, but the latter remains normal since the precursor molecule, T_4, is now normally available (22).

Studies with iodinated contrast dyes (18) and amiodarone (19), all substances that inhibit T_4 to T_3 conversion, show that these agents elicit a fall in serum T_3 levels and an elevation in serum T_4 and TSH. Although in these early studies no correction of serum T_3 was intended and part of the TSH elevation may be accounted for by the fall in serum T_3, the observation is consistent with the concept that T_4 to T_3 conversion is necessary for the feedback. If T_4 had significant intrinsic effect, its elevation would be expected to suppress TSH and the system would reequilibrate with only a minimal elevation of T_4. In more recent studies where exogenous T_3 was administered to maintain the plasma T_3, iopanoic acid increased the response to TRH (20). In unpublished studies, Silva and Michaud observed that in cirrhotic patients given large doses of iodine, a fall in the elevated free T_4 of 1 ng/dl (without return to the normal range), induced a significant increase in basal and TRH-stimulated TSH. In these protocols, the iodine was administered along with T_3 to prevent a drop in plasma T_3 resulting from the reduced secretion. The administration of iopanoic acid, along with small and repeated doses of T_3 to prevent the reduction in plasma T_3, resulted in in elevation of TSH but, in this case, free T_4 was elevated, not reduced.

A family described by Rosler et al. (21) presented with hyperthyroidism and elevated TSH. In these cases, the administration of T_3, but not of T_4, could suppress TSH; a single daily dose of 25 or 50 μg of T_3 could cause a suppression of TSH sufficient to maintain the patients euthyroid. Although in these, as in other cases described subsequently (23), there are other possible explanations, an isolated defect of pituitary 5'D-II is a clear and reasonable explanation.

I will now discuss briefly some physiological implications of the presence of 5'D in the pituitary. Since T_3 is several times more potent than T_4 and most tissues in the body can exchange T_3 rapidly with plasma, all physiological responses leading to the maintenance of plasma T_3 levels will prevent hypothyroidism when the thyroid gland fails. The fact that plasma T_4, due to the pituitary 5'D-II and other mechanisms to be discussed next, has such an important impact on TSH secretion is of tremendous adaptive advantage. Because of these mechanisms, the body can increase markedly the secretion of TSH without hypothyroidism of vital tissues such as heart, liver, or kidney. If plasma T_3 was the main or the only signal feeding back on TSH, a sufficient stimulation of the thyroid to increase the T_3 secretion to a level that compensates for the drop in extrathyroidal T_3 generation would not be possible. However, it is important to emphasize that pituitary 5'D-II is a necessary, but not a sufficient, factor for the dual feedback. Liver and kidney produce T_3 from T_4 at a much faster rate than the pituitary, but in these tissues, T_3 does not remain in the cell, it is exported. The reasons for the pituitary to retain a significant amount of the T_3 produced locally are not clear. To date, we have found that all tissues with 5'D-II produce a significant fraction of their T_3 content, but we have no other evidence of a cause-effect relationship between the presence of this enzyme and a large fraction of locally produced T_3. Studies in the central nervous system suggest that the rate of exchange of T_3 between the tissue and plasma may play an important role. In liver and kidney, the exchange of T_3 with plasma is so fast (10-20 min to reach equilibrium), that before T_3 produced *in situ* can distribute within the cell, it is "diluted" by the T_3 entering from plasma (24). Thus, either the kinetics of exchange of T_3 between the pituitary and plasma, or another factor such as some peculiarity of the subcellular distribution of 5'D-II, accounts for the fact that approximately 50% of the T_3 is locally generated.

The elucidation of this question awaits further investigation. It is interesting that in the tissues where local generation of T_3 [T_4] is a major input for the tissue T_3, the degree of nuclear receptor saturation is higher than in tissues "importer" of plasma T_3. In the pituitary gland, the receptors are approximately 80% saturated, which accounts for the low fraction of the TSH secretory capacity that is expressed in the euthyroid animal.

Another unresolved question derives from the presence of 5'D-II in the pituitary. As mentioned earlier, this enzyme increases rapidly and markedly in the central nervous system and the pituitary following thyroidectomy (26). Furthermore, plasma T_4 seems to be the most important physiological signal for the modulation of this enzyme (26). At first glance, it does not make teleological sense that thyrotrophs contain this enzyme, for the increase of its activity in hypothyroxinemia would hamper the reduction in intrapituitary T_3 that should ensue and, hence, would reduce the TSH response. Even though we do not have a definite answer, the responses of the thyrotroph 5'D-II might not be faithfully reflected by the overall responses of the whole pituitary, which is what we have examined in response to manipulations of the thyroid status. In the euthyroid rat anterior pituitary (and probably in other species as well), only a minor fraction of the cells (<10%) are thyrotrophs, becoming, at most, 40% of the cell population in chronically hypothyroid rats. The increase in 5'D-II seen following thyroidectomy might well not represent what occurs in the thyrotroph cells. The most solid piece of evidence in favor of this idea has been obtained by Koenig et al. (27). These authors have found that dispersed pituitary cells enriched in thyrotrophs respond significantly less to the removal of thyroid hormone from the medium than fractions of cells enriched in somatotrophs or lactotrophs. Moreover, a substantial part of the 5'D-II thyroid hormone dependency can be accounted for by the contamination with the other cells containing the enzyme. Secondly, even observing the overall pituitary 5'D-II response to hypothyroidism in rats given graded doses of methimazole, one sees that TSH is elevated to over 50% of the maximal response with minor reductions of T_4, whereas it takes >50% reduction in serum T_4 levels to see a significant elevation in 5'D-II (26). When one gives T_4 to chronic hypothyroid rats, 5'D-II in the pituitary requires more T_4 to be suppressed than the cerebral cortex (26). Thus, the overall sensitivity of the pituitary 5'D-II to T_4 may be less than that of the central nervous system, and that of the thyrotrophs 5'D-II even less than that of the other anterior pituitary cells.

REFERENCES

1. Oppenheimer JH, Schwartz HL, Surks MI, et al. Rec Prog Horm Res 32: 529, 1976.
2. Fukuda H, Yasuda N, Greer MA, et al. Endocrinology 97: 307, 1975.
3. Silva JE. Endocrinology 91: 1430, 1972.
4. Galton VA. In J Robbins and LE Braverman (eds), Thyroid Research, American Elsevier Publishing Co., New York, 1976, p 251.
5. Larsen PR and Frumess RD. Endocrinology 100: 980, 1977.
6. Silva JE and Larsen PR. Science 198: 617, 1977.
7. Silva JE and Larsen PR. J Clin Invest 61: 1247, 1978.
8. Sufi SB, Toccafondi RS, Malan PG, et al. J Endocrinol 58: 41, 1973.
9. Silva JE, Dick TE, and Larsen PR. Endocrinology 103: 1196, 1978.
10. van Doorn J, van der Heide D, and Roelfsma F. J Clin Invest 72: 1778, 1983.
11. Obregon MJ, Roelfsma F, Morreale de Escobar G, et al. Clin Endocrinol 10: 305, 1979.
12. Larsen PR, Dick TE, Markovitz MM, et al. J Clin Invest 64: 117, 1979.
13. Cheron RG, Kaplan MM, and Larsen PR. J Clin Invest 64: 1402, 1979.

14. Silva JE, Leonard JL, Crantz FR, et al. J Clin Invest 69: 1176, 1982.
15. Kaplan MM. Endocrinology 106: 567, 1980.
16. Visser TJ, Leonard JL, Kaplan MM, et al. Biochem Biophys Res Comm 101: 1297, 1981.
17. Visser TJ, Leonard JL, Kaplan MM, et al. Proc Nat Acad Sci 79: 5080, 1982.
18. Burgi H, Wimpfheimer C, Burger A, et al. J Clin Endocrinol Metab 43: 1203, 1976.
19. Burger A, Dinichert D, Nicod P, et al. J Clin Invest 58: 255, 1976.
20. Kleinmann RE, Vagenakis AG, and Braverman LE. J Clin Endocrinol Metab 51: 399, 1980.
21. Rosler A, Litvin Y, Hage C, et al. J Clin Endocrinol Metab 54: 76, 1982.
22. Silva JE and Silva S. J Clin Endocrinol Metab 52: 671, 1981.
23. Orgiazzi et al. 14th Annual Meeting of the European Thyroid Association, Rotterdam, 1984.
24. Silva JE and Matthews PS. J Clin Invest 74: 1035, 1984.
25. Leonard JL, Kaplan MM, Visser TJ, et al. Science 214: 571, 1981.
26. Silva JE and Leonard JL. Endocrinology 116: 2394, 1985.
27. Koenig RJ, Leonard JL, Senator D, et al. Endocrinology 115: 324, 1984.

TSH-SECRETING PITUITARY TUMORS

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TSH-secreting pituitary tumors represent one major group of the syndrome of inappropriate secretion of TSH (1). This syndrome is defined as inappropriately measurable or elevated concentrations of TSH in the presence of increased total and free serum thyroid hormones, and is comprised of both neoplastic and non-neoplastic variants. Neoplastic TSH production by a pituitary tumor may occur in the setting of either a microadenoma or macroadenoma and may be associated with the production of other hormones, particularly growth hormone and prolactin. Because of increased awareness of physicians, these tumors are being recognized with increasing frequency. Currently, over 40 cases have been reported in the literature, and we have seen or are aware of several additional unreported cases.

In diagnosing a TSH-secreting pituitary tumor, the physician must be aware of the fact that apparently elevated TSH levels in sera may result from anti-bovine TSH antibodies, anti-rabbit immunoglobulin antibodies and other substances that interfere with certain TSH radioimmunoassays. Anti-iodothyronine antibodies, elevated thyroxine-binding globulin, and abnormal albumin or prealbumin may also cause spurious elevations of total thyroid hormone levels, and, depending on the method used, false elevation of free thyroid hormone levels. Several other common thyroid problems must also be differentiated from the syndrome of inappropriate TSH secretion. Moreover, the non-neoplastic variants of this syndrome must be considered and excluded.

The non-neoplastic disorders are variants of thyroid hormone resistance which refers to sporadic or familial syndromes in which patients demonstrate variable degrees of refractoriness to the action of endogenous or exogenous thyroid hormones. Refetoff et al. (2,3) initially reported a family in which three of six children demonstrated deaf-mutism, delayed bone maturation and stippled epiphyses, goiter, elevated total and free serum T_4 and T_3, and elevated ^{131}I uptake. Despite elevated thyroid hormone levels, these patients displayed no clinical features of hyperthyroidism and also failed to respond to large exogenous doses of T_4 or T_3. These patients also demonstrated pituitary resistance to elevated serum free T_4

and T_3. Since this initial description, a number of other cases of generalized resistance to the action of thyroid hormone have been reported (1). Most of the cases have been familial but sporadic cases have also been described. In the familial cases, both autosomal recessive and autosomal dominant patterns of inheritance have been suggested, with variable expression of the disease in affected members of the same family.

It was subsequently recognized that certain patients appeared to have selective pituitary resistance to the action of thyroid hormone. Gershengorn and Weintraub (4) initially described a patient with elevated free T_4 and T_3 and inappropriately elevated TSH in the basal state and a marked TSH response to TRH. However, unlike patients with generalized resistance to thyroid hormone, this patient displayed clear clinical and biochemical features of peripheral hyperthyroidism. Moreover, exogenous T_3 caused only a partial suppression of TSH and increased peripheral hyperthyroidism. Many additional sporadic cases of selective pituitary resistance have been reported, as well as one familial description of six affected females in three generations (1). In addition, a single case of selective peripheral but not pituitary resistance to the action of thyroid hormone was reported by Kaplan et al. (5).

The distinction between neoplastic and non-neoplastic disease has significant therapeutic implications, and is determined by a constellation of clinical, biochemical, and radiologic findings. These features are likely to be modified if the patient has received thyroid ablative therapy, such as surgery, radioactive iodine, or antithyroid drugs, prior to establishing the diagnosis of inappropriate TSH secretion. In our experience, the most useful discriminator between neoplastic and non-neoplastic production of TSH is measurement of the plasma free alpha subunit (6). Normal TSH production results in equimolar serum concentrations of alpha and TSH, and an α/TSH ratio greater than one suggests neoplastic TSH secretion. Determination of the alpha subunit is not generally available, but can be obtained from several research facilities, including our laboratory at the National Institutes of Health (NIH). Radiologic evaluation usually includes a sellar computerized axial tomographic (CT) scan, but results should be interpreted carefully with respect to the clinical setting, since pituitary enlargement may occur with primary hypothyroidism, and incidental nonfunctioning pituitary adenomas or prolactinomas may be seen. The CT scan can clarify anatomic characteristics of a tumor, and the location and extent of the tumor are determinants in the choice of a surgical technique. In selected cases, further study with newer techniques may be useful. These include nuclear magnetic resonance scanning (NMR) for anatomic structure, positron emission tomography (PET) scan for functional study, and bilateral venous petrosal sinus sampling for hormonal measurement during TRH administration.

After the diagnosis of a TSH-secreting pituitary tumor has been made, treatment is usually directed toward surgical and/or radiation therapy, as pharmacologic therapy has not generally proven beneficial (7). Unfortunately there are no large, controlled clinical trials to compare various treatment modalities. Therapeutic decisions should take into consideration the age and general health of the patient, the location and apparent growth rate of the tumor, whether the tumor is previously untreated or the nature and timing of prior treatment, and the complications of the tumor, such as mass effects and altered endocrinologic function. Some of these complications are associated with significant morbidity and decreased life expectancy if left untreated. The goal of therapy is to ablate neoplastic tissue and to restore euthyroidism and normal TSH levels, while preserving pituitary function. Previous experience indicates that most TSH-secreting adenomas are invasive and are associated with significant mass effects. In our opinion, early and aggressive treatment is indicated to avoid the complications of tumor growth and endocrinopathies.

We favor the use of surgical intervention for definitive treatment when a pituitary adenoma is confirmed and preoperative assessment of other pituitary function is complete. Therapy should be undertaken immediately if there is any sign of progressive visual compromise or neurologic deterioration. The preferred surgical approach is transsphenoidal microsurgery for tumors that are intrasellar or have mainly upward extension. Transfrontal craniotomy may be necessary if there is growth outside the sella, in parasellar or retrosellar regions, or if there is extensive infiltration of brain or vascular structures by tumor. Successful resection is accompanied by rapid fall in serum TSH and, subsequently, total and free thyroid hormone levels, as well as normalization of the tumor marker, alpha subunit. There should also be improvement of other biochemical abnormalities, if present, such as elevated growth hormone or prolactin.

The complications of surgery develop most often in patients with large, locally invasive adenomas and include instances of surgical death. Postoperative complications include intracranial hemorrhage such as subarachnoid bleeding or organized hematoma; CSF rhinorrhea or meningitis; carotid artery damage resulting in bleeding, thrombosis, embolism, or cavernous sinus fistula; optic nerve damage, directly or secondary to vascular anatomic changes; nasal or sinus infection and damage; and endocrinologic dysfunction. Patients are observed postoperatively for neurologic deterioration which may require further surgery, for seizures which may require medication, and for pituitary dysfunction which may necessitate temporary or permanent treatment of hypothyroidism, hypocortisolism, hypogonadism, and diabetes insipidus. Despite the risks of surgery, one must consider the potential of developing many of the same complications related to progressive tumor growth and invasion of critical tissue if the tumor is left untreated.

Previous reports in the literature of surgical outcome dealt with macroadenomas, and in general there was residual tumor resulting in recurrent endocrine or mass effects. Therefore, careful follow-up evaluations of clinical and biochemical parameters are necessary, especially of the alpha subunit, which may be the sole indicator of recurrence. Because complete resection of these tumors is not often achieved, adjunctive radiation or pharmacologic therapy with ^{131}I, antithyroid drugs or dopamine agonists may be necessary.

Radiation therapy of pituitary tumors in general can result in improvement of mass effects of the tumor and/or decreased secretory function. It is generally preferable to surgically debulk large tumors before administering radiation therapy, depending on the ability of the patient to tolerate surgery and on a realistic appraisal of the risks and benefits of surgery, especially for large or invasive tumors.

In the case of TSH-secreting tumors, the radiosensitivity of tumor tissue has not been established, and thus far radiation has been used only as an adjunct to surgery. Radiotherapy is associated with relatively low morbidity, but a major disadvantage is the slow response. Furthermore, hyperthyroidism may persist from the continued secretion of TSH, even if some decrease in the serum level is achieved by treatment.

Conventional supervoltage radiation is the method employed at NIH and is generally available. Use of this technique requires adequate radiologic localization, so that the field size and beam direction can be established by stimulation to maximize the dose delivered to the tumor. Reports of therapy for pituitary tumors generally suggest that a dose less than 4000 rads is associated with higher recurrence rates and greater than 5000 rads with a higher incidence of complications. We recommend a tumor dose of 4500 rads fractionated at 200 rads per day. Additional treatment resulting in a

Table 1. TSH-secreting Pituitary Tumors: Biochemical Profile

Patient Age/Sex	Therapy	Date	TSH, Basal (μU/ml)	TSH, Post TRH (μU/ml)	α Subunit (ng/ml)	T_4 (μg/dl)	T_3 (ng/dl)	Diurnal TSH (μU/ml)
1 52M	1978 ^{131}I	9/82	47	65	20	10.4	182	23-57
	1982 Pituitary surgery and radiation							
	1983 Pituitary surgery	9/84	15	17	3.8	6.8	119	5.7-19.2
2 43F	1956 Pituitary surgery and radiation	8/83	9	21	3.5	13	167	7.4-11.7
	9/83 T_3 suppression	9/83	5	13	2.9	10	270	
	10/83 Pituitary surgery							
3 25F	1982 ^{131}I	10/83	45	88	17	16	350	45-69
	4/84 Pituitary surgery	5/85	4	16	1.0	9.1	142	2.7-3.2
4 35F	1976 ^{131}I	1/84	111	203	15	9.4	119	58-109
	2/84 Pituitary surgery	7/84	1.3		2.6	10.7	127	
5 31F	1978 Thyroidectomy	9/84	568	3,960	43.3	13.2	157	340-780
	1979 ^{131}I							
	9/84 Pituitary surgery	10/84	1.7	2.3	0.7	6.4	75	1.4-1.6
6 36F	1980 and 1984 Pituitary surgery, PTU	11/84	30	142	150	6.2	187	24-37
	2/85 Pituitary surgery and radiation	3/85	5.2	7.5	85	13.1	200	

Table 2. TSH-secreting Pituitary Tumors: TSH Dynamic Responses

Patient Age/sex	TRH, Acute 500 mcg IV	Dopamine agonists	Thyroid hormone	Cortico-steroids	Antithyroid drugs
1 52M	0	Minimal ↓	Minimal ↓	↓	NT
2 43F	↑	NT	Minimal ↓	NT	NT
3 25F	↑	Minimal ↑	0	NT	↑
4 35F	↑	NT	0	NT	NT
5 31F	↑	Minimal ↓	0	NT	↑
6 36F	↑	0	0	0	↑

NT = Not Tested

higher total dose might be used for recurrent tumor which had previously been treated with radiation. The dose and timing of prior radiation would need to be taken into consideration.

Immediate adverse effects such as hair loss and erythema are minimal with modern equipment, which delivers a small dose to skin and subcutaneous tissue. Late complications that have been reported with pituitary tumors occurred in the setting of the use of older equipment and less accurate localization, administration of larger doses, and less fractionation of dose. Such complications of radiation have included hypopituitarism, optic nerve damage, brain necrosis, and induction of cerebral neoplasms.

We have recently evaluated and treated six previously unreported patients with TSH-secreting pituitary tumors at the U. S. National Institutes of Health. The biochemical profile, TSH dynamic responses, and therapeutic outcome of these cases are described in Tables 1-3. In only one of these patients (No. 3) was the tumor discovered at an early microadenoma stage (0.8 cm). As is usually observed, the other patients had large tumors with extensive suprasellar extension or bony invasion. Two of these patients had associated hypersecretion of growth hormone (No. 2 and 6), two had hyperprolactinemia (No. 4 and 5), and one hypersecretion of follicle-stimulating hormone (No. 6). Typically, all of the patients had been initially misdiagnosed, and most were subjected to inappropriate treatment with ^{131}I ablation of the thyroid or antithyroid drug therapy, leading to prolonged periods of clinical or chemical hypothyroidism. We believe that in patients who have not received prior pituitary surgical or radiation therapy, such periods of hypothyroidism can lead to an aggressive transformation of the tumor, as observed following adrenalectomy for Cushing's disease in Nelson's syndrome.

It is very clear from our experience that adequate therapy of TSH-secreting pituitary tumors depends on early diagnosis. Hopefully, increased awareness of physicians, as well as the availability of TSH immunoassays with improved sensitivity and specificity, should permit earlier recognition of these tumors. Increased recognition and study of these patients are not only important clinically, but should provide fundamental insights into the regulation of TSH biosynthesis, secretion and action.

Table 3. TSH-secreting Pituitary Tumors: Therapeutic Outcome

Patient Age/Sex		Procedure *Clinical Center, NIH	Benefits	Complications
1 52M	*1982	Transsphenoidal surgery Radiation therapy	Debulking tumor Visual field improvement Biochemical improvement	-
	*1983	Transfrontal surgery	Debulking tumor Visual field stabilization	Transient epidural hematoma and seizure disorder Mild memory impairment
2 43F	1956	Transfrontal surgery Radiation therapy	Removal tumor Visual field normalization	-
	*1983	Transfrontal surgery	-	Postoperative coma, death
3 25F	*1984	Transsphenoidal surgery	Removal tumor Biochemical normalization	Transient CSF rhinorrhea
4 35F	1984	Transsphenoidal surgery	Removal tumor Biochemical normalization	Transient diabetes insipidus
5 31F	*1984	Transsphenoidal surgery	Removal tumor Visual field normalization Biochemical normalization	-
6 36F	1980	Transsphenoidal surgery	Removal prolactinoma	-
	1984	Transsphenoidal surgery	Debulking tumor	-
	*Feb 1985	Transfrontal surgery	Biochemical improvement	
	*May 1985	Radiation therapy	Debulking tumor	- CSF rhinorrhea

REFERENCES

1. Weintraub BD, Gershengorn MC, Kourides IA, et al. Ann Intern Med 95: 339, 1981.
2. Refetoff S, DeWind LT, and DeGroot LJ. J Clin Endocrinol Metab 27: 279, 1967.
3. Refetoff S. Am J Physiol 243: E88, 1982.
4. Gershengorn MC and Weintraub BD. J Clin Invest 56: 633, 1975.
5. Kaplan MM, Swartz SL, and Larsen PR. Am J Med 70: 1115, 1981.
6. Kourides IA, Ridgway EC, Weintraub BD, et al. J Clin Endocrinol Metab 45: 534, 1977.
7. Petrick PA and Weintraub BD. In DT Krieger and CW Bardin (eds), Current Therapy in Endocrinology and Metabolism, B. C. Decker, Inc., Toronto and Philadelphia, 1985, p 41.

EMERGING CONCEPTS IN INHERITED DISORDERS OF THYROID METABOLISM

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Congenital disorders of thyroid metabolism may result from complete or partial blockage at any biochemical step in hormone synthesis, storage, secretion, delivery, or utilization. Our understanding of inborn errors of thyroid awaited the development of sophisticated biochemical investigative tests pioneered by Stanbury just over 35 years ago (1). Since then, there has been an enormous growth of our knowledge of these disorders. The detailed molecular mechanism in each defect has been only partially elucidated but progress in this field has greatly assisted our knowledge of normal thyroid physiology.

A large number of specific proteins controls the biochemical reactions involved in thyroid metabolism and base pair sequence changes in their controlling genes (mutations) result in inherited or sporadic errors. Recessive patterns of inheritance have been postulated for most of these syndromes, but dominant modes have been suggested in some families with generalized resistance to thyroid hormone and an X-linked inherited thyroxine binding globulin deficiency and excess.

Clinical or phenotypic variability in a single biochemical defect depends upon whether the defect is complete or incomplete, quantitative or qualitative, and changes in the enzyme or protein involved. Early decompensation is indicated by goitrous hypothyroidism at birth with growth and mental retardation. On the other side, euthyroid goiter may be present for many years in well-compensated defects. Environmental factors may be superimposed on biochemical heterogeneity and cause further variability in clinical presentation, as hronic iodine deficiency will further exacerbate the metabolic problem caused by iodine transport defect. Finally, genetic heterogeneity may also affect clinical variability. In some kindreds with Pendred's syndrome, goiter and deafness are present, whereas others have goiter or deafness but not both, while others have only a positive perchlorate discharge test.

Inherited disorders of thyroid metabolism are best classified by the main site at which the biochemical block occurs (2). Research has shown that most categories in this classification are heterogeneous and this is indicated by subgroups. This symposium deals with recent advances on the hereditable defects in thyroid peroxidase function, abnormalities in thyroid hormone-binding proteins, the multiple defects associated with thyroglobulin synthesis, and the inherited disorders of thyroid hormone action.

The organification defect is probably the most common inherited disorder of thyroid metabolism, and 30% of the 257 patients with defective thyroid hormone metabolism studied in the Hospital das Clinicas, Sao Paulo, had an organification defect. Perchlorate-induced discharge (PDT) of greater than 50% of thyroid iodide at two hours after a tracer indicates virtually a complete absence of thyroid peroxidase activity. Partial absence or qualitative minor defects will result in less than 40% discharge of accumulated iodide and the abnormal PDT may also be found in Hashimoto's thyroiditis and in patients with deficient Tg production. Reported defects in the organification process were divided by DeGroot's group into five main categories (2). It is recognized that qualitative defects in TPO may be present in other thyroid diseases such as multinodular goiter (with a strong familial goiter prevalence) or in other thyroid metabolism defects such as iodide trapping or coupling defects, as reported in this Congress by Yamamoto et al. (3). The basic defect in Pendred's syndrome remains uncertain and may not be uniform, although a TPO reduced affinity for the phenolic substrates has been demonstrated (2). Also, the cause of sensorineural deafness and its relationship, if any, with the partial organification defect are still unclear.

The thyroid hormones T_4 and T_3 are bound to serum proteins (TBG, TBPA and albumin), TBG being the most important T_4 transport protein (80%). Inherited abnormalities regarding TBG concentration, elevations, and deficiencies have been published by several authors including the group of investigators from Rotterdam. Familial TBG excess is much less common than congenital TBG deficiency. An X-linked variant has been recently described in Aborigines and led to the question about whether polymorphism of TBG might not be due to genetically-induced alterations but caused by different TBG content of sialic acid. On the other hand, hereditary variations in TBPA are very rare. Hennemann and collaborators (4) have described two families with increased affinity of an abnormal albumin fraction for T_4, and subsequently this dysalbuminemic hyperthyroxinemia was found in other families. Much work has yet to be done about the genetic inheritance pattern in the abnormalities of thyroid hormone transport proteins.

Another heterogeneous biochemical disorder of hormonogenesis is related to abnormal Tg formation and secretion. Quantitative and qualitative abnormalities in Tg mRNA, the intracellular transport of Tg, and its glycosylation have been described in both animals and man by Vassart and his group (5). Some of the animal studies in Afrikaner congenitally goitrous cattle, sheep, and goats are very informative. Large deletions of the Tg gene do not seem to be frequently involved. Considering the exceptional size of the Tg gene, it is logical to consider that abnormal splicing might be frequently involved in congenital goiter or even in sporadic euthyroid goiter with familial clustering. Recombinant DNA technology applied to Tg studies pioneered by Vassart and his collaborators have greatly expanded our knowledge in this field.

Dr. Gross will report on the disorders related to resistance to thyroid hormone action both in sporadic, as well as inherited cases. These patients show partial or generalized resistance to thyroid hormone, first described by Refetoff et al. (6). There is evidence that there may be defects also in the 5'monodeiodinases, both in man and animal studies. Studies of cases of generalized thyroid hormone resistance may ultimately shed light on the mechanism of action of thyroid hormone. On present evidence, the most likely mode of action is via abnormal binding to a nuclear receptor protein, but it has been reported that the thyroid hormone-mediated post-transcriptional pathway may be affected.

REFERENCES

1. Stanbury JB and Hedge AN. J Clin Endocrinol Metab 10: 471, 1950.
2. Lever E, Medeiros-Neto GA, and DeGroot LJ. Endocrine Rev 4: 213, 1983.
3. Yamamoto K, Saito K, Audo K, et al. 9th ITC, Abstract 219, 1985.
4. Hennemann G, Docter R, Krenning EP, et al. Lancet i: 639, 1974.
5. Vassart G, Bacolla A, Brocas H, et al. Moll Cell Endocr 40: 89, 1985.
6. Refetoff S, et al. Metabolism 21: 723, 1972.

HEREDITABLE DEFECTS IN THYROID PEROXIDASE FUNCTION*

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INTRODUCTION

The biochemical pathway leading to secretion of thyroid hormones depends on iodide transport, iodide oxidation, and binding of tyrosyl residues present in thyroglobulin (Tg). This requires a normal thyroid peroxidase (TPO), T_2O_2 generating system, and supply of iodide and Tg. Abnormalities of this process will be reviewed. Patients with defects of hormone synthesis typically display a compensatory growth of the thyroid and hypothyroidism or cretinism depending on the severity and time of onset of the thyroid hormone deficiency. These disorders are probably all congenital, however, the underlying genetics is not fully understood.

CLINICAL EVALUATION AND PLAN OF INVESTIGATION OF PATIENTS WITH SUSPECTED METABOLIC DEFECTS

The patients with suspected metabolic defects within the thyroid are usually characterized by low T_4, T_3, and elevated TSH. The radioactive iodine scan is normal, whereas the uptake will be abnormal. In most patients with defective iodination of Tg, the 2-h radioiodine uptake is very high and often more elevated than the 24-h uptake due to the slow but continuous loss of nonoxidized iodide from the thyroid gland. Perchlorate-induced discharge of greater than 500% of thyroidal iodide at 2 hours after a tracer indicates virtually complete defect (partial defect 10-50%). A positive perchlorate discharge may also be found in Hashimoto's thyroiditis, and in patients with deficient Tg production. Furthermore, it should be noted that impaired absorption of $KSCN^-$ or $KClO_4^-$ may delay the discharge of ^{131}I, and rapid thyroidal I^- turnover may be misinterpreted as a discharge.

Elucidation of the site of the biochemical defect depends upon in vitro tests made on material obtained at thyroid biopsy.

CHARACTERISTICS OF NORMAL THYROID PEROXIDASE

TPO is a hemoprotein with a prosthetic group identified as protoporphyrin IX (1,2). It is a glycoprotein (3) but the CHO moiety has not yet

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been characterized. This enzyme is membrane-bound and its intracellular localization was recently demonstrated with monoclonal anti-TPO antibodies to be mainly in the endoplasmic reticulum and perinuclear cisternae and, to some extent, on apical cell membranes (4). The enzyme has at least two reactive sites, one for tyrosine and one for iodide; both substrates are oxidized in adjacent sites. It has been postulated that TPO, after an initial reaction with H_2O_2, oxidizes I^- to form enzyme-bound hypoiodite, which iodinates tyrosine to form iodotyrosine (5). H_2O_2 functions as the oxidant of TPO. The source of H_2O_2 is not yet fully elucidated. NADPH cytochrome c reductase, NADH cytochrome b5 reductase, xanthine-xanthine oxidase, and monoamine oxidase have each been shown to generate H_2O_2, but a definite role for one enzyme in Tg iodination has not yet been demonstrated (6). TPO has been shown to be inactivated in in vitro studies by thioureylene drugs (7) and under some conditions by high iodide concentrations (1). Free diiodotyrosine (DIT) is a positive regulator of TPO mediated coupling, but the importance of this factor in vivo is uncertain.

The normal acceptor of oxidized iodide is tyrosyl at specific sites within the thyroglobulin molecule (8). Most thyroglobulin tyrosyl groups are not available for iodination. Other intracellular proteins, including tubulin are also normally iodinated to some extent (9). In hyperactive glands and on the absence of normal amounts of peroxidase, other proteins from within the cell or from serum (including albumin and gammaglobulin) may be iodinated (10,11). Recent studies have suggested that the thyroid "microsomal antigen" is actually TPO, or a closely associated protein (12). It will be very important to characterize this antigen in patients with defects in TPO formation.

METHODS FOR EVALUATION OF TPO FUNCTION IN VITRO

Activity

TPO activity is measured mainly by two assays, one using oxidation of substrates, the other iodination of various acceptors. In the triiodide assay, I^- is oxidized to I_3^- in the presence of excess I^- (present as KI) if TPO and H_2O_2 are present. The activity is measured by the change of OD at 353 nm. Similarly, in the guaiacol assay, the rate of oxidation of guaiacol is recorded at 470 nm. The iodinating systems contain 1) peroxidase (crude, solubilized or highly purified), 2) iodine labeled with ^{131}I, 3) iodine acceptors (thyroglobulin, bovine serum albumin, free tyrosine, free monoiodotyrosine or diiodotyrosine), 4) H_2O_2 added directly or generated enzymatically by glucose plus glucose oxidase, and 5) a suitable buffer. Samples are taken after specified intervals and free and bound iodide are separated by chromatography.

Solubilization

The enzyme can be solubilized by various detergents (deoxycholate, digitonin, octyl-glucoside, etc.) alone or with addition of trypsin (2,12). The TPO activity should be measured before and after solubilization, in the solubilized material and in the pellet as well. During preparation, 10^{-5} M KI is usually added to protect enzyme activity (13).

Inhibitor

In order to remove a low molecular weight peroxidase inhibitor, dialysis in the appropriate buffer should be performed (14).

Co-factors

The effect of the addition of the co-factor of TPO is performed by preincubation of the enzyme preparation with 10^{-4} M hematin at room temperature for one hour. Hematin alone or combined with albumin should produce negligible activity. Restoration of peroxidase activity implies a normalization of the enzymatic system (15).

Receptor

Protein abnormality of the receptor can be determined *in vitro* by the incorporation of iodine into thyroglobulin. TPO of the propositus (with iodide organification defect) or control are added to propositus or control thyroglobulin and both thyroglobulins can be exposed to another peroxidase. Abnormality of the acceptor protein is thus characterized by the inability of any peroxidase to iodinate Tg of the propositus (16). Tg abnormalities can be further characterized by centrifugal sedimentation analysis, electrophoresis, immunoprecipitation, peptide mapping, and perhaps in the near future by gene analysis (17).

INHERITED METABOLIC SYNDROMES ASSOCIATED WITH PEROXIDASE DYSFUNCTION (Table 1)

Quantitative Deficiency in TPO

Patients in this category have decreased or absent peroxidase activity measured *in vitro*, usually by the guaiacol or triiodide assay (18-21). added hematin has no effect and the Tg iodine content when measured is very low. Tg is, however, qualitatively normal. If present, residual enzyme has normal function (22).

Apoenzyme Defect

An abnormal TPO causing iodide organification defect was studied extensively by Hagen, Niepomniszcze, and DeGroot (15). The subject was a euthyroid, goitrous boy with 50% perchlorate discharge. Although the patient's peroxidase must have functioned at least partially *in vivo*, *in vitro* in standard assays there was no enzyme activity. Preincubation of enzyme with hematin restored activity in the tyrosine iodinase activity to normal, although activity in the triiodide assay, which is dependent upon high levels of H_2O_2, remained low. Addition of hematin to peroxidase preparations from other normal or goitrous tissue sources usually produces minor stimulation of enzyme activity. The restored enzyme activity was not normal, as indicated by excessive lability to high concentration of H_2O_2. In another patient also studied by these authors, it was also shown that restored enzyme activity was more liable to heat than a normal peroxidase (23).

Inhibitor of TPO

Pommier et al. (14) reported six patients with organification defect in whom an inhibitor of TPO was found in association with Tg. This small, thermostable and dialyzable inhibitor has not yet been further characterized. In a patient reported by Medeiros-Neto et al. (24), the solubilized TPO sample prepared from the propositus contained a factor that inhibited iodination of protein by a normal TPO. The nature of this inhibitor was not explored.

Table 1. Main Organification Defects

Reference	FHG	Age/ sex (yr)	Clinical data	% ^{131}I discharge	Enzyme studies	Hematin response	Inhibitor	Thyroglobulin Total (N∿50-80 mg/g	Thyroglobulin Iodine content ∿0.2-0.8%)
QUANTITATIVE DEFICIENCY OF PEROXIDASE									
Valenta et al. 1973	+	52 F	Cretinism Goiter Hypothyroidism	84	ND (guaiacol, BSA iodination, thiocyanate oxidation)	No	/	6-18	0.0005
Niepomniszcze et al. 1976	+	22 F	Goiter	65	↓ (tyrosine iodinase, guaiacol)	Yes, idem control	/	20	0.15
Pommier et al. 1976	-	17 M	Goiter Hypothyroidism	100	ND (triiodide, guaiacol, Tg iodination)	No	/	25	0.0014
Medeiros et al. 1979	-	36 M	Goiter Hypothyroidism	62	7-15% of control (guaiacol, triiodide, tyrosine iodinase)	No	/	↓	0.16
Eggo et al. 1980	-	19 M	Cretinism Goiter Hypothyroidism	73	ND (guaiacol, triiodide)	No	/	/	↓
ABNORMALITY IN SUBSTRATE BINDING									
Niepomniszcze et al. 1980	-	16 F	Goiter Hypothyroidism	95	Tyrosine iodinase = ND Triiodine = 20% control Guaiacol > control	No	No	/	/
Medeiros et al. 1980	+	14 M	Goiter Hypothyroidism	86.5	Triiodide = 40% Guaiacol 4.7 mM = 11% 33.0 mM = 28% Triiodide 0.1 M = 2.6% 1.0 M = 16.0%	/	Yes	/	/

IODINE RECEPTOR ABNORMALITY								Thyroglobulin analysis
Kusakabe 1973	-	51	F	Goiter	75	Triiodide, guaiacol = normal Cytochrome b_5 reduced = normal Tg iodination reduced in vitro	/	/	50% - 3 S Normal by electrophoresis and immunoprecipitation
Niepomniszcze et al. 1977	+	13	F	Goiter	26	Triiodide = increased Guaiacol = increased	/	/	Absent by analytical centrifugation
		15	F	Hypothyroidism	20	NADPA-cytochrome c = increased reduced Catalase = normal			Absent by PAGE Absent by double immunodiffusion

FHG = Family history of goiter; BSA = Bovine serum albumin; Tg = Thyroglobulin; ND = Not detectable.

Possible Abnormal Location of TPO

Niepomniszcze et al. (22,23) and Medeiros-Neto et al. (24) have postulated the existence of an abnormal localization of TPO in three subjects. In the first two, TPO activity was quantitatively decreased but qualitatively normal (22,23). TPO activity was maximal in the 105,000 x g fraction, whereas in normal thyroid homogenate it was present in the 15,000-39,000 x g fractions. In the third subject, TPO activity was normal but maximal in the 105,000 x g fraction (24). Their data suggested a cytostructural defect separating TPO from the other components of iodination system.

Abnormality in Substrate Binding

Some goitrous TPO preparations display anomalous activities in different assays. TPO from one patient had no activity in the tyrosine iodinase assay, whereas by the guaiacol method the activity was actually higher than control (25). Another study reported an increase of activity by changing the concentration of substrate iodide (26). These results suggest that TPO of these patients binds substrate, but the affinity of the enzyme may be abnormal.

Deficiency of H_2O_2 Supply

Kusakabe (27) reported a patient with nontoxic goiter and 65% thiocyanate iodide discharge. *In vitro* studies showed iodination to occur only with added H_2O_2. Evidence was found for reduced microsomal-NADH-cytochrome b_5 reductase activity. The defect was reversed by preincubation of microsomes with flavine-adenine dinucleotide (FAD). Administration of FAD to the patient restored thyroid iodide organification to apparent normality. The author suggested that the patient had an abnormality in the biosynthesis of FAD corrected by administering this coenzyme.

Iodine Acceptor Abnormality (Pseudo-peroxidase Defect)

In patients reported by Niepomniszcze, DeGroot, and others, an *in vivo* organification defect has been associated with augmented levels of TPO, and the absence of thyroglobulin. In thyroid tissue specimens obtained from three sisters with congenital goiter studied by Niepomniszcze et al. (17), peroxidase assayed in the triiodide system was from 2 to 5 times normal. Thyroidal NADPH-cytochrome c reductase was also elevated. However, Tg was totally absent. Iodoalbumin and other iodinated proteins were found in the thyroid cytosol. It was proposed that lack of normal oxidized iodine acceptor led to accumulation of trapped iodide which was not properly bound and, thus, was perchlorate dischargeable. Kusakabe (29) considered the defect in his patient to be secondary to abnormal Tg. It should be noted that in most patients, and in animals with defective thyroglobulin formation, perchlorate discharge tests are normal and, thus, the patients do not present as an "iodination" defect.

PENDRED'S SYNDROME

Pendred's syndrome (18) is characterized by congenital sensorineural deafness, and a positive perchlorate discharge test. Most patients who have been investigated have been found to have normal TPO activity *in vitro*. A H_2O_2 generation defect is unlikely since NADPH cytochrome c reductase activity has been found to be normal in some patients. A defective TPO with very little activity for Tg iodination, and a low K_m for the oxidation of iodide to T_2, associated with a poorly iodinated Tg representing only 33% of the total soluble proteins of the thyroid gland was reported by Abdelmoumene et al. (30). It was suggested that the TPO from this patient had

a reduced affinity for the phenolic substrates, thus explaining diminished iodinating activity. Numerous other abnormalities have been postulated and the basic defect(s) remains uncertain.

MULTINODULAR GOITER

Most cases of multinodular goiter (MNG) do not have definable defects in hormogenesis, but many have strong family histories of goiter (31,32). Conversely, patients with dyshormonogenetic goiters may have relatives with nonendemic euthyroid multinodular goiters suggesting that a subtle defect in hormonogenesis is present. Overt dyshormonogenesis has been reported in cases of multinodular goiter with abnormal iodoproteins, poorly iodinated Tg in one case, and abnormal thiocyanate discharge tests in six out of nine patients in one series with a strong family history of goiter.

Rapoport et al. (32) hypothesized that the low iodination of Tg in some MNG might be due to an abnormality in the progressive iodination of Tg which must go on within the follicular lumen. Tg is presumably initially iodinated in coordination with secretion from exocytotic vesicles at the apical cell membrane into the lumen. However, Tg is probably progressively iodinated by reattaching to the apical cell membrane through specific receptors, or by undergoing micropinocytosis and a resecretion process, during which iodination continues.

GOITER AND THYROID CARCINOMA

There are at least 17 reports of the association of dyshormonogenetic goiter and development of thyroid neoplasia. The development of neoplasia has also been associated with Pendred's syndrome (33).

CONCLUSION

Inheritable abnormalities in TPO, involving each aspect of enzyme function, have been reported. Study of these abnormalities has helped elucidate the mechanism of TPO function in humans but no correlation has yet been identified between the clinical data and the specific biochemical defects. The actual frequency of TPO abnormalities in goiter is probably underestimated, perhaps in part due to great reluctance to biopsy goiters. Logically, if the defect results in inadequate thyroid hormone synthesis, the standard treatment will be the prescription of T_4, whatever the biochemical abnormality. Clearly, these defects deserve to be more extensively studied using contemporary laboratory techniques.

REFERENCES

1. Taurog A, Lothrop ML, and Estabrook RW. Arch Biochem Biophys 139: 221, 1970.
2. Ohtaki S, Nakagawa H, Nakamura S, et al. J Biol Chem 260: 441, 1985.
3. Neary JT, Koepsell D, Davidson B, et al. J Biol Chem 252: 1264, 1977.
4. Nakagawa H, Kotani T, Ohtaki S, et al. J Biochem 97: 1709, 1985.
5. Magnusson RP, Taurog A, and Dorris ML. J Biol Chem 259: 13783, 1984.
6. Bjorkman U and Ekholm R. Endocrinology 115: 392, 1984.
7. Engler H, Taurog A, Luthy C, et al. Endocrinology 112: 86, 1983.
8. Marriq C, Arnaud C, Rolland M, et al. Eur J Biochem 111: 33, 1985.
9. Santisteban P, Hargreaves AJ, Cano J, et al. Endocrinology 117: 607, 1985.
10. Jonckheer MH and Karcher CM. J Clin Endocrinol Metab 32: 18, 1971.
11. Shimaoka K and Thompson BD. Endocrinology 76: 570, 1965.

12. Portmann L, Hamada N, Heinrich G, et al. J Clin Endocrinol Metab (in press).
13. DeGroot LH, Thompson JE, and Dunn AD. Endocrinology 76: 632, 1965.
14. Pommier J, Dominici R, Bougneres P, et al. J Mol Med 2: 169, 1977.
15. Niepomniszcze H, DeGroot LJ, and Hagen GA. J Clin Endocrinol Metab 34: 607, 1972.
16. Kusakabe T. J Clin Endocrinol Metab 37: 317, 1973.
17. Niepomniszcze H, Medeiros-Neto GA, Refetoff S, et al. Clin Endocrinol 6: 27, 1977.
18. Lever EG, Medeiros-Neto GA, and DeGroot LJ. Endocrine Reviews 4: 213, 1983.
19. Valenta LJ, Bode H, Vickery AL, et al. J Clin Endocrinol Metab 36: 830, 1973.
20. Niepomniszcze H, Degrossi OJ, Scavini LM, et al. In Thyroid Research, 7th International Thyroid Congress, Excerpta Medica, New York, 1976, p 470.
21. Eggo MC, Burrow GN, Alexander NM, et al. J Clin Endocrinol Metab 51: 7, 1980.
22. Niepomniszcze H, Castells S, DeGroot LJ, et al. J Clin Endocrinol Metab 36: 347, 1973.
23. Niepomniszcze H, Rosenbloom AL, DeGroot LJ, et al. Metabolism 24: 57, 1975.
24. Medeiros-Neto G, Okamura K, Cavaliere H, et al. Clin Endocrinol 17: 1, 1982.
25. Niepomniszcze H, Coleoni AH, Targovnik HM, et al. Acta Endocrinol 93: 25, 1980.
26. Medeiros-Neto GA, Knobel M, Yamamoto K, et al. J Endocrinol Invest 2: 353, 1979.
27. Kusakabe T. Metabolism 24: 1103, 1975.
28. Leroux A, Junien C, Kaplan JC, et al. Nature 258: 619, 1975.
29. Kusakabe T. J Clin Endocrinol Metab 37: 317, 1973.
30. Abdelmoumene N, Gavaret JM, Pommier J, et al. J Mol Med 3: 305, 1978.
31. Medeiros-Neto GA and Stanbury JB. J Clin Endocrinol Metab 26: 23, 1966.
32. Rapoport B, Niepomniszcze H, Bigazzi M, et al. J Clin Endocrinol Metab 34: 822, 1972.
33. Elman DS. N Engl J Med 259: 219, 1958.

HEREDITARY GOITER WITH THYROGLOBULIN DEFICIENCY*

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INTRODUCTION

Among the various causes responsible for the development of neonatal hypothyroidism, congenital goiters secondary to a genetic defect in the mechanisms of thyroid hormone synthesis represent a minority of the cases. Only a relatively small subset of these involves anomaly (either quantitative or qualitative) in thyroglobulin gene expression. Despite their scarcity, Tg gene defects represent particularly interesting situations to study, as the identification of their mutations is expected to tell us a lot on the normal structure-function relationship at the Tg locus. It is outside the scope of this presentation to review and analyze the studies of all cases found in the literature. Excellent and comprehensive reviews have been published recently on the subject (1,2). During the last few years, considerable progress has been made in our knowledge of Tg, both as a protein and as a genetic locus (for a review see 3 and ref. therein). This was mainly accomplished by the application to Tg study of the methods derived from the recombinant DNA technology. In this paper, the problem of defective expression of the Tg gene will be analyzed in the light of recent data on Tg gene structure. Emphasis will be put on the methods and probes presently available which will permit future analysis of human cases in molecular terms.

THYROGLOBULIN GENE EXPRESSION

Thyroglobulin is defined classically as the 19S iodoprotein present in the thyroid gland (4). From the analysis of cloned cDNAs and genomic DNAs of human, rat, and bovine Tg, we now know that 19S Tg is made from two identical subunits containing 2750 amino acid residues translated from an 8.4 kb mRNA. The corresponding gene resides on chromosome 8 in man and on chromosome 7 in the rat (5-7). It is one of the largest transcription units characterized to date (8) containing probably around 50 exons and extending well over 200 kb. The primary structure of the bovine Tg protomer has been deduced from the sequence of its cDNA (9). Together with the knowledge of the precise structural organization of most of the corresponding genes (10-14), these data will allow a direct study of Tg gene defects

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Table 1. Methods and Probes Used to Detect Tg Gene Defects

Structure or Phenomenon	Methods	Probes
Gene integrity (deletions)	Southern blotting	cDNA and genomic clones
Transmission of defective gene in families	Restriction fragment length polymorphism	cDNA and genomic clones
Splicing of Tg pre-mRNA	·Northern blotting	cDNA clones
	·S_1 nuclease mapping	cDNA clones
	·Electrton microscopy of mRNA-DNA hybrids	genomic clones
Alteration of coding sequence	·in vitro translation of mRNA	anti-Tg antibodies
	·sequencing of mRNA segments	cDNA clones
Alteration of protein structure	·immunoreactions	monoclonal antibodies
	·PAGE-centrifugation-iodoaminoacid content etc.	

in patients and in animal models. The DNA region responsible for regulation of Tg gene transcription by TSH has been recently cloned and characterized in the human and bovine species (15) and its structure-function relationship is under study. Table 1 summarizes the available methods and probes which could be of use to investigate Tg gene defects. Up to now, only limited use has been made of these new tools.

Of particular interest is the identification of restriction fragment length polymorphisms within the human Tg locus (16,17). This represents a powerful tool to follow through generations of a Tg allele which would be suspected of being defective (16).

Considering the published studies of human cases with congenital defect of TG production, the great majority analyzed only the (iodo-) proteins present in the goiter tissue. As a consequence, interpretation of the data in terms of genetic lesion remained necessarily speculative. Moreover, the high susceptibility of Tg to proteolysis, which made it difficult for years to determine the true quaternary structure of the normal protein, imposes caution when comparing the findings of individual studies.

Examples of human and animal cases which have benefited from gene analyses will now be briefly reviewed.

HUMAN CASES

Using Tg cDNA obtained by reverse transcription of bovine Tg mRNA, Dinsart et al. (17) demonstrated a dramatic decrease in Tg mRNA concentration in tissue obtained from a hypothyroid goitrous patient. Only trace amounts of Tg-related antigenes were detected. Unfortunately the methodology available at that time did not allow a study of the integrity of the Tg gene.

More recently, Cabrer et al. (18) studied a sibship where virtual absence of Tg was associated with the presence of a Tg mRNA apparently normal both in size and in concentration. Overdistension of the rough endoplasmic reticulum lead to the suggestion that a mutation was responsible for the abnormal routing of the encoded protein through the cell membrane system. Similar morphological changes have been observed by others (19).

A series of five patients with defective Tg production have been investigated by Targovnik and Medeiros-Neto (unpublished) who looked for the integrity of the Tg gene in DNA obtained from peripheral leukocytes. No deletion of exonic material could be demonstrated by the Southern'-blotting method.

A family with a sibship of eight of whom four presented a goiter, has been studied by Baas et al. (16). As the family was informative for a restriction fragment length polymorphism associated with one of the Tg allele, the mode of transmission could be studied. The unexpected conclusion was that the data were compatible only with an autosomal dominant mode of inheritance. Although a rationale can be provided for such kind of transmission, it must be stressed that variable penetrance and/or coexistence in the family of two differently affected alleles could also explain the data.

As may be seen from this brief review of human cases, the situation is far from clear. The main problem consists in the difficulty to study the Tg mRNA sequence in individual human cases. This is made impractical because of the need of thyroid tissue (which is not always available), and maybe more, because of the exceptional size of Tg mRNA, making the quest for the mutation that of a needle in a haystack.

ANIMAL MODELS

Hereditary Goiter in a Herd of Dutch Goats

de Vijlder and co-workers have studied in detail this model of congenital goiter with Tg deficiency (20). It presents as an autosomal recessive hypothyroid goiter. While only traces of Tg-related antigens are found in the glands, a normal-sized Tg mRNA is definitely present, albeit at a concentration much lower than normal. Up to now, the most significant finding has been the demonstration of a relative accumulation of Tg mRNA sequences in the nucleus (21). These results have lead to the suggestion that the disease resulted from a mutation affecting Tg mRNA transport from the nucleus to the cytoplasm or decreasing dramatically its stability in this latter cell compartment (22). In spite of much effort, including the partial sequencing of goat Tg mRNA, the precise identification of the lesion is still lacking.

Hereditary Goiter of the Afrikander Cattle

This is also an autosomal recessive goiter, identified in South Africa, which has been studied by Van Jaarsveld et al. (23) and, more recently, by M. Ricketts, in collaboration with us. The affected homozygous is euthyroid

in spite of presenting a huge goiter. The iodoproteins in the goiter contain Tg-related antigens of abnormally low molecular weight. Tg mRNA sequences are present in the goiter although at a concentration lower than in normal tissue. The goiter of the homozygous animal contains, in addition to the normal-sized 8.4 kb mRNA, a 7.3 kb species (24). When translated in the reticulocyte lysate by Tassi et al. (25), goiter Tg mRNA yielded two translation products: a 250 k and a 75 k protein. No normal 300 k protein was produced. By hybridizing the goiter mRNA to a fragment of normal bovine Tg gene, it was shown that the 7.3 kb mRNA resulted from the abnormal splicing of Tg primary transcript, leading to the removal of exon n° 9 from a fraction of the mRNAs (26). More recently, the cDNA region corresponding to the borders of the deleted region has been cloned and sequenced. The results confirmed that the 7.3 kb mRNA has lost the 1,098 bases of exon 9. Interestingly, the normal reading frame is conserved in the defective message. It can, thus, be predicted that it would encode a 2,384 residue polypeptide instead of the normal 2750 amino acid subunit. While no sequence data are available yet for the normal-sized goiter mRNA, we favor the hypothesis that it harbors a mutation near the end of exon 9, resulting in a shift in the reading frame. As a consequence, it would be the longer goiter mRNA which would encode the shorter peptide described by Tassi et al. (25). The euthyroid state of the affected animals is compatible with the fact that the major T_4 hormonogenic region of Tg near the amino terminus would be present on both the short and long Tg related peptides found in the goiter. Although the complete story will only be known when the mutation is be sequenced in the genomic DNA from goitrous animals, the goiter of the Afrikander cattle is the first Tg gene defect for which a molecular explanation is presently available.

CONCLUSIONS AND PERSPECTIVES

From the available evidence, the only firm conclusions to be drawn are: 1) congenital goiters with Tg defects represent a heterogenous entity, likely to be constituted by a wide variety of different mutations, and 2) large deletions of the Tg gene do not seem to be frequently involved. Together with the invariable detection of traces of Tg antigens in all cases investigated to date, this may suggest that the complete absence of Tg would be lethal. Considering the exceptional size of the Tg gene and its numerous introns, it is logical to propose that abnormal splicing would be frequently involved in the disease. Along this line, it might be worth considering that certain cases of plain euthyroid goiter with familial clustering could represent splicing errors in the heterozygous state with minimal functional consequences. Wider use of the newly available tool on human cases will tell us whether this assumption is correct.

REFERENCES

1. Stanbury JB and Dumont JE. In JB Stanbury (ed), The Metabolic Basis of Inherited Diseases, 1983, pp 231-269.
2. Salvatore G, Stanbury JB, and Rall JE. In M De Visscher (ed), The Thyroid Gland, Raven Press, New York, 1980, pp 443-488.
3. Vassart G, Bacolla A, Brocas H, et al. Mol Cell Endoc 40: 89-97, 1985.
4. Edelhoch H. Rec Progr Hom Res 21: 1, 1965.
5. Baas F, Bikker H, Geurts van Kessel A, et al. Ann Endoc (Paris) 45: 11, 1984.
6. Berge-Lefranc JL, Cartouzou G, Mattei MG, et al. Human Genet 69: 28, 1985.
7. Brocas H, Szpirer J, Lebo RV, et al. Cytogen Cell Gen (in press), 1985.

8. Van Ommen GJB, Arnberg AC, Baas F, et al. Nucl Ac Res 11: 2273, 1983.
9. Mercken et al. Accompanying paper in this volume.
10. Targovnik HM, Pohl V, Christophe D, et al. Eur J Biochem 141: 271, 1984.
11. Parma J, Christophe D, Targovnik H, et al. Ann Endoc (abstract) 45: 62, 1984.
12. de Martynoff G. Ann Endoc (Paris) 45: 63, 1984.
13. Baas F, Bikker H, Geurts van Kessel A, et al. Hum Genet 69: 138, 1985.
14. Avvedimento V, Musti E, Obici S, et al. Nucl Acids Res 12: 3461, 1984.
15. Christophe D, Cabrer B, Targovnik H, et al. Ann Endoc (Paris) 45: 9, 1984.
16. Baas F, Bikker H, Van Ommen GJB, et al. Hum Genet 67: 301, 1984.
17. Dinsart C, Wagar G, Van Voorthuizen F, et al. Ann Endocrinol 39: 133, 1979.
18. Cabrer B, Brocas H, Perez-Castillo A, et al. (submitted), 1985.
19. Lissitzky S, Bismuth J, Jaquet P, et al. J Clin Endocr Metab 36: 17, 1973.
20. De Vijlder JJM, Baas F, Kok K, et al. In Burrow and Eggo (eds), Progress in Endocrine Research and Therapy, Vol. 2, 1985.
21. Van Voorthuizen FW, Dinsart C, Flavell RA, et al. Proc Natl Acad Sci USA 75: 74, 1978.
22. De Vijlder JJM, Van Ommen GJB, Van Voorthuizen WF, et al. J Mol Appl Genet 1: 51, 1981.
23. Van Jaarsveld PP, Sena L, Van der Walt B, et al. Verlag des Wiener Medizinischen Akademie, Vienna, 1971, pp 465-479.
24. Ricketts MH, Pohl V, de Martynoff G, et al. EMBO J 4: 731, 1985.
25. Tassi VPN, Di Lauro R, Van Jaarsveld P, et al. J Biol Chem 259: 10507, 1984.
26. Mercken L, Simons JM, de Martynoff G, et al. Eur J Biochem 147: 59, 1985.

THYROID HORMONE-BINDING PLASMA PROTEINS

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The thyroid hormones thyroxine (T_4) and 3,3',5-triiodothyronine (T_3) are bound to serum proteins for more than 99%. These proteins are thyroxine-binding protein (TBG), thyroxine-binding prealbumin (TBPA), and albumin. TBG is the most important T_4 transport protein as it binds about 75 to 80% of T_4, while both TBPA and albumin bind approximately 10% (1). There is no unanimity in the literature with regard to the exact serum distribution of T_3 over the serum proteins, however probably also here TBG binds most of it (2). In this short review some physico-chemical characteristics of these proteins will be discussed, as well as their interaction with T_4 and T_3 and in the case of TBPA also its interaction with retinol-binding protein. In addition, the genetic aspects will be reviewed.

THYROXINE-BINDING GLOBULIN

Physico-chemical Properties

TBG was discovered by Gordon et al. in 1952 (3). On conventional electrophoresis it moves between α_1 and α_2 globulin. TBG has a molecular weight of 54,000. It consists of a single polypeptide chain and contains 23% carbohydrates on a weight basis (4). With regard to the amino acid composition of TBG, different numbers of residues per molecule have been reported varying from 320 to 460 (5). By using circular dichroism and fluorescence properties, the secondary and tertiary structure was investigated (6). The relaxation time of 1.1 indicated that TBG is a compact symmetric molecule. About an equal distribution in helical and structures was found in half of the peptide groups. Later, however, the same group reports that about 55% is in the α helical form, 19% in β structure, the remaining being unordered (7). The complete amino acid sequence of TBG is not known. Only 17 N-terminal residues have been reported (12).

Interaction with T_4 and T_3

TBG has one binding site per molecule for iodothyronines and binds T_4 and T_3 with an apparent association constant of 1.0×10^{10} and 5×10^{8} M^{-1}, respectively, at pH 7.4 and 37°C (8). When TBG interacts with T_4, changes in sedimentation constant and in relaxation time indicate that TBG becomes more symmetric and compact. This change in structure is accompanied by an increase in stability of TBG (9). Despite its high affinity for T_4,

dissociation of T_4 from its binding site is very rapid. The t of T_4 dissociation from TBG is 39 seconds (10), and of T_3, four seconds (11).

Genetics

TBG normally circulates in a concentration of 280-510 nmol T_4/l or 1.5 mg per 100 ml. Inherited abnormalities, i.e., TBG concentration elevations and deficiencies, have been published. These aberrations are due to mutations at a single X-chromosome-linked locus controlling TBG synthesis (13). Results from neonatal screening programs suggest that congenital (or familial) TBG deficiency may occur in a frequency of 1 per 2,500 live births (14). Familial TBG excess is much less common; about 10 families have been reported so far. Concentrations of serum T_4 and, to a lesser extent, of T_3 vary with TBG alterations, however, the concentrations of the free hormones remain within the normal range. Microheterogeneity of TBG appears to exist. In the first place, there is a form of polymorphism not due to genetically-induced alterations in the TBG peptide structure but caused by different content of sialic acid. Using isoelectric focusing (IEF), four main bands can be discerned, the number of which is decreased to two after desialylation with neuraminidase (15). Desialylated TBG (D-TBG) moves slower on electrophoresis and, hence, is sometimes referred to as slow-moving TBG. A higher percentage of D-TBG as normal may occur in obesitas (16), pregnancy, and liver disease (17). The two bands on IEF that remain after desialylation probably differ in amino acid composition. This phenotype is designated TBG-1 or C and occurs in most individuals, including causasians. However, a variant phenotype, TBG-2 or S, also with four bands before and two after desialylation, has been described in African Pygmies, Panamanians, American blacks, and Alaskan Eskimos (15,18). This variant is unrelated to the carbohydrate moiety and constitutes an X-linked variant, probably with alterations in the peptide sequence.

Recently, another variant of TBG has been described in Australian Aborigines. This X-linked inherited variant (19) is found in approximately 40% of this population. It has about 50% reduced affinity for T_4, and affinity for T_3 is reduced to 30%. Maximal binding capacity nor its serum protein concentration are changed. The molecule is less stable during heat inactivation (20). The decreased affinity accounts for the lowered total T_4 concentration found in sera of affected subjects. Despite the fact that the abnormality probably resides in the amino acid sequence, there was no difference from normal regarding microheterogeneity.

THYROXINE-BINDING PREALBUMIN

Physico-chemical Properties

The thyroxine-binding property of prealbumin was originally described by Ingbar (21). TBPA moves in front of albumin in electrophoresis and has a molecular weight of 55,000 (22). The protein is a tetramer composed of four identical subunits, each containing 127 amino acid residues. The complete amino acid sequence has been elucidated and TBPA has a high content of aromatic amino acids (22). It contains no carbohydrates. Its regulation ratio of 1.6 indicates a globular shape (2). Important work with regard to its spatial structure has been performed by Blake and co-workers (23). The subunit has an extensive β-structure composed of eight strands organized into two four-stranded sheets. There is one short α-helix. The tertiary and quaternary structure has extensively been studied (23).

Interactions with T_4 and T_3

TBPA has two identical binding sites for iodothyronines. The K_a's for T_4 are 7×10^7 and 7×10^5 M^{-1}, and for T_3, $\sim 1 \times 10^7$ and $\sim 6 \times 10^5$ M^{-1} at

pH 7.4 and 37°C. Despite identity of the two binding sites, the affinity of the "second" one is two orders of magnitude lower than that of the "first" one because interaction of T_4 and T_3 with one binding site causes a conformational change which leads to negative cooperativity with regard to the other binding site (24). The dissociation of T_4 and T_3 from TBPA is very rapid, the t 1/2 values being 7.4 and $\sim$ 1 seconds, respectively (10,11).

Interactions with Retinol Binding Protein (RBP)

TBPA has four binding sites for RBP with an estimated K_a value of between 10^6 and 10^7 M^{-1} (25,26). The K_a is not influenced by interaction of retinol (vitamin A) with RBP (26). Although there are four binding sites for RBP on prealbumin (PA), the molar ratio of PA:RBP is 1:1 in humans *in vivo* (25). Retinol is relatively fixed to RBP and tightly bound. Addition of PA to the retinol-RBP complex renders a more stable retinol-RBP-PA complex (27,28).

Genetics

TBPA concentration in normal adults ranges between 2830 and 3860 nmol T_4/l or 16-30 mg per 100 ml. Hereditary variations in TBPA concentrations are very rare. Only a few cases of familial TBPA elevation have been reported so far. In one case in which the mode of inheritance was unclear but in which X-linked inheritance could be ruled out, there was only increased T_4 binding by PA, probably as a consequence of increased affinity (29). In another case, both total T_4 and total serum reverse T_3 (rT_3) concentrations were elevated by another type of variant PA with increased affinity for T_4 and rT_3. Here, there was no possibility to investigate the mode of inheritance (30). So far, no microheterogeneity has been found in TBPA in man. However, polymorphism due to point mutation, where a methionine has been substituted for a valine, has been found in plasma and amyloid tissue in heredofamilial amyloidosis (31). In Rhesus monkeys (Macaca mulatta), two genetic PA variants are present with an interchange of valine and isoleucine at residue 5. Both variants are very similar to human prealbumin.

ALBUMIN

Physico-chemical Properties

Albumin is known to carry many substances, e.g., fatty acids, bilirubin, calcium, and also iodothyronines. For an extensive review about isolation, structure, and function, the reader is referred to (32). Its molecular weight amounts 66,000. The protein consists of one chain. It contains no carbohydrates. The amino acid sequence has been elucidated and human albumin contains 584 residues. Its relaxation ratio is 2.2 (3), and the protein is 48% in the β-helical form and 15% in the α-form (32).

Interaction with T_4 and T_3

Albumin has one binding site for T_4 and T_3 with a K_a of 5×10^5 and 1×10^5 M^{-1}, respectively, and several (2-6) binding sites with K_a of 5×10^4 and 5×10^3 M^{-1}, respectively (3).

Genetics

In 1979 (32), two families were described with increased levels of serum total T_4 but normal T_3 and rT_3 concentrations due to autosomal inherited increased serum binding of T_4. It appeared that in purified

serum albumin of affected members, an additional protein band was visible on IEF which was not (or only barely) present in albumin fractions of non-affected members. This abnormal protein fraction constituted approximately 25% of total serum albumin (1). Serum albumin as such was not elevated in subjects positive for the trait (33). Increased affinity of the abnormal albumin fraction for T_4 explained the increased serum T_4 values. An association constant of 1×10^7 M^{-1} was found. This modified albumin was immunologically indistinguishable from normal albumin. Its binding characteristics to thyroid hormone analogues were much more comparable to that of PA than to albumin itself (34), although the protein did not share any immunogenic determinants with PA (1). It was later found (35) that, though in a concentration of only one-tenth of that in positive family members, the same "abnormal" component with similar affinity for T_4 was present in normal sera. This syndrome of euthyroid T_4 excess was termed familial dysalbuminemic hyperthyroxinemia (FDH) and has been subsequently described many times from many parts of the world (2). It was found that in FDH, T_4 binding is much more critically dependent on intact disulfide bonds as is the case with normal albumin (36). Microheterogeneity of thyroxine binding by albumin has recently been reported (37) using IEF and autoradiography. Four T_4 binding bands were found in normal albumin and albumin of FDH. In FDH there was an overabundance of T_4 binding to two bands which were less occupied in normal sera. On the basis of the fact that sometimes variant albumin besides T_4 may also bind rT_3 (38) or T_3 and rT_3 (38,39) with increased affinity, it was suggested to designate these variants as I (only T_4), II (T_4 and rT_3), and III (T_4, rT_3, and T_3) (39).

CONCLUSION

This brief review discusses, besides genetic aspects, only some but not all physico-chemical properties of thyroid hormone-binding proteins per se and of their interactions with T_3 and T_4. Other important issues regarding the occurrence in other species, the synthesis and metabolism of thyroid hormone-binding proteins, their possible function in thyroid hormone tissue delivery, induced changes in binding capacity by drugs and diseases, etc., were considered to be outside the scope of this review. A very important issue, i.e., the possible structural homology between PA and gastrointestinal hormones (40,41) and the thymic hormone-like activity of PA (42) has also not been considered.

REFERENCES

1. Docter R, Bos G, Krenning EP, et al. Clin Endocrinol 15: 363, 1981.
2. Robbins J and Bartalena L. In G Hennemann (ed), Thyroid Hormone Metabolism, Marcel Dekker, New York, in press, p 9.
3. Gordon AH, Gross J, O'Connor D, et al. Nature 169: 19, 1952.
4. Gershengorn MC, Cheng S-Y, Lippoldt RE, et al. J Biol Chem 252: 8713, 1977.
5. Hocman G. Rev Biochem Pharmacol 91: 46, 1981.
6. Gershengorn MC, Lippoldt RE, Edelhoch H, et al. J Biol Chem 252: 8719, 1977.
7. Johnson ML, Lippoldt RE, Gershengorn MC, et al. Arch Biochem Biophys 200: 288, 1980.
8. Robbins J and Rall JE. In CH Gray and VHT James (eds), Hormones in Blood, Vol. 1, Academic Press, London, 1979, p 576.
9. Grimaldi S, Edelhoch H, and Robbins J. Biochemistry 21: 145, 1982.
10. Hillier AP. J Physiol 217: 625, 1971.
11. Hillier AP. Acta Endocrinol (Kbh) 80: 49, 1975.
12. Cheng S-Y. Biochem Biophys Res Comm 79: 1212, 1977.

13. Refetoff S, Fang VS, Marshall JS, et al. J Clin Invest 57: 485, 1976.
14. Sorcini MC, Moschini L, Fiore L, et al. J Endocrinol Invest 5: 21, 1982.
15. Grimaldi S, Bartalena L, Ramacciotti C, et al. J Clin Endocrinol Metab 57: 1186, 1983.
16. Premachandra BN, Perstein IB, and Blumentahl HT. J Clin Endocrinol Metab 30: 752, 1970.
17. Gaertner R, Henze R, Horn K, et al. J Clin Endocrinol Metab 52: 657, 1981.
18. Daiger SP, Rummel DP, Wang L, et al. Amer J Hum Genet 33: 640, 1981.
19. Refetoff S and Murata Y. J Clin Endocrinol Metab 60: 356, 1985.
20. Murata Y, Refetoff S, Sarne DH, et al. J Endocrinol Invest 8: 225, 1985.
21. Ingbar SH. Endocrinology 63: 256, 1958.
22. Kanda Y, Goodman DeWS, Canfield RE, et al. J Biol Chem 249: 6796, 1974.
23. Blake CCF, Geisow MJ, Oatley SJ, et al. J Molec Biol 121: 339, 1978.
24. Irace C and Edelhoch H. Biochemistry 17: 5729, 1978.
25. Van Jaarsveld P, Edelhoch H, Goodman DeWS, et al. J Biol Chem 248: 4698, 1973.
26. Peterson PA and Rosk L. J Biol Chem 246: 7544, 1971.
27. Goodman DeWS and Leslie RB. Biochim Biophys Acta 260: 670, 1972.
28. Goodman DeWS and Raz A. J Lipid Res 13: 338, 1972.
29. Moses AC, Lawlor J, Haddow J, et al. N Engl J Med 306: 966, 1982.
30. Lalloz MRA, Byfield PGH, and Himsworth RL. Clin Endocrinol 21: 331, 1984.
31. Dwulet FE and Benson MD. Biochem Biophys Res Commun 114: 657, 1983.
32. Peters T. In VM Rosenoer, M Oratz, and MA Rotschild (eds), Albumin Structure, Function and Uses, Pergamon Press, Oxford, 1977, p 305.
33. Hennemann G, Docter R, Krenning EP, et al. Lancet i: 639, 1974.
34. Hennemann G, Krenning EP, Visser TJ, et al. In R Ekins, G Faglia, F Fensi, et al. (eds), Free Hormones in Blood, 1979, p 203.
35. Docter R, Bos G, Krenning EP, et al. Lancet i: 50, 1984.
36. Barlow JW, Csicsmann JM, White EL, et al. J Clin Endocrinol Metab 55: 244, 1982.
37. Yubu Y, Amir SM, Ruiz M, et al. J Clin Endocrinol Metab 60: 451, 1985.
38. De Nayer PH and Malvaux P. J Endocrinol Invest 5: 383, 1982.
39. Lalloz MRA, Byfield LGH, and Himsworth RL. Clin Endocrinol 22: 521, 1985.
40. Jornvall H, Carlstrom A, Petterson T, et al. Nature 291: 261, 1981.
41. Kato M, Kato K, Blaner WS, et al. Proc Natl Acad Sci USA 82: 2488, 1985.
42. Burton P, Iden S, Mitchell K, et al. Proc Natl Acad Sci USA 75: 823, 1978.

INHERITED DISORDERS OF THYROID HORMONE ACTION

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Disorders of thyroid hormone action have only been characterized by resistance to thyroid hormone (THR). This is manifest by a normal or high circulating level of thyroid hormones with an inappropriate response of the target tissues, either in terms of metabolic activity and/or TSH secretion. There are a number of comprehensive reviews (1,2) that have dealt with the subject up to 1981. I have reviewed the literature to date to try and obtain further insight into the types, frequencies, and underlying mechanisms of these disorders. Because of the small number of cases reported, I have included sporadic, as well as inherited cases. I will also mention two animal models of inherited thyroid hormone resistance.

In reviewing some 64 reported cases of thyroid hormone resistance, they could be classified into three broad categories and several subcategories as follows.

A) Inappropriate TSH secretion (51 cases) associated with:
   hyperthyroidism - 18 cases (3-9)
   euthyroidism - 23 cases (10-19)
   hypothyroidism - 11 cases (20,21)
B) Hypothyroidism with normal blood thyroxine levels - 1 case (22)
C) Nephropathic Cystinosis with inappropriate TSH secretion - 11 cases (23,24)

INAPPROPRIATE TSH SECRETION

The hormonal pattern for this group is shown in Table 1. All three subgroups show elevated levels of T_4 and T_3 in the blood. Despite this, the basal TSH levels were elevated or not depressed and there is a normal or excessive response to TRH. Thus, all three groups show pituitary resistance to thyroid hormone. The hyperthyroid group would appear to suffer from only pituitary THR. The euthyroid group would appear to be resistant to thyroid hormone both in the pituitary and in the peripheral tissues with a euthyroid balance between the two defects. The hypothyroid group seems to have a more marked resistance in the periphery than in the pituitary.

The defect in the hyperthyroid group seems to be due to reduced 5'deiodinase activity in the pituitary. This is supported by the finding that in most cases TSH secretion is depressed by normal blocking doses of T_3 and

Table 1. THR Associated with Inappropriate TSH Secretion

	T_4 µg/dl	T_3 ng/dl	TSH µU/ml Basal*	Post TRH
1) Hyperthyroid (12)	18	264	20 ± 5	49
2) Euthyroid (13)	20	265	4 ± 0.5	28
3) Hypothyroid (9)	19	297	5 ± 1	28

*Mean ± SEM. 1 vs 2 = $p<0.003$; 1 vs 3 = $p<0.005$.
The number of cases is shown in parenthesis.

the fact that in two reports this syndrome was successfully treated by long-term low dose T_3 (3) or T_3 analogue therapy (4). In the euthyroid group, normal blocking doses of T_3 inhibited TSH secretion in a majority of cases, suggesting a pituitary deiodinase defect. In the hypothyroid group, high doses of T_3 were required to reduce TSH secretion, indicating that the pituitary is resistant both to T_3 and T_4.

To test if there may be a peripheral deiodinase defect in these groups, use was made of the T_3/T_4 ratio. Using the data for T_3 production rates summarized by Weintraub et al. (2) and Gheri et al. (14), the T_3/T_4 molar ratios were calculated and were found to correlate significantly ($p<0.02$) with T_3 PR. The ratios for each of the three groups are shown in Table 2. The euthyroid group had a significantly depressed ratio which would indicate a reduced T_3 PR, possibly due to a partial defect in peripheral T_4 5'deiodinase. The other two groups showed ratios that did not differ from normal values.

Other sites of thyroid hormone action have been examined and the data are summarized in Table 3. The nuclear binding studies are equivocal, especially in the fibroblast target. The data are few on the other systems tested. LDL degradation and glycosaminoglycan synthesis in fibroblast cultures would seem to be useful indicators. More studies are needed.

Table 2. THR Associated with Inappropriate TSH Secretion

	Plasma T_3:T_4 Molar Ratio x 100
1) Hyperthyroid (11)	1.81 ± 0.09
2) Euthyroid (14)	1.52 ± 0.10*
3) Hypothyroid (8)	1.93 ± 0.09
4) Normals (4)**	2.00 ± 0.16

*Mean ± SEM; Group 2 is significantly < 1, 3, and 4.
**References, Weintraub et al. (2), Gheri et al. (14).

Table 3. THR Associated with Inappropriate TSH Secretion; Defects in Thyroid Hormone Target Systems

1) Lymphocytes	Abnormal
T_3 binding to nuclei	11/17
T_4 binding to nuclei	1/1
2) Fibroblast cultures	Abnormal
T_3 binding to nuclei	2/35
Glycosaminoglycan synthesis	4/6
Collagen & protein synthesis	0/4
LDL degradation	3/3

References 10,11,13,14,20,21,26,27.

What are the modes of inheritance of these syndromes? The distribution of cases between the sexes is shown in Table 4. The data for the kindreds of the euthyroid and the hypothyroid groups are compatible with inheritance as an autosomal dominant. This is the generally accepted view in the literature. The kindreds of the hyperthyroid group, however, show that the syndrome occurs only in females. Also, the symptoms of hyperthyroidism appeared after puberty in all but one of the cases.

HYPOTHYROIDISM WITH NORMAL SERUM T_4

There is one case of this type reported by Kaplan et al. (22). This patient had a mean serum T_4 of 5 micrograms/dl. She suffered from hypothyroidism and felt best on 250 micrograms of T_3/day. Lower doses resulted in a depression of oxygen consumption. The effective doses of T_4 were in the molecular ratio to T_3 that would be expected from their respective thyromimetic potencies. This indicated a normal peripheral deiodinase activity. Serum TSH was below the limits of detection with all doses of T_3, indicating a normal responsiveness of the pituitary. This is a case of thyroid hormone resistance in the peripheral tissues while the response of the pituitary is normal. The site of the defect in the target cells in this case is unknown.

Table 4. THR Associated with Inappropriate TSH Secretion; Sex Ratios - Females/Males

	Sporadic cases	Kindreds
1) Hyperthyroid	7/4	9/0
2) Euthyroid	1/5	10/5*
3) Hypothyroid	4/3	1/2*

*Inherited as an autosomal dominant.

Table 5. Nephropathic Cystinosis

T_4 µg/dl	10.3 ± 2.5
T_3 ng/dl	239.0 ± 21
T_3:T_4 ratio	2.7 ± 0.73
Age - years	3 - 10

Mean ± SEM (n = 11)

NEPHROPATHIC CYSTINOSIS WITH INAPPROPRIATE TSH SECRETION

Nephropathic cystinosis is a disorder inherited as an autosomal recessive. In the series reported by Bercu et al. (23,24), the children were euthyroid (see also 28) but showed the hormonal pattern in Table 5. Basal TSH was elevated despite a normal serum T_4 and a raised serum T_3. TSH was normalized with 2 micrograms of T_3 and 6 micrograms of T_4 per kg/d. This would tend to rule out a deiodinase defect in the pituitary to account for the inappropriate TSH secretion. Some of these children also showed elevated alpha subunits in the serum. This could not be accounted for by a change in gonadotrophin levels. Tentatively, the possible deposition of cystine crystals in the hypothalamic-pituitary area may cause a disorder in TSH synthesis and/or secretion.

ANIMAL MODELS

There are two animals that have been reported to be resistant to thyroid hormone action. These are the sex-linked dwarf (SLD) domestic Leghorn chicken (29,30) and the obese (ob/ob) mouse (31).

The hormonal and enzyme data of the SLD are shown in Table 6. They show an elevated serum T_4 and a depressed serum T_3. This is reflected in the low T_3/T_4 ratio. This indication that T_3 production is impaired is substantiated by the fact that liver 5'deiodinase activity is only one-fifth

Table 6. Inherited THR in the Sex-linked Dwarf Leghorn Chicken

	T_4 µg/dl	T_3 ng/dl	T_3:T_4 ratio	GH ng/ml	Malic Enz NADP/mg
Normals (DW/DW)	1.7	330	23	164	100
Heterozygotes (DW/dw)	2.2*	246*	13	207*	65*
Sex-linked dwarfs (dw/dw)	2.2*	142*	7.6	301*	72*

N = 12; *Significantly different from normal control.
Ref. - Stewart et al. (30).

that of the normal controls (29). This would also account for the lowered malic enzyme activity. The table also shows elevated GH levels in the SLD, which are also found in hypothyroid chickens and would suggest inappropriate pituitary hormone secretion. This is supported by the report (31) that the TRH-induced GH rise in SLD is effectively inhibited by T_3 but not by T_4. In light of the elevated serum T_4, this can be best explained by a defect also in the pituitary T_4 to T_3 deiodinase. It is interesting to note from Table 6 that the heterozygotes show intermediate but significant changes in serum T_4, T_3, and GH. The malic enzyme activity is also depressed. The gene may not be entirely recessive. The dynamics of TSH, the activity of the pituitary deiodinase and liver nulcear T_3 binding remain to be investigated. This chicken syndrome is quite similar to hyperthyroid and euthyroid THR groups reported above (Tables 1 and 2).

The genetically obese mouse shows a lowered body temperature and oxygen consumption of some tissues. This has been attributed by York et al. (32) to the low Na+, K+-ATPase activity of liver and kidney membranes. There was no response to T_3 treatment. On the other hand, liver glucose-3-phosphate dehydrogenase behaved normally both to the lack or excess of thyroid hormones. Thus, there may be resistance to the synthesis or activity of individual enzymes that are normally responsive to thyroid hormone.

CONCLUSIONS

There is a small group of patients that show resistance to thyroid hormone either in the pituitary, the peripheral tissues, or both. There is evidence in both man and animals that there may be defects in the 5'monodeiodinases. There is also some indication that some thyroid hormone-mediated post-transcriptional pathways may be affected. The evidence of reduced nuclear binding of T_3 is somewhat equivocal.

REFERENCES

1. Lamberg B-A and Liewendahl K. Ann Clin Res 12: 243, 1980.
2. Weintraub BD, Gershengorn MC, Kourides IA, et al. Ann Intern Med 95: 339, 1981.
3. Rosler A, Litvin Y, Hage C, et al. J Clin Endocrinol Metab 54: 76, 1982.
4. Beck-Peccoz P, Piscitelli G, Cattaneo MG, et al. J Endocrinol Invest 6: 217, 1983.
5. Tisell L-E, Caidahl K, Lindstedt G, et al. Acta Endocrinol 103: 61, 1983.
6. Scott SM, Brasel JA, and Klein AH. Clin Res 31: 793A, 1983.
7. Takamatsu J, Mozai T, and Kuma K. J Clin Endocrinol Metab 58: 934, 1984.
8. Spitz IM, Sheinfeld M, Glasser B, et al. Postgrad Med J 60: 328, 1984.
9. Vance ML, Evans WS, Thorner MO, et al. Clin Res 30: 278A, 1982.
10. Eil C, Fein HG, Smith IJ, et al. J Clin Endocrinol Metab 55: 502, 1982.
11. Cooper DS, Ladenson PW, Nisula BC, et al. Metab 31: 504, 1982.
12. Lindstedt G, Lundberg P-A, Sjogren B, et al. Scand J Clin Lab Invest 42: 85, 1982.
13. Chait A, Kanter R, Green W, et al. J Clin Endocrinol Metab 54: 767, 1982.
14. Gheri RG, Bianchi R, Mariani G, et al. J Clin Endocrinol 58: 563, 1984.
15. Bajorunas DR, Rosner W, and Kourides IA. J Clin Endocrinol Metab 58: 731, 1984.

16. Sisson JC, Hopwood NJ, Sauder SE, et al. J Clin Endocrinol Metab 58: 1188, 1984.
17. Takamatsu J, Miki K, Isaji H, et al. Endocrinol Japon 31: 435, 1984.
18. Kleinhaus N, Schneer JH, Kahana L, et al. Isr J Med Sci 20: 557, 1984.
19. Gharib H and Klee GG. Mayo Clin Proc 60: 9, 1985.
20. Murata Y, Refetoff S, Horowitz AL, et al. J Clin Endocrinol Metab 57: 1233, 1983.
21. Menezes-Ferreira MM, Eil C, Wortsman J, et al. J Clin Endocrinol Metab 59: 1081, 1984.
22. Kaplan MM, Swartz SL, and Larsen PR. Am J Med 70: 1115, 1981.
23. Bercu BB, Orloff S, and Schulman JD. J Clin Endocrinol Metab 51: 1262, 1980.
24. Bercu BB and Schulman JD. Isr J Med Sci 20: 179, 1984.
25. Kvetny J. Clin Endocrinol 18: 251, 1983.
26. Vandalem J-L, De Rycker C, and Hennen G. Ann Endocrinol 45: 36, 1984.
27. Ichikawa K, DeGroot LJ, Refetoff S, et al. Proc Endocrine Soc 213, 1985.
28. Kollipara S, Conors M, Segal LD, et al. Clin Res 32: 134A, 1984.
29. Scanes CG, Marsh J, Decuypere E, et al. J Endocr 97: 127, 1983.
30. Stewart PA, Washburn KW, and Marks HL. Growth 48: 59, 1984.
31. Denver RJ, Bowen SJ, and Scanes CG. J Steroid Biochem 20: 1539, 1984.
32. York DA, Bray GA, and Yukimura Y. Proc Natl Acad Sci USA 75: 477, 1978.

EXPECTATIONS IN THE STUDY ON GROWTH REGULATION OF THYROID EPITHELIAL CELLS IN VITRO

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The life cycle of an individual cell is known to be composed of a mitotic (M) phase, a DNA synthetic (S) phase, and two gaps between M and S, which we denoted G_1 and G_2. Recent studies indicated that proliferation of normal cultured cells is under the control of exogenous growth factors, and in the absence of the proper growth factors, the cells become arrested in the G_1 phase. Serum added to the culture medium is considered to be a source of growth factors.

Table 1 lists the well-characterized growth factors classified into three groups, the platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and insulin families. These growth factors indicate DNA synthesis and division of cultured cells derived from various tissues. Recently, the thyroid has been found to be included in the list of tissues under the control of these factors.

The culture of thyroid cells was first reported by Pulvertaft et al. in 1959 as shown in Table 2. In the early 1970's, Fayet and others established a primary culture system in which TSH induced the reconstruction of three-dimensional and functional follicles. This confirmed the earlier work by Kerkoff and Chaikoff in which cultured thyroid cells were reorganized and showed a follicle-like appearance under the influence of TSH. These findings stimulated studies on morphological and biochemical characteristics of thyroid cells in primary cultures and TSH effects on them during the 1970's. On the other hand, the stimulation of cell growth by TSH was controversial in that period. Although the proliferation of dog and rat thyroid cells had been reported to be stimulated by TSH, that of sheep and human cells was apparently not influenced by this hormone. In addition, differences in culture conditions, and the purification level of TSH interfered with attempts to answer the question of whether TSH is a mitogen or not.

In the early 1980's, Ambesi and others established a continuous line of normal rat thyroid cells, FRTL, which grew in the presence of TSH and other growth-promoting hormones in a defined culture medium containing a low concentration of serum. Besides this established rat cell line, Dumont's group found that the primary culture of dog thyroid cells responded to TSH by maintaining their growth in a defined medium.

The establishment of these two culture systems and the use of defined media opened a new way to investigate the mechanisms of growth regulation

Table 1. Some Growth Factors for Cultured Cells

Growth factors	m.w.
PDGF Family	
Platelet-derived growth factor	28K-31K Da
Osteosarcoma-derived growth factor	31K Da
Glioma-derived growth factor	31K Da
Fibroblast-derived growth factor	
Transforming protein of SSV	24K Da
EGF Family	
Epidermal growth factor	6K Da
Transforming growth factor	7K Da
Insulin Family	
Insulin	5.7K Da
Insulin-like growth factor I	6K Da
Insulin-like growth factor II	6K Da
Multiplication-stimulating activity	6K Da

of thyroid cells. The TSH effect on cell growth was confirmed in the rat and dog cell cultures, although a species difference was still obvious.

Among the studies done in the 1980's, the response of thyroid cells to EGF and other growth factors is of great importance, since these growth factors or their receptors have been found to relate to oncogene action. Structural studies on growth factors and their receptors in other tissue cells had already provided some ideas to understand growth regulation mechanisms in general.

The effects of growth factors have been found in cell cultures. The effective concentration of EGF on thyroid cells was in a physiological range and the growth factor was able to induce growth of thyroid cells in culture in any animal species previously examined, which suggest that EGF is responsible for the proliferation of thyroid cells, not only in culture but also in vivo. On the other hand, the EGF effect was enhanced synergistically by a low concentration of serum, while TSH induction was enhanced by insulin. These facts raised a question as to how many growth factors are involved in the growth regulation of thyroid cells.

Both TSH and EGF are known to act at the plasma membrane. Involvement of cAMP in the growth regulation by TSH has been shown by Dumont's group. Burrow's group suggested that EGF action was mediated by metabolites derived from phosphatidylinositol. However, these findings indicate only the first step of a long reaction cascade consisting of either tyrosine kinases or threonine or serine kinases. No information is presently available on their substrates and processing, in spite of their great importance for understanding growth regulation mechanism, not only of thyroid cells, but also of tissue cells in general.

Another point of interest is the relationship between growth regulation and cell differentiation.

Table 2. Brief History of Studies on Cultured Thyroid Cells

1959	First report on cultured thyroid cells.
1964	TSH-dependent reorganization of cultured cells.
1971	TSH-dependent reconstruction of three-dimensional follicles.
1970's	Morphology and biochemistry of cultured cells. TSH-induced recovery of the morphology and differentiated functions. Controversy. Is TSH mitogenic?
1980	TSH-dependent strain of rat thyroid cells, FRTL family. TSH-depencent primary culture of dog thyroid cells.
1980's	Biochemistry of cell functions and growth control. TSH receptor and the adenylate cyclase system. Localization of functional apparatus. TSH is mitogenic but there are species differences. EGF and other growth factors control thyroid cells. Growth control and differentiation. Application for assay systems.

EGF stimulates cell growth and simultaneously inhibits hormone formation. TSH stimulates cell growth, as well as the reconstruction of functional follicles. Insulin stimulates cell growth and also enhances TSH-induced morphogenesis.

These facts suggest that growth regulation systems, differentiation-inducing systems, and systems regulating cell functions are correlated in some way. Still another point, other than intracellular messenger system or the reaction cascades mentioned above, is the relationship between oncogene products and growth factors or their receptors. Analysis of gene structure has disclosed some structural homology between retrovirus oncogenes and chromosomal genes. In addition, some of the oncogene products were identified as active components of the host cell. Sis gene produces a part of platelet-derived growth factor, and erb B products are very similar to a part of the EGF receptor. The structure of the insulin receptor has partial homology with the EGF receptor and, hence, the oncogene products Since TSH is included in the growth factor families, we may consider the possibility that the TSH receptor has some structural relation to oncogene products. On the other hand, another aspect of TSH activity was raised by B. Rapoport who found that TSH induced the expression of two oncogenes, c myc and H-ras.

In any case, if thyroid cell growth is regulated by TSH as well as other growth factors that are in some way related to oncogene activity, some phenotype expressions of TSH and oncogenes are also expected to be somewhat similar. This is true in some respects; for example, enhanced transport of metabolites, high production of plasminogen activator, excessive blebbing of plasma membrane, round morphology, and failure of actin filaments to organize into large bundles. Some of these changes occur not only under conditions which give growth promotion by TSH, but also in the case when TSH induces morphological and/or functional differentiation. These facts, again, suggest a rather close relation in mechanism between growth regulation and differentiation promotion by TSH.

The study of growth regulation of thyroid cells has just been started. At present, as mentioned in this brief comment, we still have many problems unsolved. However, the burst of new findings from cell-biological and molecular-biological studies gives us hope to solve the problems in the near future.

GROWTH CONTROL OF FOLLICULAR CELLS IN VITRO*

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Thyroid follicular cells represent a fine example of endocrine and hormone-regulated cells. Similar to other types, both cell growth and differentiation are exactly adjusted in vivo throughout the life of the organism. Regulation of cell proliferation and acquisition and maintenance of the differentiated phenotype seem part of the unique program coordinated under normal conditions and subverted by pathological conditions leading to tumor growth and anaplastic dedifferentiation.

Regulatory mechanisms affecting cell functions have been and continue to be investigated in vivo in animal and clinical studies. These studies, however, can be difficult to interpret because of the complex and often obscure interactions between different cell types, tissues, and organs. The effects and mode of action of various hormones or hormone-like substances have been described in clinical and biological thyroid studies and are also reported elsewhere in this book. Nevertheless, mitogenic stimulation of the thyroid gland has been difficult to explain in physiological or pathological conditions. For example, the TSH-like activity present in sera from patients with Graves' disease, earlier described as "Long Acting Thyroid Stimulator (LATS)" and later discovered to be an immunoglobulin, is more precisely a thyroid-stimulating antibody (TSAb) or an autoantibody against TSH receptor component(s) (1).

Isolated thyroid follicular cells may be studied in vitro, free of undefined interactions. In such a system, homogeneous cell strains can be cultured in chemically and hormonally defined media, under standardized conditions. Thus, putative regulatory molecules can be tested for their biochemical effects on cell functions and proliferation. Ideally, cell cultures should also be genetically homogeneous (cloned from a single cell), continuously growing in culture while retaining their normal phenotype and differentiation. Such cultures should maintain their features over prolonged periods of time so as to provide consistent results at different times and in different laboratories. Unfortunately, such an ideal system is extremely difficult and probably impossible to obtain.

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Table 1. Culture Systems Employed in the Study of Follicular Cell Growth Control

- Freshly trypsinized cells in suspension
- Primary monolayer cultures
- Primary follicles in suspension
- Long-term monolayer cultures

Despite the rapid progress in cell biology and in culturing differentiated cells achieved in the last few years, several difficulties are still encountered by investigators trying to grow differentiated, normal cells in long-term cultures. The field of thyroid biology, however, is particularly fortunate in having developed several valid in vitro cell culture systems. Probably none of these possess all the characteristics of an ideal system, but, knowing their limitations, they may be used to address several questions in cellular endocrinology.

Various thyroid experimental systems have been applied to the in vitro studies of hormonal control of thyroid follicular cell functions. Those systems may be classified in four categories (Table 1).

Freshly trypsinized cells in suspension. As early as 1962, Tong used freshly trypsinized sheep thyroid cells to study iodide transport and the influence of TSH on this differentiation marker (2). Thyroid cells were found to be particularly resistant to treatment but, due to the short life of suspended cells, growth regulation could not be studied (Table 2).

Primary monolayer cultures. A better definition of culture media and conditions now permit the maintenance of primary thyroid cell cultures. Little or no cell proliferation occurs, but cells are kept under physiological conditions and express normal metabolism and characteristics for at least 5-7 days. Growth is usually accompanied by rapid dedifferentiation, and the possibility of selective mitogenic stimulation of non-follicular contaminant cells should be taken into account. Several important aspects of thyroid biology have been studied in primary cultured cells.

Various TSH effects, such as stimulation of cAMP metabolism (3-7), or active iodide transport (8), have been extensively investigated in primary cultures. In 1979, the mitogenic effect of TSH was evaluated in cultures of human adenoma cells. The results led the authors to conclude that TSH was not mitogenic for those cells (9). At variance with this conclusion, several other studies reported TSH mitogenic activity in primary cultures of various animal thyroids (5,10-12). TSH also stimulated thyroid differentiation during the quiescent state of the cells (13). Primary cultures have also been used to investigate the effect of growth factors other than TSH (12,14,15). This is particularly important in view of the recently

Table 2. Freshly Trypsinized Cells in Suspension

Parameter studied
- TSH stimulation of iodide uptake

Table 3. Primary Monolayer Cultures

Parameters studied
- TSH/receptor interactions
- TSH stimulation of cAMP metabolism
- TSH stimulation of iodide metabolism
- Expression and TSH regulation of Tg synthesis
- Trophic effect of TSH
- Mitogenic role of TSH
- Mitogenic and differentiative role of other growth factors

demonstrated homologies between different receptors (16-18), and between receptors and oncogenes (19,20). These homologies suggest very important involvement of normal regulatory pathways in unregulated, neoplastic growth (Table 3).

Primary follicles in suspension. Suspension cultures of thyroid follicles are also short-term but may be considered a very physiological *in vitro* model reproducing the three-dimensional thyroid fractional unit, i.e., the follicle. Follicles in suspension may be heterogeneous in cell composition, with the follicular cells comprising the vast majority of the cell population. This was also a characteristic of the monolayer short-term culture system.

Although most of the studies performed on primary suspension cultures dealt with cell polarity (21-23) and follicle formation, the mitogenic role of TSH was demonstrated in 1980 (24). In primary follicles of rat thyroids, Nitsch and Wollman showed by electron microscopy studies that significantly more mitotic figures were present in sections of TSH-stimulated vs unstimulated cultures in serum-free media (Table 4).

Long-term monolayer cultures. In 1980, a cell culture system was developed in our laboratory from Fischer rat thyroids. Differentiated cells from normal adult glands have been adapted to grow either in low (0.5%) serum (25) or high (5%) serum (26) concentrations. Both cell strains (FRTL and FRTL5, respectively) not only respond to TSH mitogenic stimulation but their growth *in vitro* depends on the continuous presence of the hormone in the culture medium. Both FRTL and FRTL5 require, for optimal growth, a mixture of six hormones and growth factors (25), among which TSH is certainly the most important. Because of the purity of both TSH (National Hormones and Pituitary Program, USA, 21 mU/ml) and of the cell population,

Table 4. Primary Follicles in Suspension

Parameters studied
- Cell polarity
- Follicle formation
- Mitogenic role of TSH

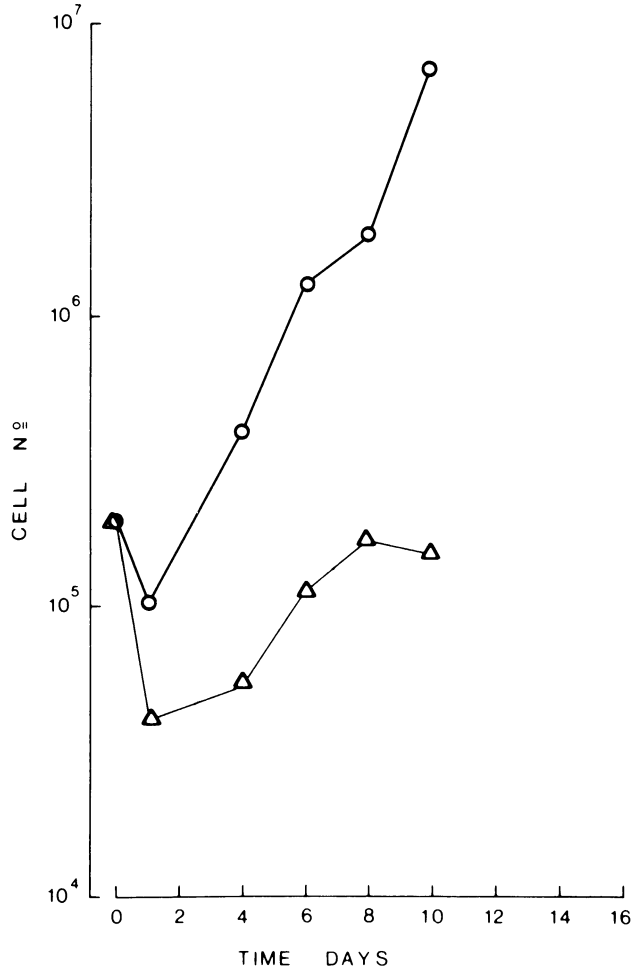

Fig. 1. Growth curve of FRTL5 cells in full hormonal complement (o) and in 5H, lacking TSH (Δ).

the mitogenic effect of the hormone is clearly demonstrated. Moreover, this cell system appears to be very useful in studying the mechanism of action of TSH, particularly as a growth factor. The long-term growth of FRTL5 cells, their fast duplication rate, and their nonmalignant and differentiated phenotype, make these cells a close-to-ideal system for growth control experiments.

A simple growth curve demonstrates the essential role of TSH (Fig. 1). Upon reapplication of the hormone to starved cells, several events such as TSH receptor clustering and internalization (27), cytoscheleton reorganization (28), and adenylate-cyclase stimulation occur within the first 1-2 hours, whereas mitogenic stimulation occurs only after a lag-phase of 24-36 hours (29). The interval between early and late events in TSH stimulation demonstrates the existence of separate TSH bioeffects in thyroid cells and permits speculation on possible intracellular pathways. Work in our laboratory is now largely devoted to the elucidation of such pathways following two approaches.

The genetic approach. TSH-independent mutants have been obtained from FRTL5 cells. Such mutants (Endocrinology, in press) still have TSH-stimulatable adenylate-cyclase but grow in medium lacking TSH. Furthermore, addition of the hormone does not produce any mitogenic effect. In analogy to other prokariotic and eukariotic mutant cells, the characterization of the genetic lesion may lead to identification of biochemical steps in hormonal studies.

The cellular endocrinology approach. It is possible that complex interactions between hormones and growth factors may be involved in proliferation control in vivo. In our cellular system it is now possible to study these processes. EGF, a growth factor very active on several cell types, has been tested on FRTL5 cells. EGF added alone did not show significant growth stimulation. However, preliminary experiments show that EGF, added together with TSH, displays cooperativity with the hormone. EGF concentrations from 0.1 pg/ml to 100 ng/ml shift the TSH dose-response to lower concentrations by more than two log units. Such EGF-TSH cooperativity is effective only at submaximal TSH concentrations, a situation that probably occurs physiologically in vivo.

The finding of cooperative effects between EGF and TSH suggests that other hormonal interactions may be involved as regulatory mechanisms for thyroid follicular cells, as well as for other differentiated cell types. In our opinion, these complex interactions and mechanisms can be best approached in studies of endocrine cell biology in vitro since they offer the possibility of detailed analysis of individual, as well as multiple ligand effects of homogeneous cell populations. It is also our opinion that control of cell proliferation may be regarded as one aspect of cell differentiation and tissue-specific gene expression, genetically programmed in the developmental stage and maintained in normal conditions through the entire life of the organism.

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REFERENCES

1. Doniach D, Bottazzo GF, and Khoury EL. In A Pinchera, D Doniach, GF Fenzi, et al. (eds), Autoimmune Aspects of Endocrine Disorders, Academic Press, London, 1980, p 25.
2. Tong W, Kerkof P, and Chaikoff IL. Biochim Biophys Acta 60: 1, 1962.
3. Rapaport B and Adams RJ. J Biol Chem 251: 6653, 1976.
4. Rapaport B. Endocrinology 98: 1189, 1976.
5. Winand RJ and Kohn LD. J Biol Chem 250: 6534, 1975.
6. Ollis CA, Munro DS, and Tomlinson S. J Endocrinol 95: 237, 1982.
7. Fayet G, Hovsepian S, Dickson JC, et al. J Cell Biol 93: 479, 1982.
8. Wadeleux PA, Etienne-Decerf J, Winand RJ, et al. Endocrinology 102: 889, 1978.
9. Westermark B, Karlsson FA, and Walinder O. Proc Natl Acad Sci USA 76: 2022, 1979.
10. Roger PR and Dumont JE. FEBS Lett 144: 209, 1982.
11. Roger PP, Servais P, and Dumont JE. FEBS Lett 157: 323, 1983.
12. Bachrach LK, Eggo MC, Mak WW, et al. In MC Eggo and GN Burrow (eds), Thyroglobulin - The Prothyroid Hormone, Raven Press, New York, Vol. 2, 1985, p 263.
13. Roger PR and Dumont JE. J Endocrinol 96: 241, 1983.
14. Roger PR and Dumont JE. Mol Cell Endocrinol 36: 79, 1984.

15. Westermark K, Karlsson FA, Ericson LE, et al. In MC Eggo and GN Burrow (eds), Thyroglobulin - The Prothyroid Hormone, Raven Press, New York, Vol. 2, 1985, p 255.
16. Ullrich A, Bell JR, Chen EY, et al. Nature 313: 756, 1985.
17. Ebina Y, Ellis L, Jarnagin K, et al. Cell 40: 747, 1985.
18. Perrotti N, Taylor SI, Richert ND, et al. Science 227: 761, 1985.
19. Robbins KC, Antoniades HN, Devare SG, et al. Nature 305: 605, 1983.
20. Downward J, Yarden Y, Mayes E, et al. Nature 307: 521, 1984.
21. Inoue K, Horiuchi R, and Kondo Y. Endocrinology 107: 1162, 1980.
22. Nitsch L and Wollman SH. Proc Natl Acad Sci USA 77: 472, 1980.
23. Mauchamp JA, Margotat M, Chambard B, et al. Cell Tissue Res 204: 417, 1979.
24. Nitsch L and Wollman SH. Proc Natl Acad Sci USA 77: 2747, 1980.
25. Ambesi-Impiombato FS, Parks LAM, and Coon HG. Proc Natl Acad Sci USA 77: 3455, 1980.
26. Ambesi-Impiombato FS, Picone R, and Tramontano D. Cold Spring Harbor Conf on Cell Prolif 9: 483, 1982.
27. Avivi A, Tramontano D, Ambesi-Impiombato FS, et al. Mol Cell Endocrinol 25: 55, 1982.
28. Tramontano D, Avivi A, Ambesi-Impiombato FS, et al. Exp Cell Res 137: 269, 1982.
29. Valente WA, Vitti P, Kohn LD, et al. Endocrinology 112: 71, 1983.

CONTROL OF PROLIFERATION AND OF THE EXPRESSION OF DIFFERENTIATION IN THYROID CELLS IN CULTURE

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In pluricellular organisms, the biology of individual cells is tightly controlled to follow the constraints and requirements of the organism as a whole. These controls are mostly expressed through extracellular signal molecules (hormones, local hormones, neurotransmitters, etc.). The cellular processes submitted to such controls can be divided into three broad categories: those dealing with the quantitative regulation of function, of growth and proliferation, and the qualitative expression of differentiation. Perversion of the two latter processes leads to tumorigenesis. The study of growth and differentiation expression has mostly used cells in culture as experimental models. In this short review, we wish to report some recent data on the control of dog thyroid cells in primary cultures and compare them to parallel results obtained with other systems and species.

The proliferation and growth of cells is controlled mainly by two classes of extracellular signal molecules, hormones and growth factors. In the case of dog thyroid cells, the following agents have been shown to directly modulate proliferation and expression of differentiation: a) hormones: thyrotropin (TSH), insulin, somatomedin, hydrocortisone; b) growth factors: epidermal growth factor (EGF), fibroblast growth factor (FGF); c) serum; d) pharmacological probes of intracellular signal systems: phorbol esters, forskolin, cholera toxin.

Dog thyroid cells were cultured in serum-free, insulin-supplemented medium as described elsewhere. Cell proliferation has been measured by four methods: cell counting, DNA content of the culture, ^{3}H thymidine incorporation into DNA, and the counting of labeled nuclei after ^{3}H thymidine addition to the medium. Most results have been obtained using three of these methods. In general, treatments were generally applied after four days of culture to cells that were quiescent (1 to 2% labeled nuclei per 24 hours). Total ^{3}H thymidine incorporation into DNA which is sometimes used as the only measurement, is open to the criticism that it may reflect variations in deoxyribonucleoside phosphate pool and specific activity. The expression of differentiation has been measured by iodide transport at equilibrium (C/M), iodide binding to proteins in the cells, and thyroglobulin mRNA levels.

The first question asked concerns the kinetics of proliferation after addition of various stimulating agents to the quiescent cells. Several agents, such as thyrotropin (TSH), epidermal growth factor (EGF), serum and phorbol esters, can induce, after a prereplicative phase of 15-18 hrs, the DNA synthesis of such cells, only a minority of which has proliferated in vitro before stimulation (start of DNA synthesis G_1/S transition). Added alone in the presence of insulin (5 µg/ml), these different growth factors are sufficient to induce a wave of DNA synthesis in a part of the cell population, while the combination of TSH, EGF, and fetal calf serum (10%) induces a maximum DNA synthesis rate (>90% of the cells were labeled within 48 hrs) and an exponential proliferation (doubling time: 31 hrs) until confluence is reached. This shows that in our cultivated cells, the proliferative response to maximal stimulation is general and that under such conditions there is no distinction between a population of stem cells and a population of nonproliferating finally differentiated cells. If such a distinction is justified in vivo, it does not reflect on the behaviour of the cells in vitro. A percentage labeled mitoses curve from exponentially growing cells treated with EGF, TSH and serum together showed that the minimum time of cycle is 27 h (<G2 + M>: 3.5 h; <S>: 7 h). From these data, the G1 period is estimated to be at least 16 h which closely corresponds to the duration of the prereplicative phase of quiescent cells. This indicates that the quiescent thyroid cells are arrested in an early G1 stage rather than in an out of cycle G0 phase, which supports our first conclusion.

Cell counting or DNA measurement shows that our dog thyroid cells are only able to divide four to five times. This limit is reached before confluency, which suggests an endogenous limitation. Whether this limitation reflects the in vivo situation or an artefact of the culture system is not yet known.

In the presence of insulin, TSH triggers a 30-60% cumulative labeling index within 48 h of exposure. The effect of TSH on proliferation is mimicked by cholera toxin and forskolin, whose sole known biochemical action is to activate adenylate cyclase, and by dibutyryl cyclic AMP, an analog of cyclic AMP. It is, therefore, sufficient to increase cAMP levels to induce DNA synthesis in a significant part of the thyroid cell population. This clearly shows that this effect of TSH in these cells is mediated by cyclic AMP. Forskolin is particularly interesting for such work, as its effect (cyclic AMP accumulation) is very fast and disappears in a few minutes after washing of the cells. It is, therefore, possible to apply pulses of cyclic AMP accumulation or to interrupt continuous stimulation by pulses of normal resting cyclic AMP concentrations. The modulation of the length of cAMP accumulation by exposure of the cells for increasing or discontinuous periods to forskolin shows that a continuous cAMP rise for most of the prereplicative phase is necessary to support the progression in this phase until the commitment to DNA synthesis (2 hours before the start of DNA synthesis = restriction point). Interruptions in the cAMP rise as short as 2 h delayed the onset of DNA synthesis but did not postpone it by another 17 h period. These delays depend on both the moment and the duration of the cAMP interruptions. This shows that the system has a memory but that the cascade of events leading to the decision of DNA synthesis is reversible until the restriction point. Similar excessive delays in the onset of DNA synthesis are also produced by reversibly blocking protein synthesis with cycloheximide. This suggests that the induction by TSH of a commitment to DNA prereplication strictly depends on peculiarly labile cAMP-dependent events which might well be the induction by cAMP of the synthesis of a key labile protein.

The second question asked concerns the role and action of various extracellular and intracellular signal molecules in the control of proliferation and differentiation expression in our cells. After a few hours, TSH

causes the reaction and rounding up of the thyroid cells. This effect is also mimicked by cholera toxin, forskolin, and cyclic AMP analogues and, therefore, mediated by cyclic AMP. The fact that it involves all cells in the culture demonstrates the homogeneity of the culture and is further proof that these cells are follicular. After one day, TSH through cyclic AMP induces proliferation in quiescent cells, but it also maintains or reinduces the expression of differentiation. The fact that these effects are mimicked by combinations of cyclic AMP analogues which specifically activate cyclic AMP-dependent protein kinases indicates that they are mediated by these kinases. Several protein substrates of these kinases have been identified on 2D gel electrophoregram, but their nature and role are still unknown. On the other hand, the fact that the proliferation effects of TSH are not mimicked by the short-acting activator of adenylate cyclase prostaglandin E, nor by pulses of forskolin, shows again that prolonged accumulation of cyclic AMP is needed. It has been shown that in porcine thyroid cells, TSH enhances the availability of EGF receptors. However, this could not explain the proliferative effect of TSH in dog cells, as it occurs with the same kinetics as the EGF effect and in the absence of serum or EGF.

Epidermal growth factor (EGF), like TSH, stimulates the proliferation of thyroid cells. Since EGF does not enhance cyclic AMP accumulation, its effect is not mediated by cyclic AMP. On the other hand, EGF accelerates the disappearance of differentiation markers in control cells, still decreases their low expression at equilibrium, and inhibits TSH reinduction of these markers. TSH and EGF act in a competitive manner on this parameter. The effects of EGF, as those of TSH, are delayed. Similar results have been obtained by Westermark and by Eggo and Burrow in porcine and ovine cultures. As the dedifferentiation action of EGF is observed in cells at quiescence at the end of proliferation, it cannot be ascribed to the acceleration of proliferation. Bachrach has shown in ovine cells that EGF also decreases cyclic AMP accumulation. However, in short-time incubation (4 hours) we have observed no such effect in dog thyroid slices.

It is interesting that the stimulation of proliferation and the extinction effect on differentiation markers of EGF is mimicked by phorbol esters (e.g., TPA). These agents have been shown to be probes of the diacylglycerol-regulated protein kinase C. As EGF has been shown in other cells to activate phosphoinositide hydrolysis and diacylglycerol generation, it is possible that the action of EGF is mediated by this intracellular signal. However, contrary to EGF, the action of phorbol esters decreases after 4-6 days. Moreover, phorbol esters decrease to their level of stimulation the effects of EGF concentrations added concomitantly. Similar effects of TPA have been observed by Bachrach in ovine cells.

It should be emphasized that both EGF and TPA decrease the expression of differentiation markers in the thyroid cells, but do not truly dedifferentiate them. Indeed, even when differentiation markers are no longer evident, these cells can still be induced to express the markers by TSH or forskolin. Thus, these cells have retained their identity of thyroid follicular cells and, thus, respond to their trophic hormone TSH by reexpressing their differentiated characteristics. It is rather striking that the whole EGF circuit has apparently been lost in the FRTL cell line.

The DNA synthesis induction by TSH (cAMP) or by EGF depends on, or is potentiated by, insulin. Strikingly, nanomolar concentrations of insulin are already sufficient to support a stimulation of DNA synthesis by TSH, while micromolar concentrations of insulin are absolutely required for the action of EGF. This suggests that insulin supports the action of TSH by acting on its own high-affinity receptors, while the effects of EGF are dependent on the somatomedin-like effects of high concentrations of insulin. Insulin always supports the progression in the prereplicative phase

stimulated by TSH, but it is not absolutely necessary for the initiation of the prereplicative phase by TSH in all the experiments. A marked synergism is also observed, in the presence of high insulin concentrations, between the increase of cAMP and EGF, for the induction of DNA synthesis. Thus, in the stimulation of proliferation by EGF, the observed effect of insulin is probably mediated by somatomedin (IGF_1) receptors, while in the support of the action of TSH, both insulin and somatomedin receptors may be involved.

Fibroblast growth factor (FGF) and serum induce dog thyroid cell growth as EGF, but they do not affect the expression of differentiation or the TSH-cyclic AMP action on this expression. However, both in the presence of FGF and serum, the expression of differentiation markedly decreases during the proliferation phase. It is interesting that platelet-derived growth factor (PDGF) has no effect on porcine or dog thyroid cells.

Hydrocortisone greatly enhances the effect of TSH on the expression of differentiation markers in dog thyroid cells. A complementary effect on the induction of growth has also been observed in an ovine thyroid cell line (OVNI).

A third question we have asked to our system concerns the mechanism of the induction of proliferation by various agents or, more precisely, which newly synthesized proteins could be involved. Our aim was to appreciate whether different mitogenic stimuli acting by different intracellular signals could finally converge to activate the synthesis of a unique set of key proteins before commitment to DNA synthesis. Protein synthesis in the G1 period of the cell cycle has been investigated using 2D electrophoresis in the quiescent cultures induced to proliferate by the combined action of thyrotropin (TSH) (acting through cAMP), epidermal growth factor (EGF), and serum (through cAMP-independent mechanisms), or by each of these agents acting alone. The analysis of the proteins pulse-labeled for 3 h with (^{35}S) methionine in quiescent cells deprived of serum and in cells that had been stimulated for various periods of time by the addition of TSH, EGF, and serum showed maximal modifications before entry into S phase; the labeling of at least 10 proteins was enhanced while labeling of at least six proteins was decreased. The synthesis of one of these proteins (protein 1; Mr 81000) showed some peculiarity; it was maximal 9-12 h after stimulation by the proliferative agents but began to decrease at 15-18 h and was still decreased at 29-32 h. The study of the effects of each of the proliferative agents alone on the labeling of these 16 proteins showed that TSH specifically stimulated the labeling of eight polypeptides (proteins 2 to 9) and that, in contrast, EGF and serum specifically increased the labeling of two other proteins (proteins 1 and 10). The labeling of one protein was decreased by each of the different agents (protein 6'), while TSH specifically decreased the labeling of four polypeptides (protein 1' to 4') and increased the labeling of one polypeptide (protein 5') whose synthesis was decreased by EGF and serum. Cholera toxin perfectly mimicked all these effects of TSH. The labeling of two of these proteins was no more stimulated when the cells no longer proliferated at confluency (proteins 1 and 7). Protein 7 has a Mr and an isoelectric point similar to those of cyclin/PCNA (37 kD, pI 5).

We have, therefore, not identified one unique protein, the labeling of which is increased by each of the different mitogenic stimuli. Consequently, no one specific increase in the synthesis of proteins can be considered as a marker of the entry of thyroid cells into S phase. However, the effect of TSH on protein 7 labeling is specifically potentiated by EGF and serum, while the effect of EGF and serum on protein 1 is specifically potentiated by TSH. Thus, the synthesis of these two proteins shows a correlation with the stimulation of DNA synthesis which is much greater when the mitogenic agents are acting in combination.

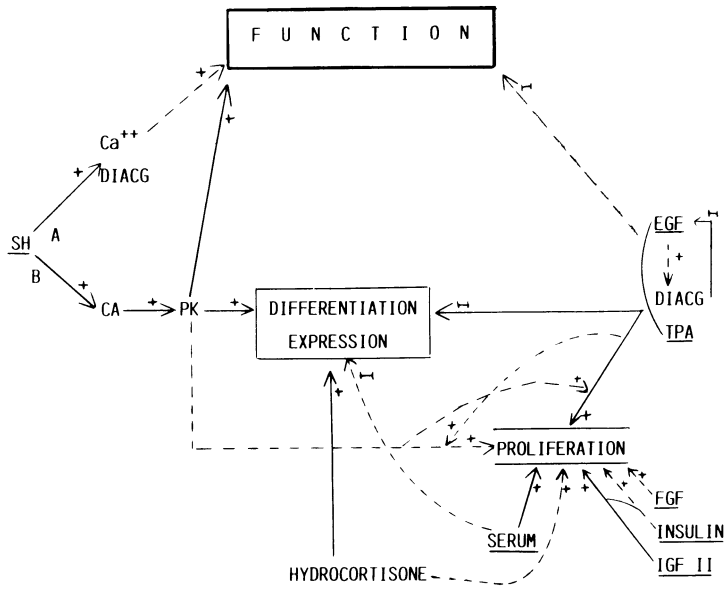

Fig. 1. Diagram of extracellular and intracellular signals controlling proliferation and expression of differentiation in thyroid cells.

A possible interpretation of our data is that in thyroid cells, cAMP-dependent and cAMP-independent mitogenic stimuli induce not one, but at least two essentially independent sequences of events, each one culminating with the synthesis of one peculiar protein before DNA synthesis. In order to definitively test such a hypothesis, we are currently developing antibodies against proteins 1 and 7.

Our present concept of the role of various extracellular and intracellular signals in the control of proliferation and of the expression of differentiation in thyroid cells is summarized in Fig. 1.

REFERENCES

1. Ambesi-Impiombato FS, Parks LAM, and Coon HG. Proc Natl Acad Sci USA 77: 3455, 1980.
2. Ambesi-Impiombato FS, Picone R, and Tramontano D. In GH Sato, AB Pardee, and DA Sirbasku (eds), Cold Spring Harbor Conferences on Cell Proliferation, Vol. 9, Cold Spring Harbor, 1982, p 483.
3. Avvedimento VE, Tramontano D, Ursini MV, et al. Biochem Biophys Res Commun 122: 472, 1984.
4. Bachrach K, Eggo MC, Mak WW, et al. Endocrinology 116: 1603, 1985.
5. Dumont JE, Roger P, Van Heuverswyn B, et al. Adv Cyclic Nucleotide Res 17: 337, 1984.

6. Eggo MC, Bachrach LK, Fayet G, et al. Mol Cell Endocrinol 38: 141, 1984.
7. Errick JE, Eggo MC, and Burrow GN. In MC Eggo and GN Burrow (eds), Progress in Endocrine Research and Therapy, Vol. 2, Raven Press, New York, 1985, p 271.
8. Fayet G and Hovsepian S. In MC Eggo and GN Burrow (eds), Progress in Endocrine Research and Therapy, Vol. 2, Raven Press, New York, 1985, p 211.
9. Fayet G, Hovsepian S, Dickson JG, et al. J Cell Biol 93: 479, 1982.
10. Lamy F, Roger PP, Lecocq R, et al. Eur J Biochem (submitted).
11. Magnusson RP and Rapoport B. Endocrinology 116: 1493, 1985.
12. Nitsch L and Wollman SH. Proc Natl Acad Sci USA 77: 2743, 1980.
13. Passareiro H, Roger PP, Lamy F, et al. Eur J Biochem 147: 263, 1985.
14. Roger PP and Dumont JE. FEBS Lett 144: 209, 1982.
15. Roger PP and Dumont JE. J Endocrinol 96: 241, 1983.
16. Roger PP and Dumont JE. Mol Cell Endocrinol 36: 79, 1984.
17. Roger PP, Reuse S, Servais P, et al. Cancer Res (in press).
18. Roger PP, Servais P, and Dumont JE. FEBS Lett 157: 323, 1983.
19. Roger PP, Van Heuverswyn B, Lambert C, et al. Eur J Biochem 1985 (in press).
20. Westermark K, Karlsson FA, and Westermark B. Endocrinology 112: 1680, 1983.
21. Westermark K, Karlsson FA, and Westermark B. Mol Cell Endocrinol 40: 17, 1985.
22. Westermark K and Westermark B. Exp Cell Res 138: 47, 1982.

POST-RECEPTOR EVENTS IN GROWTH CONTROL*

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TSH regulates thyroid function and growth. However, both clinical observations and experimental results have suggested that TSH is necessary for, but not the trigger of, gland growth. Several findings support this hypothesis:

1- Glands with decreased iodine stores are hypersensitive to TSH (1,2).
2- Serum TSH is normal in some endemic goiter areas (3) and in most thyroid cancers (4,5).
3- Experimental goiter (LID, MTU, etc.) develops before an increase in serum TSH is detected (6,7).
4- Goiter size in some endemic areas is inversely related to iodine content (8).
5- Compensatory thyroid growth, after hemithyroidectomy, may occur in the absence of TSH (9,10).

These results led to the idea that some factors, intrinsic to the thyroid, modulate the response of the gland to TSH (or other factors). Further studies have shown that the concentration of organic iodine in the gland is related to changes in the response to stimulators or inhibitors. A decrease in this parameter is associated with increased sensitivity to TSH, while the opposite is observed when excess iodine is administered. This constitutes the so-called autoregulatory mechanism (11,12). In human benign tumors, the concentration of total iodine, T_3, and T_4 is significantly decreased (13) (Table 1), a finding that supports the hypothesis that the autoregulatory mechanism may play a role in the onset of thyroid growth.

Excess iodine inhibits many thyroid parameters, through the iodo-organic intermediate (11,12), whose exact nature is not known. Iodothyronines have been proposed to be such intermediates, since they mimic some, but not all, of the inhibitory effects of iodine on the gland (12), and nuclear T_3 receptors have been found in calf thyroid (14). However, their role is still controversial.

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†Established investigators from the CONICET.

Table 1. Thyroid Content of Total Iodine, T_3, and T_4

	Normal (n=7)	Adenoma (n=6)	Adenomatous goiter (n=5)
Iodine			
µg/g tissue	472 ± 40	126 ± 24[a]	62 ± 18[a]
µg/mg protein	5.6 ± 1.2	1.6 ± 0.4[a]	0.6 ± 0.2[a]
µg/mg DNA	104 ± 18	38 ± 2[a]	14 ± 4[a]
Thyroxine			
µg/g tissue	174 ± 27	38 ± 16[b]	6 ± 3[b]
µg/mg protein	1800 ± 300	510 ± 210[b]	70 ± 30[b]
µg/mg DNA	38.6 ± 6.3	8.8 ± 3.2[b]	1.3 ± 0.7[b]
Triiodothyronine			
µg/g tissue	4.5 ± 0.6	0.6 ± 0.1[b]	0.2 ± 0.1[b]
µg/mg protein	40 ± 5	7 ± 2[b]	3 ± 1[b]
µg/mg DNA	1020 ± 210	128 ± 27[b]	51 ± 35[b]

Each value represents the mean ± SEM; a: $p<0.01$; b: $p<0.001$.
Reproduced from (13) with permission.

It is known that, besides thyroid hormones, iodine may be incorporated into lipids in glands from different species (15-21). Boeynaems and Hubbard (22) have shown that iodine is incorporated into an iodolactone and derived compounds. Recent results from our laboratory have provided additional information concerning lipid biosynthesis and regulation (23):

1- Lipid iodination correlates in parallel with hormone biosynthesis.
2- ^{125}I incorporation into iodolipid is inhibited by PTU and MMI.
3- Thyroid iodolipids are not bound to protein.
4- They are mainly localized in the mitochondrial-microsomal fraction.
5- Most important compounds are: a) iodinated free fatty acids (around 50%); b) iodinated neutral lipids (20%).
6- Radioiodine incorporation into iodolipids is increased by TSH, T_3, and indomethacin, and decreased by mepachrine (inhibitor of phospholipase A_2).

These data, together with that previously obtained at different laboratories, have suggested that arachidonic acid might undergo different pathways in the thyroid (Fig. 1): a) biosynthesis of prostaglandins and related compounds (cyclo oxygenase); b) biosynthesis of leukotrienes (lipoxigenase); c) biosynthesis of iodinated derivatives of arachidonic acid (peroxidase). It is not known whether the peroxidase involved in this latter pathway corresponds to the enzyme which catalyzes thyroid hormone formation.

In calf thyroid, we have observed the biosynthesis of ^{125}I-labeled 15-iodo-14-hydroxy and 15-hydroxy-14-iodo-eicosatrienoic acid (I-OH-A) and the corresponding iodolactone (to be published). These compounds were prepared by organic synthesis and their effects on thyroid function were examined. In previous results, we have shown that a non-purified preparation inhibits *in vitro* different parameters, such as iodine uptake and organification, and

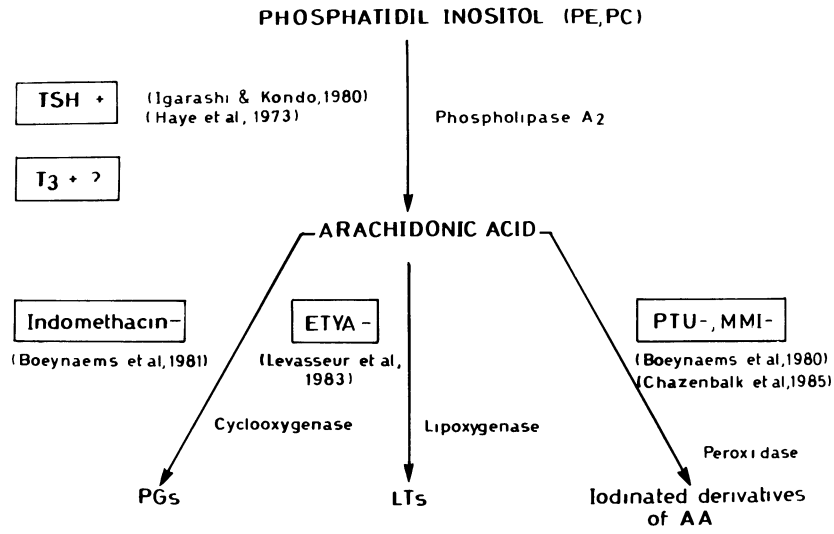

Fig. 1. Possible pathways of arachidonic acid in the thyroid.

^{3}H-uridine incorporation into RNA (24). When purified preparations were examined, we found that I-OH-A is a potent inhibitor of iodine metabolism and of cyclic AMP formation (25). In vivo studies showed that I-OH-A inhibits the goitrogenic action of MMI. When rats were treated with MMI, thyroid weight increased by around 120-130%. This could be prevented by the simultaneous administration of 5 µg/day of I-OH-A. Arachidonic acid was without effect. When a dose of KI, equivalent to the iodine that would be generated

Table 2. Prevention of MMI-induced Goiter in the Rat

Treatment	Thyroid weight mg/100 g b.wt.	% Variation vs control	vs MMI
Control	9.66 ± 0.34		
MMI 6 mg/day	21.52 ± 0.68 (a)	+ 122	
I-OH-A 5 µg/day	10.12 ± 0.59	+ 4	
AA 5 µg/day	11.46 ± 0.88 (b)	+ 19	
T_3 5 µg/day	7.11 ± 0.23 (a)	- 20	
KI 1.25 µg/day	10.76 ± 0.99	+ 11	
MMI + I-OH-A	14.85 ± 0.78 (c)		- 57
MMI + AA	22.50 ± 0.73		+ 4
MMI + T_3	10.30 ± 0.24 (c)		- 95
MMI + KI	20.36 ± 1.66		- 5

Groups of six rats were injected with the compounds during 10 days. Each value is the average ± SEM. a: $p<0.01$ vs control; b: $p<0.02$ vs control; c: $p<0.001$ vs MMI.

Table 3. Serum TSH During Goiter Induction and Prevention

Treatment	Days	Thyroid weight mg/100 g b.wt.	Serum TSH μU/ml
Control	3	6.87 ± 0.35	21 ± 1
MMI	3	16.26 ± 0.81 (a)	190 ± 18
MMI + I-OH-A	3	11.94 ± 0.38 (b)	208 ± 12
Control	25	4.62 ± 0.23	21 ± 3
MMI	25	16.04 ± 0.98 (a)	234 ± 29
MMI + I-OH-A	25	9.22 ± 0.82 (b)	180 ± 30

Each value vs the average of six rats ± SEM. a: $p<0.0005$ vs controls; b: $p<0.0005$ vs MMI.

from the complete dehalogenation of I-OH-A, was injected, no action was found. These data indicated that I-OH-A exerts its effect per se and not through its possible metabolites. This is further supported by the fact that I-OH-A caused an inhibition of thyroid growth in the presence of MMI which blocks iodine organification. T_3 at a dose of 5 μg/day, also caused an inhibition of gland growth (Table 2), but I-OH-A appears to have a different mechanism of action. The administration of this latter compound did not alter the serum levels of T_3, T_4 (not shown), and TSH (Table 3). When thyroid cyclic AMP content was measured, we found that it increased after MMI treatment. The injection of I-OH-A caused a significant decrease in both basal and in MMI-stimulated values (Table 4). Therefore, we may conclude that I-OH-A has a direct inhibitory effect on thyroid growth.

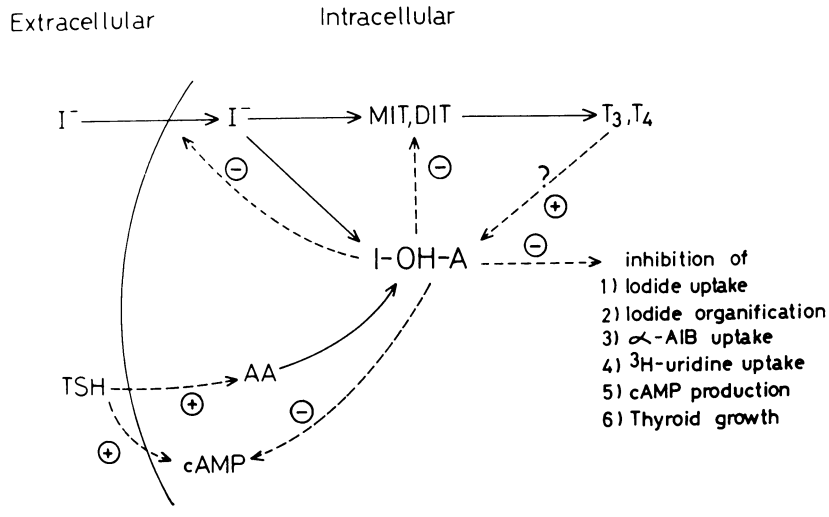

Fig. 2. Diagram illustrating how iodinated lipids in the thyroid gland mimic the effects of excess iodine.

Table 4. Thyroid Cyclic AMP Content During Goiter Induction and Prevention

Treatment	Thyroid weight mg/100 g b.wt.	% Variation vs control	% Variation vs MMI	Thyroid cAMP pMoles/mg prot.	% Variation vs control	% Variation vs MMI
Control	7.1 ± 1.3			13.6 ± 1.9		
MMI (5 mg/day)	22.0 ± 1.0 (a)	+ 210		25.0 ± 1.1 (a)	+ 83	
I-OH-A (5 µg/day)	7.7 ± 0.2			9.2 ± 1.0 (b)	- 32	
MMI + I-OH-A	14.3 ± 0.4 (c)	+ 101	- 52	11.4 ± 0.3 (c)		- 100

Each value is the average of 10 rats ± SEM. The animals were treated during 10 days. a: $p<0.0005$; b: $p<0.05$ vs control; c: $p<0.0005$ vs MMI.

It has been shown that KI inhibits TSH stimulation of cAMP generation in the thyroid through an organic intermediate (26,27). The results herein presented, and those observed in vitro (25), demonstrate that I-OH-A mimics the effects of KI on cAMP production. Since cAMP is a well-known stimulator of thyroid growth (28), we may explain the inhibitory action of I-OH-A through an effect on cAMP generation, without excluding other sites of action.

In summary, the present available information supports the hypothesis that an alteration in the autoregulatory mechanism would be linked to the initiation of thyroid growth in the presence of normal levels of TSH. Although the exact nature of the organic intermediates involved is not disclosed, the data herein discussed demonstates, for the first time, that some iodinated lipids, synthetized by the gland, mimic the effects of excess iodine, thus partially fulfilling the requirements to be such intermediates (Fig. 2). Further studies are being performed in our laboratory in order to clarify this point.

REFERENCES

1. Bray GA. J Clin Invest 47: 1640, 1968.
2. Agrawal R and Furth E. 48th Annual Meeting of the American Thyroid Association, Abstr. 50, 1972.
3. Pisarev MA, Utiger RD, Salvaneschi JP, et al. J Clin Endocrinol Metab 30: 680, 1970.
4. Mayberry WE, Gharib MM, Bilstad MM, et al. Ann Int Med 74: 471, 1971.
5. Greenspan FS, Lowenstein JM, West MN, et al. J Clin Endocrinol Metab 35: 795, 1972.
6. Berthier C and Lemarchand-Beraud T. Acta Endocrinol (Kbh) 89: 567, 1978.
7. Naejie R, Vanhaelst L, and Goldstein J. Horm Metab Res 10: 521, 1978.
8. Hellstern P, Keller HE, Weinheimer B, et al. Clin Endocrinol (Oxf) 9: 351, 1978.
9. Lewinsky A, Bartke A, and Smith KR. Endocrinology 113: 2317, 1983.
10. Romeo H, Cardinali DP, and Boado RJ. Neuroendocrinology, in press.
11. Pisarev MA and Kleiman de Pisarev DL. J Endocrinol Invest 3: 317, 1980.
12. Pisarev MA. J Endocrinol Invest, in press.
13. Krawiec L, Kleiman de Pisarev DL, Pisarev MA, et al. Acta Physiol Pharmacol Latinoamer 34: 375, 1984.
14. Pisarev MA, Juvenal GJ, Kleiman de Pisarev DL, et al. Horm Metab Res, in press.
15. Taurog A, Tong W, and Chaikoff IL. J Biol Chem 213: 119, 1955.
16. Taurog A, Tong W, and Chaikoff IL. J Biol Chem 235: 1390, 1960.
17. DeGroot LJ and Carvalho E. J Biol Chem 227: 759, 1957.
18. Mauchamp J, Nunez J, and Roche J. C R Soc Biol (Paris) 157: 971, 1963.
19. Rodesch F and Dumont JE. Exp Cell Res 47: 386, 1967.
20. Shah DH, Thakare UR, Showmkeen RC, et al. Acta Endocrinol (Kbh) 74: 461, 1973.
21. Rousset B, Poncet C, Dumont JE, et al. Biochem J 192: 801, 1980.
22. Boeynaems JM and Hubbard WC. J Biol Chem 255: 9001, 1980.
23. Chazenbalk GD, Pisarev MA, Juvenal GJ, et al. Acta Endocrinol (Kbh) 108: 72, 1985.
24. Chazenbalk GD, Pisarev MA, Krawiec L, et al. Acta Physiol Pharmacol Latinoamer 34: 367, 1984.
25. Pisarev MA, Chazenbalk GD, Krawiec L, et al. Ninth Internat Thyroid Congress, Abstr., 1985.
26. Van Sande J and Dumont JE. Biochem Biophys Acta 313: 320, 1973.
27. Filetti S and Rapoport B. Endocrinology 113: 1608, 1983.
28. Pisarev MA, DeGroot LJ, and Wilber JF. Endocrinology 83: 339, 1970.

GROWTH CONTROL AND FOLLICULAR CELL NEOPLASIA*

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In the previous lectures of this symposium we have been informed of the actual state of knowledge in the fields of growth control of follicular cells in vitro (Dr. Ambesi-Impiombato), of growth factors other than TSH (Dr. Dumont), and of metabolic events evolving in the growing cell (Dr. Pisarev). It now remains to be seen how the normal thyroid grows, how it is transformed into a goiter within the intact organism, and how a neoplasia can possibly develop from a normal gland.

Intense acute growth stimulation by TSH or thyroid-stimulating immunoglobulins invariably produces hyperplasia of all thyroid follicles and of all cells within a follicle, together with depletion of the colloid stores. The best known example is that of experimental goiters produced by chronic iodine deficiency. However, two different thyroid glands exposed to the same degree of stimulation acting for an identical length of time may respond with a very different growth pattern, if the local intrathyroidal environment is not strictly identical.

A striking example is provided by the goiters produced in rats by diphenylthiohydantoin (DPTH)-feeding together with either high or low iodine supply (1). DPTH is a goitrogen that does not interfere with organic binding of iodine, but rather acts through increasing biliary loss of thyroxine. We have previously demonstrated (1) that DPTH produces the classic type of hyperplastic goiters, devoid of colloid, if insufficient iodine is available. In contrast, large colloid-filled lumina with less columnar epithelial shells are produced if ample iodine is provided. Thus, the growth pattern of these goiters strikingly depends on the availability of iodine.

Similarly, the growth of Graves' goiters greatly depends on local intrathyroidal factors. While acute Graves' goiters invariably grow in a thoroughly homogeneous macroscopic and microscopic pattern, this is no longer true in the more chronic disease. Not only may gross macroscopic nodules appear in long-standing Graves' goiters, but a microscopic work-up of the gland may reveal entirely different growth patterns within the same gland. When transplanted into nude mice, the different patterns may be faithfully reproduced by the grafts (Fig. 1), thus indicating that the

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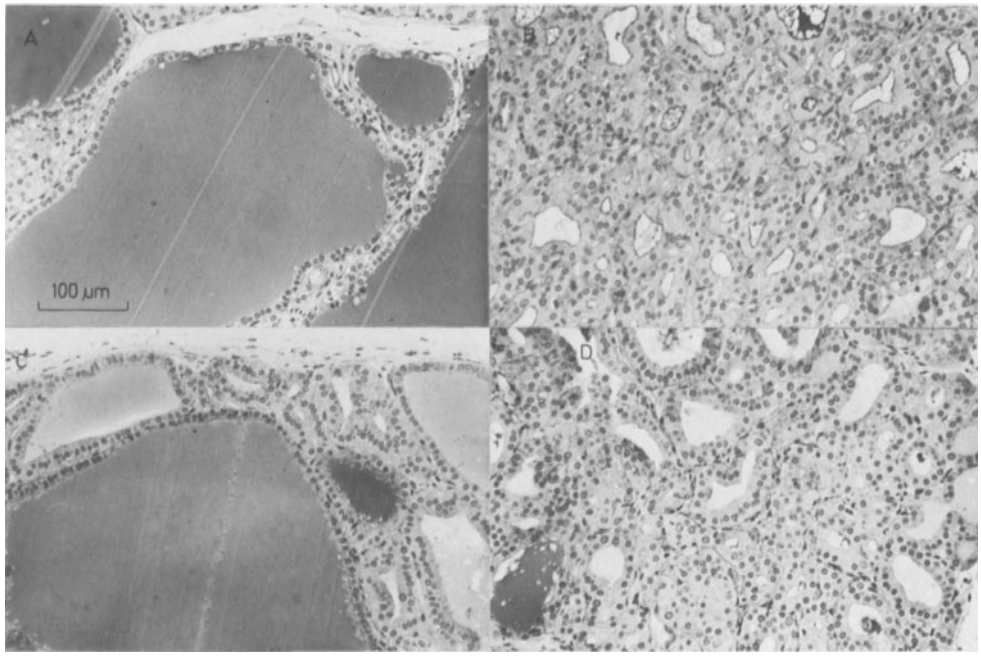

Fig. 1. A,B: Graves' goiter with a macrofollicular (A) and a microfollicular and solid growth pattern (B).
C,D: The same goiter as in Fig. 1 A,B growing as a transplant in a nude mouse injected with the patients own serum. The heterogeneous structures of the donor tissue are reproduced by the growing transplant.

particular structures did not depend on some microenvironmental factors, but were rather an inheritable trait of individual cells (2-4).

From these observations we conclude that local intrathyroidal factors, such as the availability of iodine (5,6) and other yet unknown events may greatly modify the structure of a growing gland and may possibly cause regionally differing growth patterns. Perhaps even more important, different thyroid cells, when forced to replicate, may not necessarily build up identically structured tissue for reasons that depend on an inheritable individuality in expressing some of their traits (3).

We shall now consider the particular growth response of a thyroid gland exposed to chronic low-grade growth stimulation such as low levels of TSH or growth-stimulating immunoglobulins (7). Knowledge of the two following basic characteristics of normal follicular epithelia are taken for granted.

1) Not every thyroid cell has the same intrinsic growth potential (8,9). While some cells divide even in the absence of the classic growth factor TSH, other epithelial cells require long exposition to TSH before entering the mitotic cycle (3).

2) An intrinsically high growth potential is an inborn, inheritable trait of some thyroid cells. It is, therefore, transmitted from one cell generation to the next (2,3).

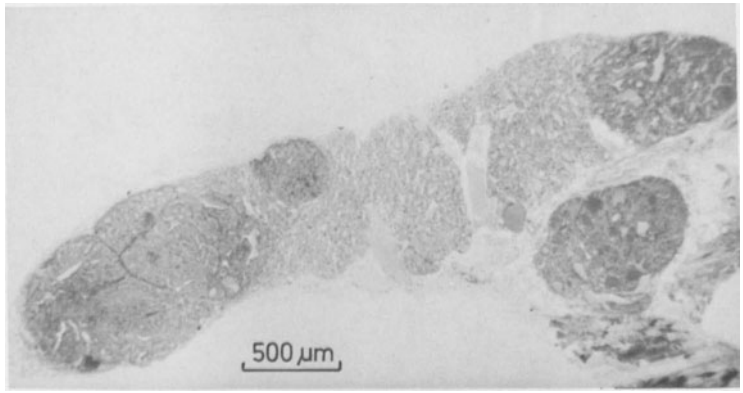

Fig. 2. PAS stained section of a mouse goiter produced by feeding perchlorate for 16 weeks. Four clearly delimitated tumors have formed.

The evidence for this concept is based on three pillars. First, a homogenous population of cells growing in tissue culture invariably contains some cells with very short cell-cycle times, while other cells replicate only at long or even infinite intervals (10). Second, colonies of cells growing in tissue culture in the presence of growth factors attain a widely varying size in any preset time period (11). Third, if the normal intact thyroid gland should also contain epithelial cells with a higher than average intrinsic growth rate, then any chronic growth stimulation would have to produce focal accumulations of rapidly growing cell nests. This is, indeed, the case. It is classic knowledge that chronic feeding of a goitrogenic regimen will produce multiple benign and eventually even malignant tumors in most species of animals (12,13). An illustration from our own experiments is given in Fig. 2. Multiple adenomas have formed in a mouse thyroid stimlated by perchlorate feeding for 16 weeks (14). Yet another citation classic is Mortensen's observation that even normal human thyroid glands strongly tend to become nodular with advancing age of their bearers (15).

It is tempting to wonder whether there is any discernible link between the cell that apparently has a high inborn growth rate in cloned tissue culture and the progenitor cells of adenomas in the growing thyroid. We believe that such a link does, indeed, exist.

First of all, newly generated cells are not randomly scattered all over the growing thyroid, but cells of successive generations strongly tend to remain adjacent to each other so as to form large cohorts. This is shown in Fig. 3. The newly generated cells may be used in either one of three ways (Fig. 4). They may remain within the monolayered epithelium like cell islands inserted into the expanding follicular shell, or they form buds protruding either into the follicular lumen or outwards into the interstitial space. Examples of sprouting cell buds forming new follicles have been illustrated previously (3,8,9).

Some little cell humps proliferate with particularly short cell-cycle times. In this case, a true adenoma will soon be forming (13).

All cells of these early adenomas are dividing at the same fast pace. This is in line with their presumed monoclonal origin. However, the growth pattern of the multiple adenomas appearing late in the course of experimental goitrogenesis is different. While the fraction of actively dividing

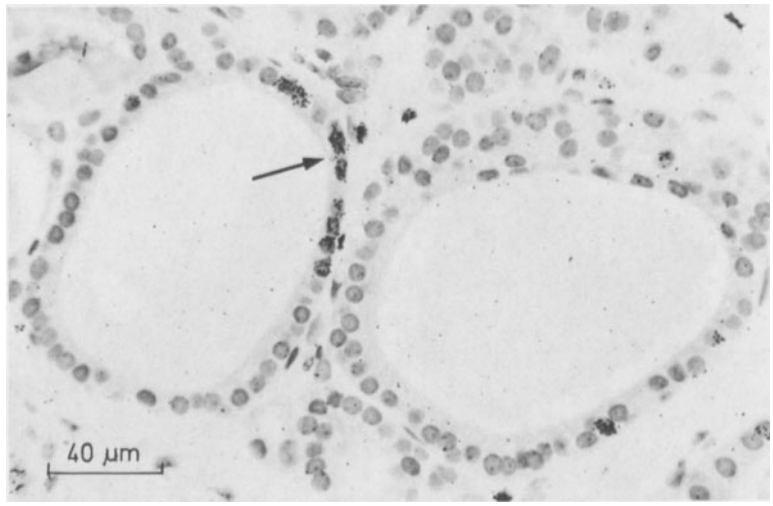

Fig. 3. Normal human thyroid growing as a transplant grafted onto a nude mouse. The mouse was fed methimazole for 3 weeks and ^{3}H-thymidine was injected 3 times daily during weeks 2 and 3. Highly proliferating cells are not scattered randomly but form large, coherent families within the shell of some follicles (3) (arrow).

cells is always much higher in the adenoma than in the nontumorous gland, there is now a considerable minority of cells which divide at a slower pace or not at all (Fig. 5) (14). How, then, can the emergence of asynchronous replication rates in an apparently clonal cell line be interpreted? A possible explanation comes from the work of Wynford-Thomas and Williams (16).

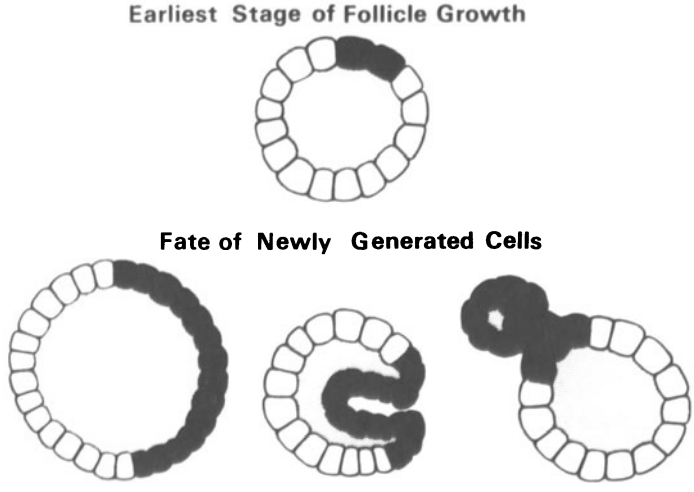

Fig. 4. Schematic drawing representing the three possible ways in which newly generated thyroid cells are built into the existing tissue.

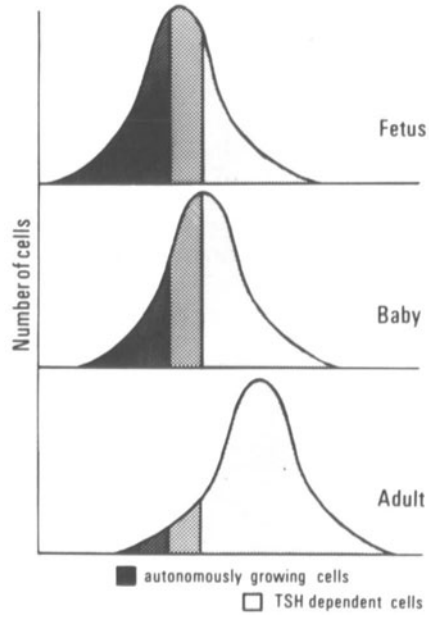

Fig. 5. Schematic representation of the declining number of autonomously growing, rapidly dividing cells during maturation of the thyroid gland. Most important is the persistence, in the adult gland, of a small fraction of these highly growth-prone cells. (Abscissa: Increasing TSH-independency is to the left, increasing TSH-dependency to the right).

The authors have shown that the high mitotic rate of thyroid cells characterizing the early growth response to TSH is not maintained with chronic stimulation. Rather, most cells will become refractory (through some still unknown mechanism) to chronically persisting growth stimuli. The emergence of growth-refractory cells in apparently clonal adenomas may, therefore, be (somewhat surprisingly) but the expression of a normal regulating mechanism characterizing healthy thyroid cell.

A similar down-regulation of cellular growth propensity naturally occurs during maturation of multicellular organisms. In fact, the growth rate of fetal organs is incomparably higher than that of adult tissue. For example, in the fetal thyroid gland, a large fraction of all cells have a high TSH independent growth rate. To cite but one experimental result, in one thyroid gland of a 10 week old human fetus transplanted onto a nude mouse, as much as 30% of all cells proliferated in spite of TSH suppression in the recipient mouse. In contrast, in adult human glands, not more than 1% of all cells are growing autonomously (3). Likewise, in the thyroid gland of one week old mice, as much as 25% of all cells keep replicating even under TSH suppression, while the ^{3}H-thymidine labeled cells again amount to less than 1% of the adult thyroid (14). Thus, the fetal thyroid and, to a lesser extent, the newborn's thyroid behave rather like autonomously growing thyroid nodules or benign adenomas. During maturation toward adulthood, more

than 99% of all cells lose their aggressive intrinsic autonomous propensity to replicate, but an occasional cell will retain its original inherited high high replicating power in the adult gland (Fig. 5). We have produced evidence that these particular cells are at the origin of thyroid nodules and adenomas, since their inbuilt high growth rate enables them to proliferate faster than any other neighboring cell and, therefore, to form locally expanding tumors (14).

All existing theories on the pathogenesis of benign and malignant tumors assume that the fast growing cells must either acquire a new trait or lose some normally existing control mechanism. In contrast, the view exposed here considers the benign tumor as the late consequence of the persistence, in adult organs, of a few normal cells endowed with the relentless autonomous growth potential of fetal cells. The mechanisms which switch off the high growth potential in the overwhelming majority of all fetal cells during maturation are not known at the present time.

One last comment concerns the transition of the common, TSH-dependent adenomas in experimental goiters into autonomously growing, transplantable adenomas and eventually into frankly malignant tumors. This sequence of events has first been deomonstrated some 30 years ago (12). The data presented here (and in 14 and 17) readily explain the phenomenon of autonomous growth. Indeed, it is, in our mind, but the necessary consequence of the inevitable overgrowth in chronically stimulated glands of the rapidly dividing, intrinsically autonomous cells described above. Although we have no data on malignant transformation, it has been demonstrated experimentally (what might be expected on theoretical grounds) that the insertion into the genome of oncogenic DNA sequences is greatly enhanced by accelerating the cell's replication rate (18).

We may conclude from this review that there is barely any other organ more suitable than the thyroid gland which allows investigation of the basic mechanisms of growth and formation of benign and possibly malignant tumors.

REFERENCES

1. Gerber H, Stocker R, and Studer H. Ann Endocr (Paris) 45: 78, (abstract 140), 1984.
2. Gerber H, Peter HJ, and Studer H. In D Reinwein and P Scriba (eds), Treatment of Endemic and Sporadic Goiter, Schattauer Verlag, Stuttgart and New York, 185, in press.
3. Peter HJ, Gerber H, Studer H, et al. J Clin Invest, in press.
4. Studer H, Peter HJ, and Gerber H. (accompanying paper in this volume).
5. Rentsch HP, Studer H, Frauchiger B, et al. J Clin Endocrinol Metab 53: 515, 1981.
6. Gerber H, Studer H, Conti A, et al. J Clin Invest 68: 1338, 1981.
7. Drexhage HA, Bottazzo GF, Doniach D, et al. Lancet II: 287, 1980.
8. Studer H and Ramelli F. Endocr Rev 3: 40, 1982.
9. Peter HJ, Studer H, Forster R, et al. J Clin Endocrinol Metab 55: 941, 1982.
10. Alberts B, Bray D, Lewis J, et al. Molecular Billogy of the Cell, Garland Publ., New York, 1983. p 618.
11. Westermark K, Karlsson FA, Ericson LE, et al. In MC Eggo and GN Burrow (eds), Thyroglobulin - The Prothyroid Hormone, Progress in Endocrine Research and Therapy, Vol. 2, Raven Press, New York, 1985, p 255.
12. Axelrad AA and Leblond CP. Cancer 8: 339, 1955.
13. Money WL, Fitzgerald PJ, Godwin JT, et al. Cancer 6: 111, 1953.
14. Gerber H, Studer H, Peter HJ, et al. (accompanying paper in this volume).

15. Mortensen JD, Woolner LB, and Bennet WA. J Clin Endocrinol Metab 15: 1270, 1955.
16. Wynford-Thomas D, Stringer BMJ, and Williams ED. Acta Endocrinol 101: 210, 1982.
17. Peter HJ, Gerber H, Studer H, et al. (accompanying paper in this volume).
18. Goyette M, Petropoulos CJ, Shank PR, et al. Science 219: 510, 1983.

CLINICAL PROBLEMS IN THYROID DISEASE

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Several interesting and controversial topics have been grouped together in this symposium. These topics are not related, but each is clinically relevant and represents an area where significant advances have occurred over the past few years. Experts will review our current knowledge of the effects of transplacental passage of maternal immunoglobulins on neonatal thyroid function; the nature of the binding protein abnormalities in familial dysalbuminemic hyperthyroxinemia (FDH) and kindred syndromes, and their effects on thyroid function; the diverse effects of the antiarrhythmic agent amiodarone on thyroid function; and the experimental evidence relating to the efficacy of radiolabeled antithyroglobulin antibodies in the detection and treatment of metastatic thyroid cancer. The latter will not be discussed further, as it is preliminary data and will be fully reviewed in the symposium.

TRANSPLACENTAL ANTIBODY-INDUCED NEONATAL SYNDROMES

Placental transfer of thyroid-stimulating immunoglobulin from mother to fetus inducing neonatal thyrotoxicosis is a well-recognized, but fortunately rare, complication of maternal Graves' disease. In a recent comprehensive study of this problem, Zakarija and McKenzie (1) confirmed the general clinical impression of the decline in maternal thyroid-stimulating antibody (TSAb) during pregnancy associated with the amelioration of clinical features of thyrotoxicosis. Their data, and that of others, suggest that prognostication of neonatal thyrotoxicosis is not possible by reliance on maternal clinical features, but can be made by serial measurements of maternal TSAG late in pregnancy.

It is now apparent that at least three different functional classes of immunoglobulins may be transferred to the fetus of a mother with autoimmune thyroid disease. The effects of these immunoglobulins are thyroid stimulation (neonatal thyrotoxicosis), thyroid inhibition (transient neonatal hypothyroidism), or thyroid enhancing (delayed neonatal thyrotoxicosis). Furthermore, there is now good evidence that thyroid growth-stimulating or inhibiting antibodies (TGI) occur which are quite distinct from antibodies blocking TSH binding to specific thyroid receptors (2). The recent finding of TGI block in 80% of 104 cases of sporadic congenital hypothyroidism suggests that the transplacental passage of these immunoglobulins may be involved in the pathogenesis of this disorder (3). Thus, the past few years

have seen the emergence of new experimental data implicating placental transfer of maternal immunoglobulins in most forms of neonatal thyroid disease.

LABORATORY DIAGNOSIS AND MISDIAGNOSIS IN STATES OF ALTERED THYROXINE BINDING

The clinical features of thyroid disease may be so subtle or atypical that reliance upon thyroid function tests is frequently necessary to obtain a diagnosis. Measurement of total serum thyroxine (T_4), with either indirect or direct assessment of the free T_4 fraction, is the most commonly employed front line test. Alterations in thyroxine-binding globulin (TBG), and hence total T_4 concentrations, are frequent, especially in females taking estrogen medication. More recently, it has become apparent that euthyroid hyperthyroxinemia is a common occurrence. This condition is defined as an elevated serum T_4, either total or free, occurring in the absence of hyperthyroidism. The T_4 excess may be transient or persistent and may be associated with elevated, normal, or even low concentrations of circulating serum T_3. Table 1 lists the more common causes of euthyroid hyperthyroxinemia (4).

Recently, much attention has been focused on the syndrome of familial dysalbuminemic hyperthyroxinemia (FDH). The features of this disorder are autosomal dominant inheritance, elevated serum total T_4, normal total T_3, elevated free T_4 index, but normal free T_4 by equilibrium dialysis, normal TSH response to TRH stimulation and normal serum concentrations of TBG, TBPA, and albumin. The importance of the syndrome rests in the frequency of misdiagnosis often resulting in inappropriate treatment for hyperthyroidism. Conflicting evidence for qualitative abnormalities in serum albumin await resolution. In a recent communication, Yabu and co-workers (5) have produced evidence for the presence of a quantitative increase in a normally occurring albumin which has a particularly high affinity for T_4. The effect of this albumin on commonly used laboratory tests will be discussed during this symposium.

Abnormal binding of T_4 to TBPA has also been reported as a cause of euthyroid hyperthyroxinemia (6,7). This appears to be a much rarer condition than FDH and further studies are required to define and characterize

Table 1. Common Causes of Euthyroid Hyperthyroxinemia (4)

1.	Binding protein abnormalities
	- TBG*
	- Dysalbuminemia
	- TBPA excess
	- Autoantibodies*
2.	Hormone resistance*
3.	Drug induced
4.	Acute psychiatric illness
5.	Hyperemesis Gravidarum

*T_3 may also be elevated.

Table 2. Effects of Amiodarone on Thyroid Function

Serum T_4
Serum rT_3
Serum T_3
Serum TSH
Response to TRH enhanced

the clinical features and laboratory abnormalities. Other conditions which produce abnormal serum T_4 and/or T_3 levels, such as peripheral thyroid hormone resistance, are discussed in these proceedings.

AMIODARONE AND THYROID FUNCTION

Amiodarone is a benzofuramic acid derivative, containing 37.2% iodine by weight, used widely in the treatment of cardiac arrhythmias. Besides providing pharmacological quantities of iodine to the patient, it also has other thyroidal effects not readily explained by iodine excess. These effects are summarized in Table 2. Inhibition of 5'-deiodinase is the principal cause of the decline in plasma T_3 and rise in plasma reverse T_3 with treatment. The rise in plasma total and free T_4 has been attributed to decreased clearance of T_4. The enhanced TSH response to TRH stimulation, in the face of elevated T_4 concentrations, has been attributed to inhibition of conversion of T_4 to T_3 within the pituitary thyrotroph. With prolonged therapy, the serum TSH level returns to normal. To date, there is no convincing evidence tht amiodarone has a direct effect on thyroid hormone sensitive tissues other than the pituitary, either by inhibiting conversion of T_4 to T_3 within cells or antagonizing T_3 binding to nuclear receptors.

The known effects of amiodarone on peripheral thyroid hormone metabolism do not explain the common clinical disturbances in thyroid function observed in patients taking prolonged courses of drug therapy. Whether these disturbances are due solely to iodine-induced thyroid dysfunction remains to be determined. Paradoxically, chronic amiodarone therapy is associated with a high incidence of both hyperthyroidism and hypothyroidism. The nature of the abnormality in thyroid function has been associated with environmental iodine intake, with hyperthyroidism occurring in 9.6% of patients where iodine intake is relatively low and hypothyroidism in 22% of patients where iodine intake is relatively abundant (8). Underlying autoimmune thyroiditis, as judged by the presence of thyroid autoantibodies, may be an important determining factor in the precipitation of amiodarone-induced hypothyroidism. Further studies are needed to clarify these observations. It is important that all patients receiving chronic therapy with amiodarone have baseline thyroid function tests and measurements of thyroid autoantibodies and serial examinations at regular intervals during therapy.

REFERENCES

1. Zakarija M and McKenzie JM. J Clin Endocrinol Metab 57: 1036, 1983.
2. Van der Gaag RD, Drexhage HA, Wiersinga WM, et al. J Clin Endocrinol Metab 60: 972, 1985.

3. Van der Gaag RD, Drexhage H, Doniach D, et al. Proc Int Cong Endocrinol Abst. 64, 1984.
4. Stockigt JR and Barlow J. Aust & NZ J Med 15: 277, 1985.
5. Yabu SM, Amir M, Ruiz LE, et al. J Clin Endocrinol Metab 60: 451, 1985.
6. Moses AC, Lawlor J, Haddow J, et al. N Engl J Med 306: 966, 1982.
7. Lalloz MRA, Byfield PGH, and Himsworth RL. Clin Endocrinol (Oxf) 21: 331, 1984.
8. Martino E, Safran M, Aghini-Lombardi F, et al. Ann Int Med 101: 28, 1984.

TRANSPLACENTAL ANTIBODY-INDUCED NEONATAL SYNDROMES*

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This review will be divided into the following segments:

1. Overview of what is known regarding the effects on the fetus of the transplacental passage of circulating antibodies that occur in autoimmune thyroid disease.

2. Description of what is known about the progression of development of the fetal thyroid-pituitary axis; relevant comparison will be made of the time frame for fetal and maternal contributions to the concentration of IgG in the fetal circulation.

3. Descriptions, with references to the above reviews, of fetal and neonatal thyroid disorders based on maternal autoimmune thyroid disease, collating published reports with experience garnered in this laboratory.

1. Maternal antibodies (all typically TgG and, therefore, likely to reach the fetal circulation) that might cause fetal thyroid disorders are listed in Table 1. As indicated there, the antibodies most commonly identified in the adult, antithyroglobulin and antibody to the thyroid microsomal antigen, are considered to have no effect on the fetus or neonate. This is despite recognition for at least 25 years that they readily cross the placenta and an early concept that they might be the cause of athyreotic cretinism (1). In fact, Blizzard and his colleagues in 1960 showed that although these could be found in the neonates of mothers with autoimmune thyroid disease, there appeared to be a distinct maternal thyrocytotoxic factor associated with sporadic cretinism (1).

Antibodies to T_4 and/or T_3 occur in autoimmune thyroid disease, typically late in a clinical course (2,3). Presumably, they would have effects in the fetus and neonate similar to what is seen in the adult, namely a high or low value for the hormone measurement depending upon the RIA method used, but no clinical thyroid disease due to the antibody per se. This circumstance apparently has not yet been reported.

An analogous situation, abnormal laboratory data but no thyroid disturbance, has been reported as due to the transplacental passage of a

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**Kathleen and Stanley Glasser Professor of Medicine.

Table 1. Antibodies in Autoimmune Thyroid Disease and Neonatal Syndromes

	Antibody in the mother	Effect on neonate
1.	Anti-thyroglobulin	None
	Anti-microsomal	None
2.	Anti-T_4 and/or T_3	Euthyroidism
3.	Anti-TSH	Euthyroidism
4.	Anti-TSH receptor:	
	a) TSAb (thyroid-stimulating)	Hyperthyroidism
	b) TBIAb (TSH-binding inhibiting)	Hypothyroidism
	c) TBEAb (TSH-binding enhancing)	Hyperthyroidism
	d) Interaction of 2 or 3 antibodies	Hypo-, hyper-, euthyroidism
5.	TGBAb (thyroid growth blocking)	? Hypothyroidism
6.	TGSAb (thyroid growth stimulating)	? Euthyroidism + goiter

presumed antibody to TSH (4). In this instance, a neonatal screening program reported a high TSH in a child who was euthyroid with a normal T_4 concentration. Studies with the mother's serum indicated she almost certainly had an antibody to human TSH with no other evidence of autoimmunity or thyroid disease; the cause for development of the antibody was not apparent and the child had a normal TSH measurement at seven months of age. Other reports identified falsely elevated TSH levels in paired maternal and neonatal sera due to heterophilic antibodies that bound TSH; at least in some there were antisera against rabbit proteins related to prior therapeutic maneuvers unconnected with thyroid disease (5). The "lesion" from these reports is that a high TSH, especially in a neonatal screening program, should not reflexly result in a diagnosis of hypothyroidism requiring replacement therapy and that mothers' sera should be checked for the same abnormality.

Neonatal Graves' disease was first described with a patient seen in 1910 (6) and that it was probably due to transplacental passage of TSAb was indicated by studies of LATS bioassays in 1964 (7). TSAb is now accepted as one of several antibodies to the TSH receptor occurring in Graves' dis- and as indicated in Table 1, these antibodies have been recognized as caus-causing neonatal thyroid disorders through transplacental passage to the fetus. There are many reports of TSAb measurements in association with neonatal Graves' disease and we have shown that a high level of tne antibody in the third trimester of pregnancy forecasts the neonatal syndrome (8), a disorder that in the uncomplicated situation is self-limited and is related directly to the time it takes the neonate to metabolize the maternal IgG; the clinical state, in consequence, usually lasts no more that 2-3 months.

"Classical" TSAb, depending on its potency in stimulating the thyroid, will inhibit binding of TSH to its receptor and is, therefore, variably positive in TBIAb assay. However, other antibodies that do not stimulate the thyroid occur in autoimmune thyroid disease but also inhibit TSH binding and both TSH and TSAb bioactivity. These TBIAbs have been reported to cross the

placenta and cause temporary hypothyroidism in the neonate (9,10). This may have been the "thyrocytotoxic factor" of Blizzard et al. (1), in which the inhibition of thyroid function was severe enough in utero to result in per-developmental damage. Some recently observed examples of neonatal disorders related to the action of TBIAb are outlined below.

As will be described in more detail, we have reported studies with another antibody, the characteristic of which is enhancement of TSH-binding, and this has been associated with prolonged neonatal hyperthyroidism (11, 12). As also indicated in Table 1, there have been instances where explanation of a neonatal clinical syndrome, e.g., euthyroidism followed by hyperthyroidism, are best explained by the action of a mixture of these antibodies.

The latest contribution to the saga are the results of studies of antibodies that may be unrelated to TSAb and TBIAb but either block the growth-promoting action of TSH or, by themselves, stimulate growth and, thus, by their actions in the fetus may lead to thyroid agenesis and hypothyroidism (13) or, at least in concept, euthyroid goiter. Data related to these considerations are reviewed below.

2. Fisher has well reviewed, and contributed much to, current understanding of the development of the fetal thyroid-pituitary axis (14). The cardinal features are as follows:

a) Thyroid visible - 16 to 17 days embryo
b) Intracellular colloid - 10 to 12 weeks
c) ^{125}I organic binding and synthesis of iodothyronines - 10 to 12 weeks
d) RIA evidence of TSH in the pituitary - 12 weeks
e) ^{125}I uptake, TSH, T_4 and fT_4 undetectable or very low before 18 to 20 weeks
f) Full thyroid function - 22 to 26 weeks

Emphasis may be given to the fact that thyroid and pituitary thyrotrope function are identifiable as early as 10-12 weeks of gestation, but significant hormone levels appear only at 18-20 weeks with full function only about the beginning of the third trimester. These facts have to be borne in mind and correlated with the rate of appearance of maternal IgG in the fetal circulation.

The transplacental passage of maternal IgG is reflected in the composite diagram of Fig. 1. These data, culled from published reviews (14-16), indicate that fetal serum IgG is only 5-8% of the adult concentration until about 16 weeks, only 10-20% until 17-22 weeks, and then, due to an abrupt change in placental permeability, increases rapidly in the third trimester to approach the maternal concentration. Throughout gestation, the contribution of IgG by the child is minimal, but steadily increases from early in the postpartum period as the maternal IgG is metabolized.

3. Consideration of influences of maternal antibodies on the fetus and neonate obviously has to take cognizance of the above reviewed facts. General development is apparently largely independent of fetal thyroid function until the third trimester. Therefore, a maternal antibody inhibiting TSH binding, and consequently action, will be expected to influence only that final period and, indeed, would do this progressively as the concentration in the fetus increases. This concept is compatible with the data published by such as Matsuura et al. (9) and Iseki et al. (10) who showed that maternal TBIAb inhibited TSH binding and action, was present in the neonate early in life, and was cleared between three and 10 months of age: after replacement therapy for variable periods (up to 34 months) the children were

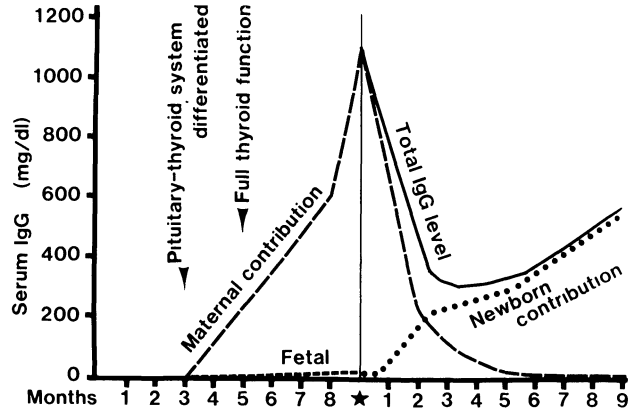

Fig. 1. Development of pituitary-thyroid system and IgG levels with age. Adapted from Stites (16) (who modified from Allansmith, et al., J. Pediat. 72: 289, 1968) with permission from the publishers of J. Pediat., C.V. Mosby Co.

apparently normal in subsequent physical and mental development. What conceivably was a similar circumstance, described in 1960 (17) and further evaluated in 1973 (18), apparently led to permanent residual defects related to the early phase of hypothyroidism. It seems obvious that a variable degree of thyroid hypofunction is to be expected, related to the concentration of the inhibiting antibody, and thus differing from consequences in terms of mal-development of the child.

It is clear that there is potential for misdiagnosing permanent hypothyroidism, particularly in a neonatal screening program, when the child's state is temporary and due to maternal TBIAb (see below).

Time of onset of fetal hyperthyroidism due to transplacental passage of TSAb will depend on the concentration and affinity of the antibody. One might expect fetal signs, particularly tachycardia (well documented by Cove and Johnston) (19), at the earliest at about 16 weeks (12), increasing in frequency towards term as the passage of IgG increases. On the other hand, autoantibodies, including TSAb, tend to decline in women in the third trimester (8) and this probably reduces the incidence and severity of neonatal hyperthyroidism (as it similarly may be associated with clinical improvement in the mother) (8). Attempts to treat intrauterine hyperthyroidism, occurring in the fetus of a woman who is not herself hyperthyroid, so far have been very few (12,19,20). The clinical outcome of the neonate in this series was uniformly good, warranting the use of antithyroid drugs in such circumstances, despite recognized potentially harmful effects (21). The guidelines applicable to women with overt hyperthyroidism, i.e., a use of a minimal effective dose of the drug that will maintain eu- or slight hyperthyroidism, are suggested, except that the parameter to be closely monitored is fetal heart rate. In women who are ot hyperthyroid, e.g., previously treated with either ^{131}I or surgery, or spontaneously hypothyroid (8), antithyroid drug may be given more readily in a dose dictated by the change in fetal status. In either situation, after birth, neonates should be monitored for signs of hyperthyroidism after the effect of the antithyroid drug wears off (19).

Apart from what are, by now, the reasonably clear-cut neonatal syndromes of hyperthyroidism due to TSAb and hypothyroidism from maternal

TBIAb, more confusing clinical patterns are being documented. One has been described by us in a series of publications (11,12,22) which, in summary, may be viewed as follows: the mother was diagnosed in her teens as having Hashimoto's thyroiditis and hypothyroidism requiring permanent therapy with T_4; there was a family but not a personal history of hyperthyroidism. Reconstructing the histories of four pregnancies and three neonatal events (12,22), with detailed studies of the mother's IgG (11), it can be judged that she had at least two antibodies (TBEAb and TBIAb, Table 1) that led tointrauterine hyperthyroidism (TBEAb), euthyroidism at birth and for six weeks (TBIAb inhibiting TBEAb), and subsequent hyperthyroidism (reflecting the action of TBEAb). In short, the fetal and neonatal clinical states reflected the effects of the mixture of these antibodies, and the doubtless varying affinities for, as well as the capacities of, their corresponding antigens. The changing concentration of maternal IgG in the second half of gestation in the fetus and the first six months of life (Fig. 1) undoubtedly played an important role.

Another patient had one miscarriage and three children (to be published by Drs. Francis and Riley, Gainesville, FL) were all found on neonatal screening to be hypothyroid; one was subjected to thyroid scanning with pertechnetate and was said to have "thyroid dysgenesis". Two of the children, including the "thyroid dysgenesis" infant, at five and four years of age, had T_4 therapy discontinued and subsequently remained euthyroid; the third has still to be so tested. The mother had Graves' disease at age eight, at 25 became spontaneously hypothyroid, and was started on replacement therapy; her IgG, recently elevated, has TSAb, but is overwhelmingly potent as TBIAb.

The most recent, and most controversial, area is the question of another type of antibody, TGBAb (Table 1) causing thyroid atrophy and hypothyroidism (13). The beginning of this concept is the description of thyroid-growth assays, responsive to TSH or to an antibody, apparently distinct from TSAb, that promotes thyroid growth (TGSAb) (23). [As reported at this Congress (Neylan et al., this Congress) we have reservations about the disparate nature of TSAb and TGSAb.] The growth-stimulating assay depends upon a measure of DNA-replication, namely, the assessment by cytochemistry of the proportion of cells in S phase in guinea pig thyroid in vitro after exposure to the test IgG or TSH for five hours. Using this procedure, Van der Gaag et al. (13) found that the growth stimulus provided by TSH could be inhibited by variable concentrations of certain maternal IgG, i.e., from some mothers whose children had been identified as hypothyroid on neonatal screening. Acknowledging the concepts illustrated in Fig. 1, the authors postulated that initial thyroid growth was not inhibited but perhaps there was, later in gestation, interference with the maturation of relevant (TSH) receptors. A surprising feature was that most of the mothers had TGBAb in the serum but, apart from two, had no other feature of thyroid disorder or autoimmunity; this is in contrast to mothers associated with neonatal syndromes of hyper- or hypothyroidism who had TSAb or TBIAb in the blood and who personally presented with autoimmune thyroid disease (8-10). Since, of the seven children so far tested by scintiscanning, three had thyroid agenesis and four had ectopic glands, it is difficult to relate the anatomical findings to this thesis. It seems clear that this is an area of investigation that deserves, and will receive, greater attention and attempts obviously will be made to confirm the findings.

Regarding the TGBAb assay described above, Drexhage and colleagues (23) and others (24,25) have reported TGSAb in instances of goitrous autoimmune thyroid disease and nontoxic, simple (i.e., otherwise considered to be nonautoimmune) goiter. If this is a concept and goiter-pathogenesis mechanism that becomes thoroughly established, one should expect neonatal goiter to be a common consequence of the transplacental passage of TGSAb in a woman with autoimmune or simple goiter, frequently encountered maladies. In fact, this does not appear to be a recognized syndrome. Since

fetal goiter is liable to result in malpresentation at the time of labor, it seems unlikely that this syndrome, if it exists, is at all common. Consequently, either TGSAb is an antibody entirely different from TSAb and TBIAb in that it cannot affect the fetal thyroid, or the bioassays used to identify it (23-25) are detecting a different species of bioactive molecule. The other TGSAb assay recently developed (25) is based upon ^{3}H-thymidine thymidine incorporation into DNA in $FRTL_5$ cells; this is much less sensitive than the cytochemical procedure (23) in terms of the minimal effective concentration of TSH but has a considerably wider range of response. In our experience (Neylan et al., this Congress), with this procedure, IgG from goitrous individuals without Graves' disease has uniformly been negative.

From this review of fetal and neonatal consequences of maternal autoimmune thyroid disease, it may be concluded that there is justification for screening certain clinical categories of pregnant women. The first emphasis should be on the need to consider thyroid disease in the circumstance of second or third trimester fetal tachycardia; when this is recognized, the mother's status and history regarding a possible autoimmune thyroid disorder should be reviewed. Positive features should lead to testing maternal serum for anti-TSH receptor antibodies. Obviously, women with known Graves' disease and those with a history of children with neonatal hypo- or hyperthyroidism ought to be checked. It would be doubtfully cost-effective, at present, for every woman with Hashimoto's thyroiditis to be tested, but the alert physician might recognize a strong family history of autoimmune thyroid disorders and pay special attention to such a patient.

The final question is, which test should be used for monitoring a pregnant woman - TSAb in human thyroid or $FRTL_5$ cells or TBIAb in human thyroid or guinea pig fat cell membranes, or other preparations that are now in use? In Koizumi et al. (this Congress) we illustrate the fact that false information may be provided by assay of a single concentration of IgG in any of four representative assay systems, i.e., examples are given there of apparently minimal or no response at a particular point, whereas full analysis showed a different interpretation. Consequently, outside of the research setting, the advice is - monitor by any available assay, but be aware of the intricacies of the assays and the potential for the provision of misleading data.

For the infant, it would appear from the experience reviewed above that, if maternal antibody is thought to have caused neonatal hypothyroidism, full reevaluation later in infancy is indicated. Considering the experience quoted above of an erroneous diagnosis of thyroid dysgenesis by radionuclide scanning shortly after birth, the Quebec practice of delaying scanning until three years of age (13) would seem to be highly commendable.

REFERENCES

1. Blizzard RM, Chandler RW, Landing HB, et al. N Engl J Med 263: 327, 1960.
2. Premachandra BN and Blumenthal HT. J Clin Endocrinol Metab 27: 931, 1967.
3. Moroz LA, Meltzer SJ, and Bastomsky CH. J Clin Endocrinol Metab 56: 1009, 1983.
4. Lazarus JH, John R, Ginsberg J, et al. Brit Med J 286: 592, 1983.
5. Czernichow P, Vandalem JL, and Hennen G. J Clin Endocrinol Metab 53: 387, 1981.
6. White C. J Obstet Gynaec Brit Emp 21: 231, 1912.
7. McKenzie JM. J Clin Endocrinol Metab 24: 660, 1964.
8. Zakarija M and McKenzie JM. J Clin Endocrinol Metab 57: 1036, 1983.
9. Matsuura N, Yamada Y, Nohara Y, et al. N Engl J Med 303: 738, 1980.

10. Iseki M, Shimizu M, Oikawa T, et al. J Clin Endocrinol Metab 57: 384, 1983.
11. Zakarija M, Garcia A, and McKenzie JM. J Clin Invest 76: 1885, 1985.
12. Zakarija M, McKenzie JM, and Hoffman WH. J Clin Endocrinol Metab (in press).
13. Van der Gaag RD, Drexhage HA, and Dussault JH. Lancet 1: 246, 1985.
14. Fisher DA and Klein AH. N Engl J Med 304: 702, 1981.
15. Gitlin D. In BM Kagan and ER Stiehm (eds), Immunologic Incompetence, Year Book Medical Publishers, Inc., Chicago, 1971, p 3.
16. Stites DP. In HH Fudenberg, DP Stites, JL Caldwell, et al. (eds), Basic and Clinical Immunology, Lange Medical Publishers, Los Altos, 1980, p 343.
17. Sutlerland JM, Esselborn VM, Burket RL, et al. N Engl J Med 263: 336, 1960.
18. Goldsmith RE, McAdams AJ, Larsen PR, et al. J Clin Endocrinol Metab 37: 265, 1973.
19. Cove DH and Johnston P. Lancet 1: 430, 1985.
20. Volpe R, Ehrlich R, Steiner G, et al. Am J Med 77: 572, 1984.
21. Cheron RG, Kaplan MM, Larsen PR, et al. N Engl J Med 304: 525, 1981.
22. Zakarija M, McKenzie JM, and Munro DS. J Clin Invest 72: 1352, 1983.
23. Drexhage HA, Bottazo GF, Doniach D, et al. Lancet 2: 287, 1980.
24. Chivato L, Hammond LJ, Hanafusa T, et al. Clin Endocrinol 19: 581, 1983.
25. Kohn LD, Valente WA, Alvarez FV, et al. In PG Walfish, JR Wall, and R Volpe (eds), Autoimmunity and the Thyroid, Academic Press, Inc., Toronto, 1985, p 217.

LABELED ANTIBODIES IN THE LOCALIZATION AND THERAPY OF THYROID CARCINOMA

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Radioimmunodetection, or radioimmunoimaging, detects tumors by using labeled antibodies against tumor-specific or tumor-associated antigens. This technique has been employed widely to detect carcinoembryonic antigen (1,2), alpha feto protein (3), and chorionic gonadotropin-producing tumors (4). Application of specific monoclonal antibodies has also been investigated (5-7).

Since serum levels of thyroglobulin (Tg) increase in many patients with thyroid carcinoma and it has been known that Tg is secreted from thyroid carcinoma into the circulation, the present studies were undertaken to investigate the accumulation of radioiodinated anti-Tg antibody by thyroid carcinoma.

MATERIALS AND METHODS

Studies with Nude Mice

Radioiodinated anti-Tg antibody. In order to produce radiolabeled anti-Tg antibody, serum was obtained from a patient with Hashimoto's disease whose anti-Tg antibody titer was 8×10^7 by thyroid test, hemagglutination test of Fuji Zohki Co. Following purification, it was radioiodinated using lactoperoxidase and a glucose-glucose oxidase system.

Procedures. Papillary adenocarcinoma, follicular adenoma, Graves' and normal thyroid tissues were transplanted into nude mice. Four weeks after transplantation, 25 μCi of ^{125}I anti-Tg antibody were injected intravenously and scintigrams were taken at three and seven days after injection of the antibody. Mice were sacrificed immediately after the last scintigram, and transplanted thyroid tissues and other tissues of the nude mice were removed and analyzed for ^{125}I-labeled compounds.

Analysis of radioiodine-labeled compounds. In order to analyze radioiodine-labeled compounds in tissues and sera, transplanted thyroid tissues, livers, and kidneys of nude mice were homogenized with phosphate buffer and centrifuged to separate soluble fractions. Sera and supernatants of the above mentioned tissues were filtered through Sephacryl S 300. The peaks of gel filtration were analyzed by affinity chromatography for anti-IgG-antibody and anti-Tg-antibody.

In Vitro Experiments

Monolayer culture. Thyroid tissues from subjects with Graves' disease and from normal subjects obtained at the time of surgery were digested with collagenase and dispase. Cells were cultured in the plates with Ham's F12 containing 10% FCS. On the sixth day of culture, ^{125}I anti-Tg antibody was added to the media and the cells were incubated for one hour. After washing with fresh culture media, the cells were digested with trypsin and the radioactivity of the hydrolyzed digestion media was measured.

Suspension culture. Graves' thyroid tissues were digested with collagenase and dispase. Cells were then cultured in Ham's F12 containing 0.5% or 10% FCS in culture tubes rotated vertically at 25 rpm (8). On the second and fifth days of culture, fluorescence isothiocyanate (FITC) labeled anti-Tg antibody and tetramethyl rhodamine isothiocyanate (TRITC) labeled anti-microsomal antibodies (9,10) were added to the media and incubated for 30 minutes. After washing the cells carefully, stained cells were examined under a fluorescence microscope. Microsomal antibody was obtained from a patient with autoantibodies against microsomes but not Tg.

Clinical Studies

Following an explanation of the purpose and the potential benefits of the studies, 1.5 mCi of ^{131}I-labeled anti-Tg antibody was injected intravenously into patients who consented. Scintigrams were taken one, three, five, and seven days after injection of the antibody.

RESULTS

Studies with Nude Mice

Fig. 1 shows the scintigrams of nude mice seven days after the injection of ^{125}I anti-Tg antibody. Thyroid tissues were transplanted into the

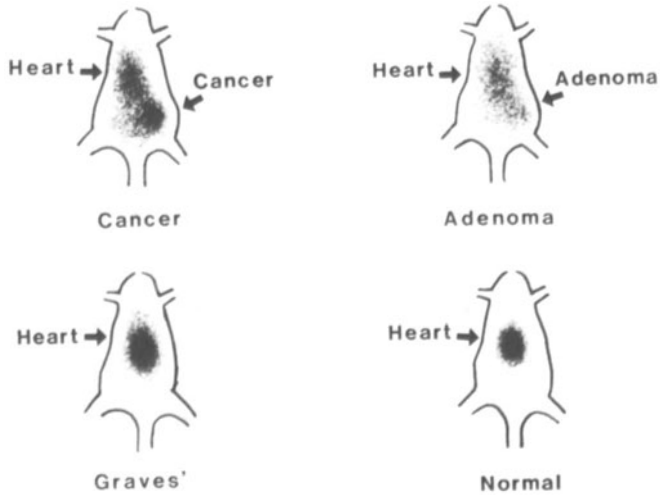


Fig. 1. Scintigrams of nude mice transplanted with various human thyroid tissues and given ^{125}I anti-Tg antibody. Thyroid tissues were transplanted five weeks and ^{125}I anti-Tg antibody was given seven days before scintigraphy.

right lumbar area. As can be seen, papillary carcinoma shown in the upper left and follicular adenoma in the upper right parts of the figure accumulated ^{125}I anti-Tg antibody, while Graves' and normal tissues did not.

Fig. 2 shows radioactivity in various tissues expressed as the ratio of tissue to blood. Radioactivity due to ^{125}I anti-Tg antibody is shown on the left and that due to labeled normal IgG is shown on the right. The results in nude mice with transplanted normal thyroids, Graves' thyroids, follicular adenoma, and adenocarcinoma are shown from the top to the bottom. Each bar represents radioactivity in each tissue. Although normal thyroid and Graves' thyroid tissues could accumulate ^{125}I anti-Tg antibody, as shown by the transplanted tissue of each group, the tissue/blood ratios of ^{125}I were only approximately 0.5. In contrast to the findings in normal and Graves' thyroid tissues, transplanted adenoma and adenocarcinoma accumulated ^{125}I anti-Tg antibody radioactivity to concentrations greater than 2 to 5 times that found in blood. As shown on the right, no significant accumulation was observed when normal IgG was injected.

In carcinoma and adenoma, approximately 70% of the radioactivity was soluble and 80% was soluble in normal and Graves' tissues. These soluble fractions were further analyzed by Sephacryl S 300 gel filtration.

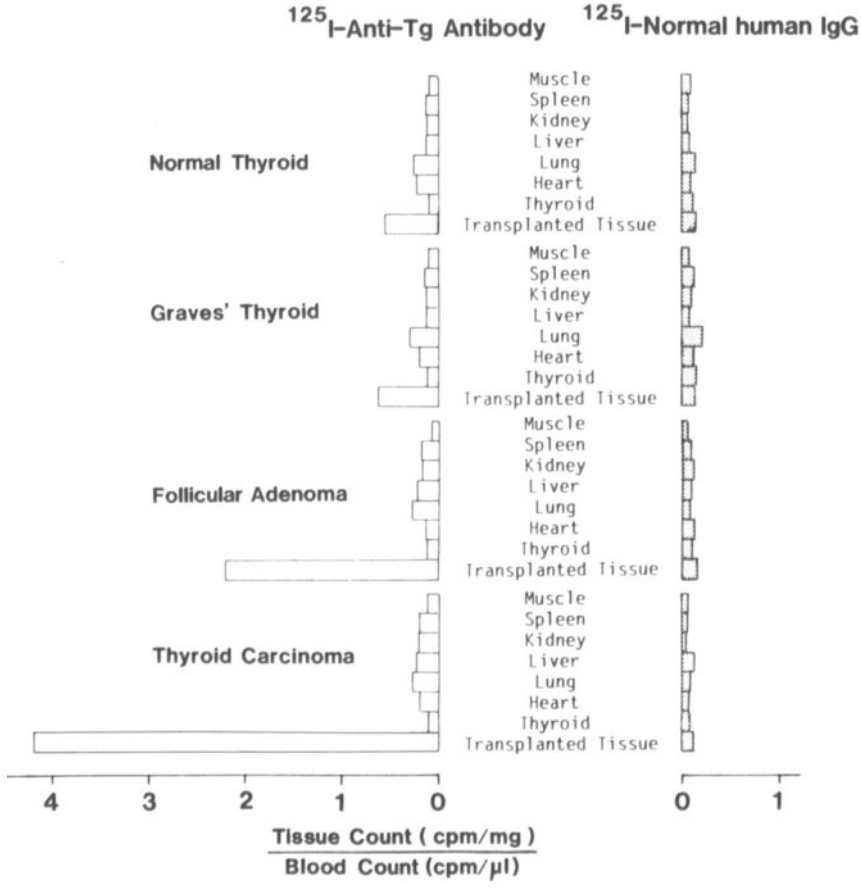


Fig. 2. Radioactivity of various tissues of nude mice transplanted with human thyroid tissues and given ^{125}I anti-Tg antibody or ^{125}I normal human IgG.

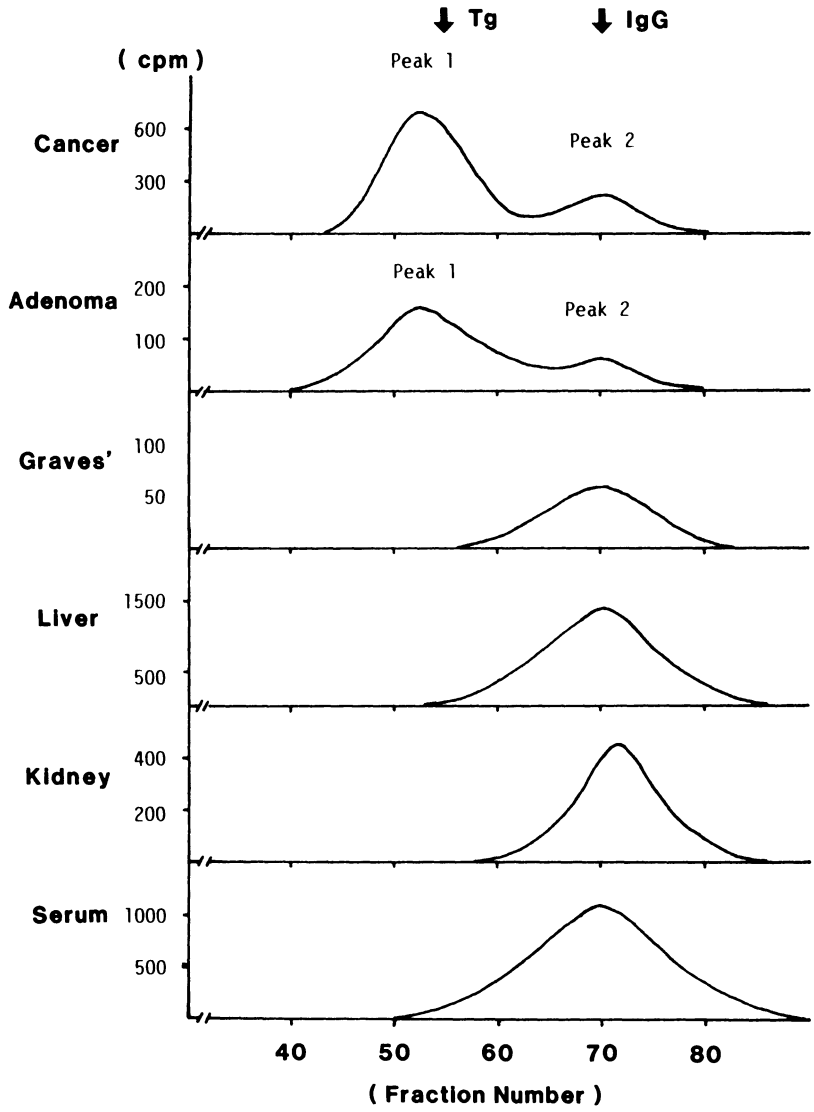


Fig. 3. Patterns in gel filtration of supernatants from various tissues of nude mice transplanted with human thyroid tissues and given ^{125}I anti-Tg antibody.

As shown in Fig. 3, supernatants of transplanted carcinoma and adenoma showed two distinct peaks, whereas supernatants of transplanted Graves' thyroid tissue, liver, and kidney had only one peak which were similar to that of serum IgG, indicating that the radioactivity in Graves' tissues, liver, and kidney could be due to contamination with serum-labeled IgG.

Fig. 4 shows the results of affinity chromatography of peak I and peak II of gel filtration shown in Fig. 3. Values are the radioactivity bound to affinity columns expressed as percent of total radioactivity.

In peak I obtained from transplanted thyroid carcinoma, more than 90% of the radioactivity was bound to anti-IgG and more than 60% to anti-Tg, indicating that most of the radioactivity present in peak I was anti-Tg

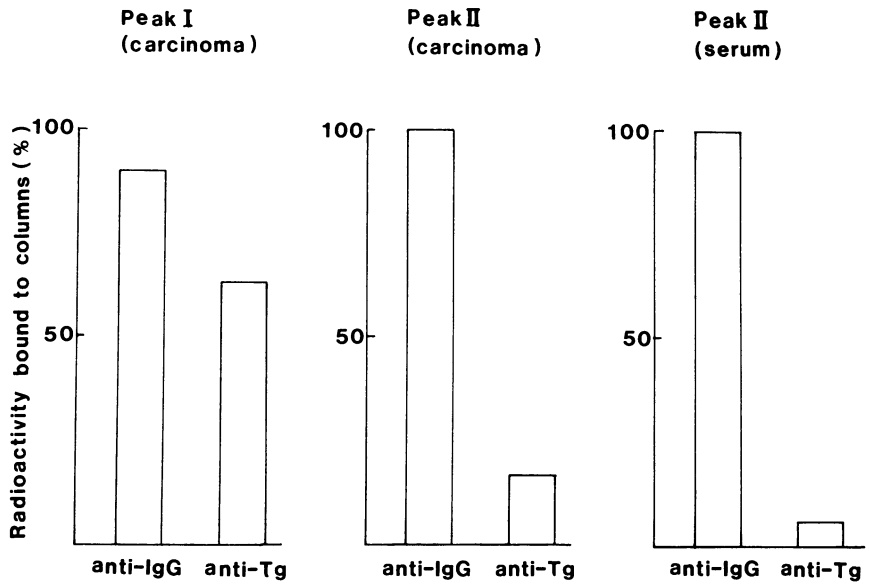


Fig. 4. Affinity column chromatography of peak I and peak II of gel filtrations of supernatants from transplanted thyroid carcinoma into nude mice.

antibody and Tg-immune complex. However, in peak II, most of the radioactivity was bound to anti-IgG and very little to anti-Tg. The results were very similar to those seen in peak II from sera of nude mice, indicating that most of the radioactivity in peak II was labeled anti-Tg antibody.

In Vitro Experiments

Monolayer culture. Fig. 5 shows the accumulation of ^{125}I anti-Tg antibody to Graves' thyroid cells and fibroblasts. Values shown are the radioactivity of cells incubated one hour with ^{125}I anti-Tg antibody alone (with additional 0.1 mg/ml of Tg), cold anti-Tg antibody, or with normal IgG. Open bars represent values of thyroid cells and solid bars represent those of fibroblasts as control. ^{125}I anti-Tg antibody was bound to Graves' thyroid cells significantly greater than to fibroblasts. The binding was inhibited by large amounts of Tg and cold anti-Tg antibody, but not by normal IgG.

Suspension culture. Fig. 6 shows the results of immunohistological staining. Thyroid cells were cultured in 0.5% FBS and the majority of cells should have normal polarity because fluorescence-labeled antibodies were incubated for only 30 min when the cells were still living. Therefore, antibodies (or at least most of the antibodies) should exist on the cell surface and not within intracellular components. Here, anti-Tg antibody was bound to the cell surface of some thyrocytes, but not to colloid. Anti-microsomal antibody, which is believed to bind only to apical membranes and not to Tg, was also found on the cell surface of some thyrocytes, as shown in the middle part of the photograph.

Although it would be difficult to conclude that the binding sites of anti-Tg and anti-microsomal antibodies are identical, the photograph on the

right, in which both anti-Tg and anti-microsomal antibodies are seen simultaneously, suggests that binding sites of anti-Tg were not greatly different from those of anti-microsomal antibody.

It is believed that in cultures consisting of 10% FBS, many thyroid cells reverse their polarity (11), so that more apical membranes are being exposed to the incubation medium.

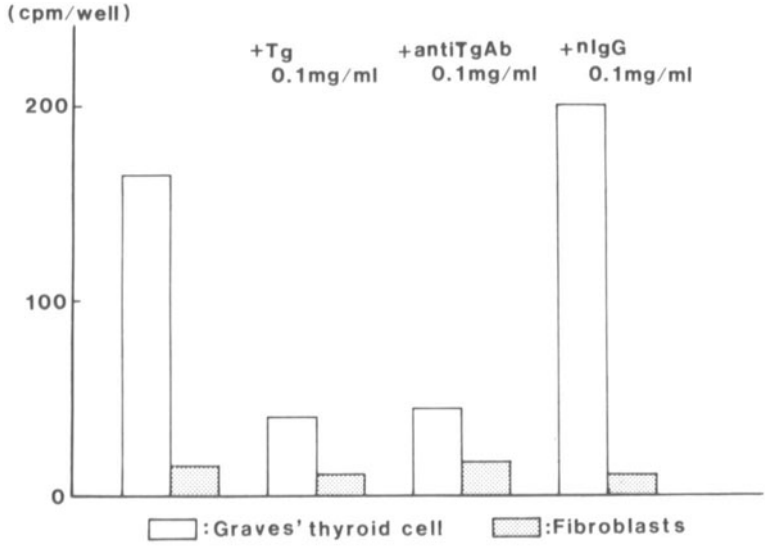
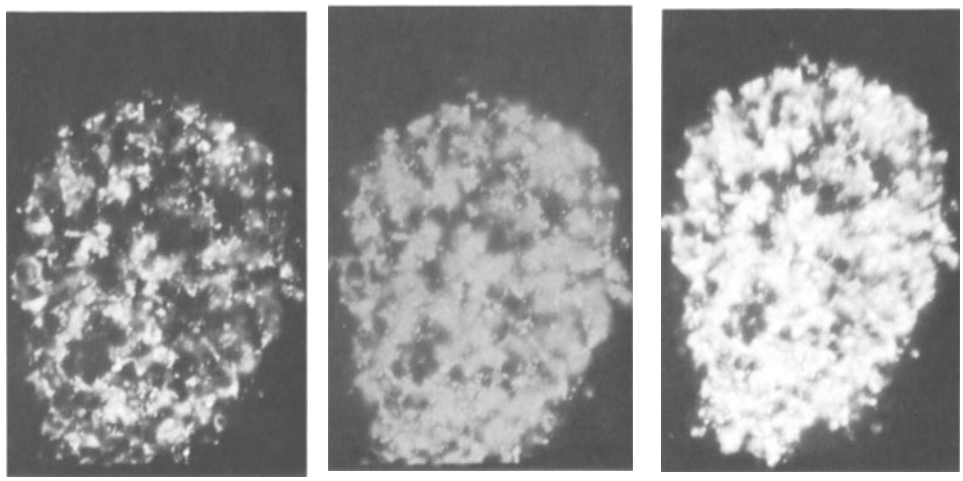


Fig. 5. Accumulation of ^{125}I anti-Tg antibody by Graves' thyroid cells and fibroblasts in monolayer cultures.

Immunohistological staining of Graves' thyroid cells in suspension culture (0.5%FCS)



FITC-antiTgAb TRITC-antiMsAb Double

Fig. 6. Immunohistological staining of Graves' thyroid cells in suspension cell cultures. Left: FITC-labeled anti-Tg antibody. Middle: TRITC-labeled anti-microsomal antibody. Right: both.

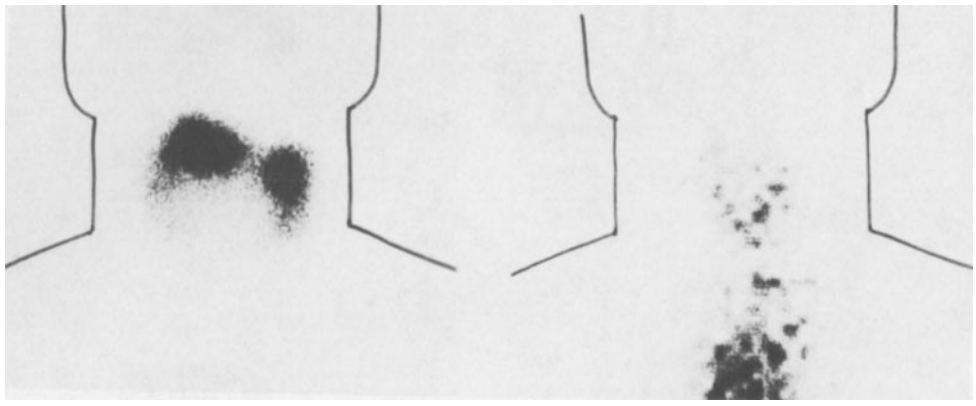


Fig. 7. Scintigrams of the thyroid of a patient with Graves' disease and an adenoma. Left: inorganic ^{123}I. Right: ^{131}I anti-Tg antibody.

The results in 10% FBS were essentially the same as those in 0.5% FBS except that the number of cells to which antibodies were bound appeared to be greater than in 0.5% FBS.

Clinical Studies

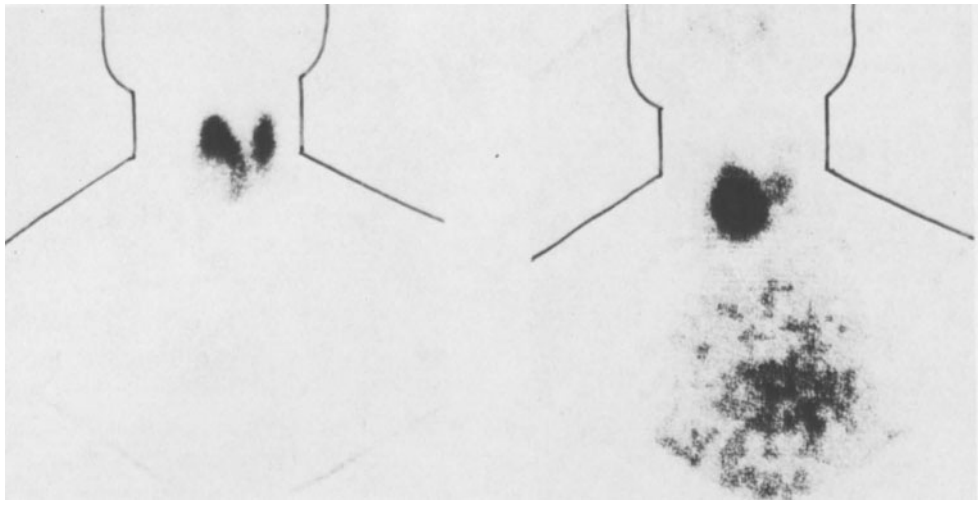
Fig. 7 shows the scintigrams of a patient with Graves' disease and a large adenoma. The scintigram with inorganic ^{123}I on the left shows an enlarged thyroid and a large cold area. In the scintigram with ^{131}I-labeled anti-Tg antibody, ^{131}I was not accumulated in Graves' thyroids, but was accumulated by the adenoma. Since the subtraction technique was not employed, ^{131}I was also found in the area of the heart. The scintigram was so definite that the subtraction method was not necessary.

Fig. 8 shows scintigrams of a patient with a solitary adenocarcinoma. Accumulation of ^{123}I in the normal thyroid tissue and a cold area can be seen on the left. In the right scintigram, ^{123}I-labeled anti-Tg antibodies were not accumulated by normal thyroid tissue, but were accumulated by thyroid carcinoma.

Fig. 9 shows scintigrams of a patient who had a total thyroidectomy. He had elevated serum Tg levels, indicating that he had metastases; however, scintigrams with inorganic ^{123}I or ^{131}I did not show any hot areas. When ^{131}I anti-Tg antibody was injected, the scintigram on the left (obtained 24 hours after injection) showed no hot areas except in the area of the heart. However, the scintigram on the right, taken five days after injection, showed a hot area in the right side of his neck. Lymph node metastases were confirmed at operation, and serum Tg levels decreased to normal after resection of the node.

DISCUSSION

From studies in nude mice, it is clear that labeled anti-Tg antibody was accumulated by transplanted thyroid carcinoma and adenoma, but not by normal and Graves' thyroid tissues. Approximately 70% of the radioactivity in carcinoma and adenoma was in the soluble fraction as Tg-anti-Tg-antibody immune complex.



^{123}I

^{131}I Anti-thyroglobulin Antibody

Fig. 8. Scintigrams of the thyroid of a patient with a solitary adenocarcinoma. Left: inorganic ^{123}I. Right: ^{131}I anti-Tg antibody.

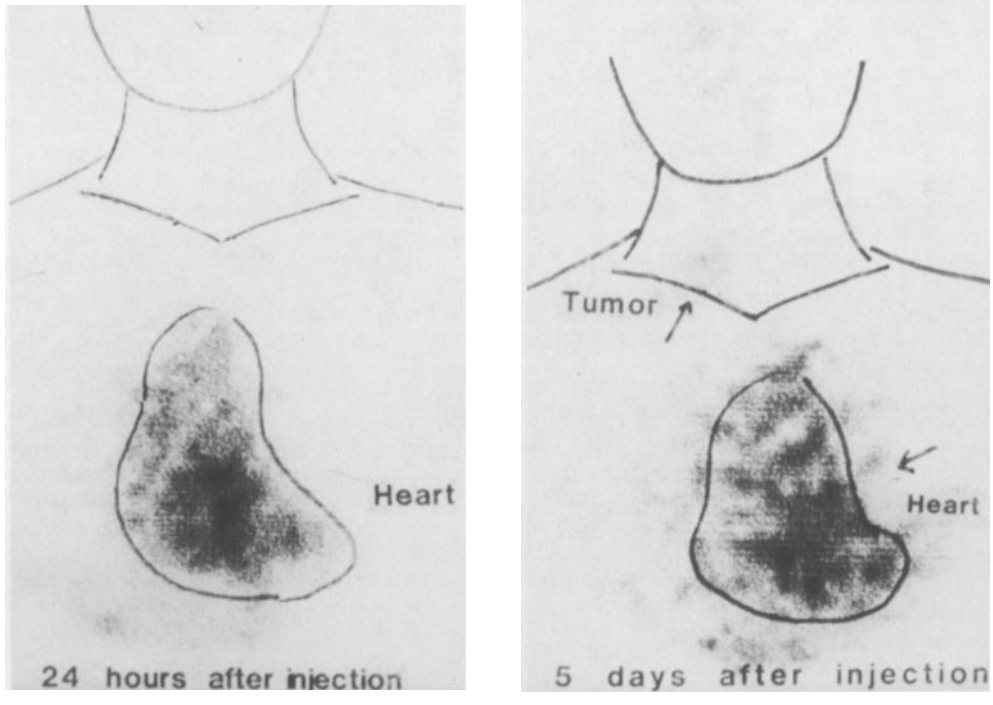


Fig. 9. Scintigrams of a thyroidectomized patient with thyroid carcinoma metastases to the right neck region. Left: 24 hours after ^{131}I anti-Tg antibody. Right: Five days after ^{131}I anti-Tg antibody.

Since accumulation of radioiodinated anti-Tg antibody was found only in carcinoma and adenoma, but not in normal and Graves' thyroid tissues, in vitro experiments were undertaken to investigate the difference in antibody accumulation, using monolayer cell cultures of normal and Graves' thyrocytes and suspension cell cultures of Graves' thyrocytes in 0.5% and 10% FBS, the latter to change the polarity of thyrocytes (12,13).

The difference of the results between the in vitro nude mice study and the in vitro monolayer culture could be considered as follows, since labeled anti-Tg antibody must reach the site where Tg molecules exist.

In the in vivo studies, the reason why labeled antibody was not accumulated by normal or Graves' thyroids is that Tg molecules are not present on the cell surface exposed to circulating antibodies. In contrast, in in vitro experiments, labeled anti-Tg antibody was able to reach membranes through which Tg is secreted (14), because it is well known that thyroid cells lose their polarity in monolayer culture (15). Another possible explanation is that labeled anti-Tg antibody was able to reach the colloid of monolayer cultured cells, since the binding of contiguous cells could be quite loose in monolayer culture.

In experiments of suspension culture using FITC-anti-Tg antibody and TRITC-anti-microsomal antibody, it was shown that anti-Tg antibody was bound on the cell surface, as was anti-microsomal antibody.

From these in vitro experiments, it is very likely that thyroid carcinoma and adenoma lose their polarity and secrete Tg directly into the circulation, and labeled anti-Tg antibody binds to Tg on the cell surface to produce Tg-anti-Tg-antibody immune complex which is gradually internalized and remains in the cytosol.

The results of animal experiments and in vitro studies clearly indicated that radioimmunodetection could be useful for the localization and treatment of thyroid cancer. Clinical studies were carried out in patients who agreed to cooperate after explanation of the purpose of our study. The results of the clinical studies have shown that ^{131}I-labeled anti-Tg antibody was accumulated by thyroid adenoma and carcinoma, but not by normal and Graves' thyroids. Furthermore, metastases of thyroid carcinoma which secreted Tg but did not concentrate radioiodide, accumulated ^{131}I anti-Tg antibody.

Although similar results have been presented or published (16,17), these results of basic and clinical studies clearly showed the usefulness of the radioimmunodetection of thyroid carcinoma.

In conclusion, although the techniques for detection must be improved, radioimmunodetection may be useful in the localization and treatment of thyroid carcinoma because of the following reasons: 1) Anti-Tg antibody binds to thyroidal carcinoma and adenoma tissues but not to normal and Graves' thyroids. Therefore, a) ^{131}I anti-Tg antibody can detect carcinomas as hot nodules and can be used to preferentially treat the tumors, and b) labeled antibody can detect and treat metastases without total thyroidectomy, thus avoiding complications. 2) Anti-Tg antibody can bind to metastases which secrete Tg but do not concentrate radioiodide. Therefore, labeled antibody can detect and treat the less well differentiated metastases than can be possible by radioiodide alone.

ACKNOWLEDGMENT

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REFERENCES

1. Goldenberg DM, Kim EE, DeLand FH, et al. Cancer Res 40: 2984, 1980.
2. Mach JP, Forni M, Ritschard J, et al. Oncodev Biol Med 1: 49, 1980.
3. Ishii N, Nakata K, Muro T, et al. Ann NY Acad Sci 417: 270, 1983.
4. Goldenberg DM, Kim EE, and DeLand FH. Proc Natl Acad Sci 78: 7754, 1981.
5. Mach JP, Buchegger F, Forni M, et al. Immunology Today 2: 239, 1981.
6. Farrands PA, Perkins AC, Pimm MV, et al. Lancet II 8295: 397, 1982.
7. Ishii N, Munehisa T, Koji T, et al. In H Peeters (ed), Protides of the Biological Fluids, Volume 31, Pergamon Press, Oxford and New York, 1984, p 305.
8. Inoue K, Horiuchi R, and Kondo Y. Endocrinology 107: 1162, 1980.
9. Kawaoi A. An Illustrated Technology of Immunofluorescence: Its Principles, Practice and Applications, Soft Science, Inc., Tokyo, 1983, p 7.
10. Mckay IC, Forman D, and White RG. Immunology 43: 591, 1981.
11. Nitsch L and Wollman SH. J Cell Biol 86: 875, 1980.
12. Hanafusa T, Pujol-Bomell R, Chiovato L, et al. Clin Exp Immunol 58: 639, 1984.
13. Khoury EL, Bottazzo GF, and Roitti LM. J Exp Med 159: 577, 1984.
14. Davies TF, Platzer M, Schwartz AE, et al. Clin Endocrinol 21: 239, 1984.
15. Chambard M, Gabrion J, and Mauchamp J. J Cell Biol 91: 157, 1981.
16. Hirayu H, Izumi M, Morita S, et al. Oncodevel Biol Med 4: 63, 1983.
17. Fairweather DS, Bradwell AR, Watson-James SF, et al. Clin Endocrinol 18: 563, 1983.

AMIODARONE AND THE THYROID

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Amiodarone is a benzofuranic derivative containing 37.2 mg iodine per 100 mg of active drug. It is widely used for the treatment of cardiac arrhythmias and angina pectoris (1,2), and has a prolonged half-life up to 2-4 months. Amiodarone inhibits the outer ring deiodination (5'-deiodinase) of thyroxine (T_4) and 3,3',5'-triiodothyronine (rT_3) resulting in an increase in the concentration of serum T_4, a decrease in the concentration of serum triiodothyronine (T_3) and an increase in the concentration of serum rT_3 (3-12). These changes are associated during the first few months of therapy by an increase in basal serum thyrotropin (TSH) concentration and the TSH response to thyrotropin-releasing hormone (TRH) (3,5). Although a tendency toward normalization after more prolonged therapy is usually observed, changes in these parameters of thyroid function may persist to some extent. Moreover, the development of hyperthyroidism and hypothyroidism may also occur with variable frequency (4,6,13-21). The mechanisms by which thyroid dysfunction is induced are not completely clear and the reasons why some patients become hyperthyroid and others hypothyroid remain to be elucidated. In view of the multiple and variable changes of thyroid function tests occurring in euthyroid, hyperthyroid, and hypothyroid amiodarone-treated patients, the precise evaluation of thyroid status often represents a difficult clinical challenge. Moreover, difficult therapeutic problems arise in the presence of amiodarone-associated thyrotoxicosis (AT) and, to a lesser extent, in the presence of amiodarone-associated hypothyroidism (AAH).

These problems will be discussed in the present report on the basis of our experience (6) resulting from studies carried out in an area where iodine intake is moderately low (West Tuscany, Italy) and in an area where iodine intake is sufficient (Worcester, MA, USA).

INCIDENCE OF THYROID DYSFUNCTION

The incidence of thyroid dysfunction was evaluated in 239 patients submitted to chronic treatment with amiodarone for various cardiac arrhythmias and/or angina pectoris. Clinical and laboratory evidence of hyperthyroidism was found in 18 of 188 patients (9.6%) residing in Italy and only in 1 of 41

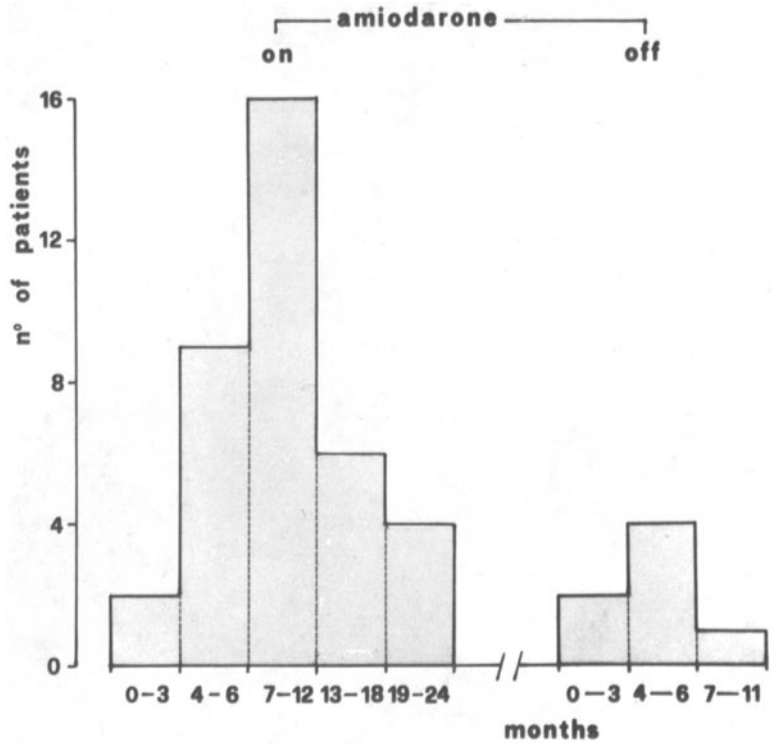

Fig. 1. Time intervals between the onset of AAT and the institution or withdrawal of amiodarone therapy.

patients (2%) living in the USA. In contrast, hypothyroidism was more common in the USA (9 of 41 patients, 22%) than in Italy (10 of 188 patients, 5%). Environmental factors such as iodine intake may well account for these differences. The higher incidence of AAT in West Tuscany, a region of low iodine intake, is not surprising because iodine-induced thyrotoxicosis is much more common in areas of endemic iodine deficiency when supplemental iodine is administered or when iodine-containing compounds are given (16). Conversely, the higher incidence of hypothyroidism during amiodarone therapy observed in Worcester may be explained by the higher environmental iodine intake in the USA (6). It is of interest that AAT may occur at any time during chronic amiodarone treatment and even several months after withdrawal of therapy. The time interval between the onset of disease and the institution of withdrawal of therapy observed by us in a recent series of 44 AAT patients is reported in Fig. 1.

EUTHYROID AMIODARONE-TREATED PATIENTS

In the majority of clinically euthyroid patients, the serum T_4 concentration was within the normal range, but the mean value was slightly higher than in control subjects, the difference being significant in Worcester but not in West Tuscany. Serum concentrations of free T_4 were significantly increased in both areas. The mean serum concentration of total T_3, but not of free T_3, was significantly decreased in patients from West Tuscany, while both parameters were significantly reduced in Worcester. A significant increase in serum rT_3 was noted in both areas. Serum thyroglobulin concentration was similar to that found in control subjects. With the exception of two cases with slightly elevated values, basal serum TSH was within the

normal range. Interestingly, absent or blunted TSH responses to TRH were frequently observed (43% in West Tuscany and 24% in Worcester). Serum total and free T_4 and T_3 concentrations did not differ between patients with absent, blunted, or normal responses from either area.

The above changes in serum T_3 and rT_3 are in keeping with the known effects of amiodarone on 5'-deiodinase. The slight increase in serum T_4 could also be explained by a decreased metabolic clearance rate of T_4 (9, 22) resulting from an inhibition of the entrance of T_4 into the cell (23) and by a compensatory rise in the T_4 production rate in response to the decreased serum T_3 concentration. The absent or blunted TSH response to TRH occurring in clinically and biochemically euthyroid patients may be difficult to explain. These abnormalities may result from a previous transient mild thyrotoxic state or may herald impending thyrotoxicosis as suggested in a recent report (24). As an alternative explanation, a weak agonistic effect of amiodarone on the pituitary cell should be considered. Evidence for such an effect has recently been documented in hypothyroid rats in which the acute administration of amiodarone was associated with a partial inhibition of TSH secretion associated with a further decrease in serum T_4 and T_3 (25). In contrast, the finding that amiodarone stimulates *in vitro* the release of TSH from cultured rat pituitary cell remains to be explained (12).

Serum thyroglobulin and thyroid microsomal antibodies had an incidence (7%) similar to that of controls, indicating that amiodarone treatment per se does not induce thyroid autoimmunity.

AMIODARONE-ASSOCIATED THYROTOXICOSIS (AAT)

The serum free T_4 concentration was elevated in most (15/18) patients with ATT observed in West Tuscany, but the serum T_4 concentration was often (10/18) in the range seen in euthyroid amiodarone-treated patients. The serum free T_3 concentration was elevated in all and the serum total T_3 concentration was elevated in 16. Serum TSH was significantly decreased and the TSH response to TRH was absent in all patients. Similar changes were observed in the single AAT patient from Worcester. Thus, the serum total and free T_3 concentrations appear to be the most useful indicators of thyroid function in the diagnosis of hyperthyroidism associated with chronic amiodarone therapy (6,26). The serum thyroglobulin concentration was frequently elevated, as in other hyperthyroid conditions except for thyrotoxicosis factitia (27). In a separate study, 24 hr thyroid radioactive iodine uptake (RAIU) was determined in 35 patients with AAT. Markedly reduced values ($< 4\%$) were found in all 12 patients who had no thyroid abnormalities by physical exam and ultrasound. In contrast, values greater than 8% were observed in most of the AAT patients with either diffuse (9/11, mean $\pm$ SE = 26 $\pm$ 5%) or nodular (8/12, 16 $\pm$ 5%) goiter. There was no correlation between RAIU values and serum concentration of thyroid hormones or urinary iodine excretion (28). The RAIU was always undetectable in euthyroid amiodarone-treated patients, irrespective of the presence or absence of goiter. goiter. It would appear that the goitrous patients with AAT had an underlying disorder leading to failure of the thyroid to adapt to an excess iodine load by decreasing the active transport of iodide from plasma into the thyroid. This may explain why the incidence of spontaneous remission of thyrotoxicosis is much lower in the AAT patients with goiter than in those without goiter. Studies on circulating thyroid antibodies were carried out in patients with AAT. Thyroglobulin and/or microsomal antibodies were found in the majority (5/7) of the patients with toxic diffuse goiter, in one of seven patients with nodular goiter, and in two of nine patients with no apparent thyroid abnormality. In AAT patients with diffuse goiter, TSH receptor antibodies were found in all cases (7/7) when assayed by TSH-binding inhibition assay and in 57% (4/7) cases when assayed by adenylate

cyclase stimulation assay. Positive results with both assays were found only in one case with nodular goiter and in no patient without goiter. As discussed in an accompanying report published elsewhere in this volume (29), the absence of thyroid autoantibodies in the AAT patients with no evidence of underlying thyroid disorders excludes a pathogenic role of thyroid autoimmunity in these cases and provides indirect support to the concept that their thyrotoxicosis could be accounted for by the iodine load. This also applies to the AAT patients with nodular goiter. In contrast, the high incidence of typical autoimmune humoral features of Graves' disease in the AAT patients with diffuse goiter suggests that in these patients the thyroid autoimmune disorder was already present albeit clinically not apparent. It would appear that in these patients, amiodarone plays a triggering role in clinical expression of hyperthyroidism rather than including Graves' disease (30).

THERAPY OF AAT

AAT should be regarded as a serious complication, since it develops in patients with cardiac disorders and because conventional methods of therapy are often ineffective. Spontaneous remission of AAT after withdrawal of amiodarone has been observed in some patients, but several months may be required for this to occur, resulting in the danger of a worsening of their cardiac disease. Thionamide drug therapy is usually less effective in this condition and the use of radioiodine therapy is often prevented by the low thyroidal ^{131}I uptake. The risk of thyroidectomy in cardiac patients with uncontrolled thyrotoxicosis cannot be considered. Recently, effective control of hyperthyroidism by administration of corticosteroids has been reported in a small number of patients with thyrotoxicosis induced by amiodarone (31) or other iodine-containing compounds (19). No other effective medical treatment of AAT has so far been described. We recently (32) proposed the use of the simultaneous administration of potassium perchlorate ($KClO_4$) and methimazole (MMI). The effectiveness of this therapeutic method was evaluated in 23 patients with AAT, nine of whom were untreated, six treated with 40 mg MMI daily, and eight treated with 40 mg MMI and 1 g $KClO_4$ daily for up to 40 days and then MMI alone. Amiodarone was withdrawn in all cases. Five untreated patients had no goiter and all of them became clinically and biochemically euthyroid within 2-4 months; the remaining four patients were goitrous and all of them were still hyperthyroid 6-7 months after discontinuation of amiodarone. Five of the six patients receiving MMI alone had goiter and all of them remained clinically and biochemically hyperthyroid during the time of therapy (3-6 months); one patient of this group had no goiter and became euthyroid within three months of therapy. In seven patients (five with and two without goiter) combined therapy with MMI and $KClO_4$ resulted in a rapid nomalization of serum thyroid hormones and restoration of clinical euthyroidism within 16-36 days. In the remaining patient (with goiter) of this group, $KClO_4$ was discontinued after 40 days because of mild neutropenia and euthyroidism was achieved about 100 days later with MMI therapy alone (Fig. 2). A marked increase in the urinary iodine excretion was observed during the first two weeks of treatment in the patients treated with $KClO_4$ and MMI. These data indicate that AAT is more likely to spontaneously remit in patients with no underlying thyroid disorder. Treatment with MMI alone is generally unsuccessful in inducing euthyroidism, especially in patients with underlying thyroid disease. In contrast, combined therapy controls hyperthyroidism in almost all cases irrespective of the presence of underlying thyroid disease. The mechanism of the synergistic action of $KClO_4$ and MMI in the therapy of AAT remains unclear. $KClO_4$ could exert its action by inducing a rapid depletion of excess intrathyroidal iodine whose organification is blocked by the concomitant administration of MMI. $KClO_4$ would also block further entrance of iodine from the plasma into the thyroid.

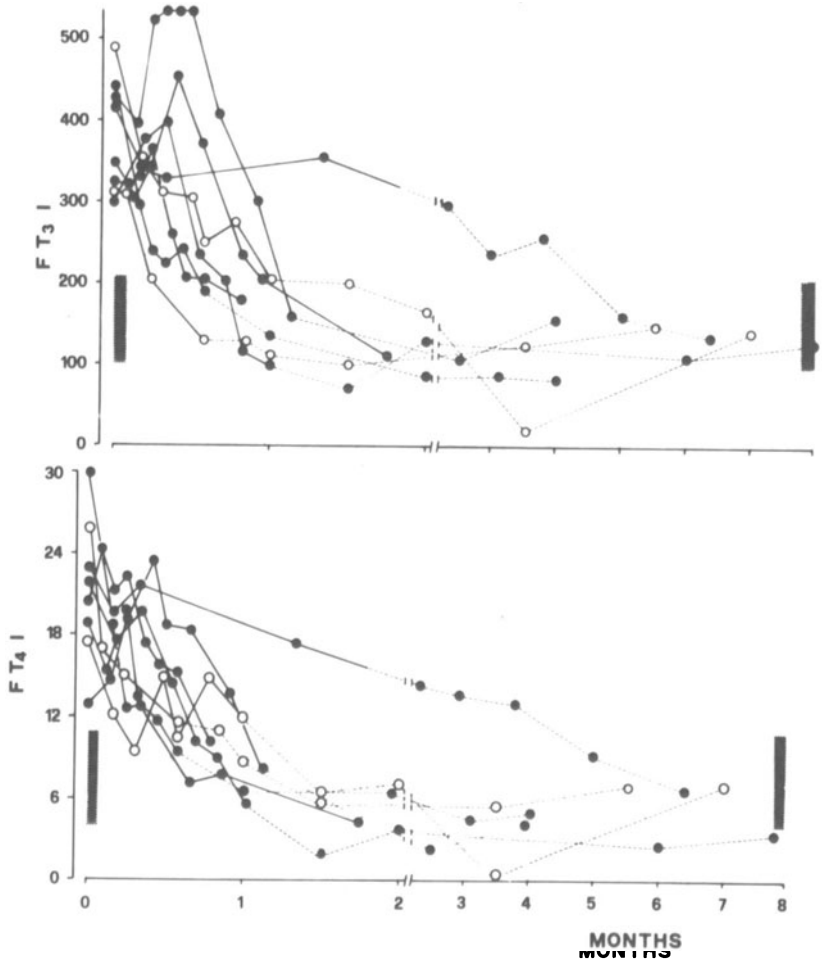

Fig. 2. Serial FT_4I and FT_3I values in patients with amiodarone-associated thyrotoxicosis treated with methimazole plus $KClO_4$. The duration of $KClO_4$ is represented by the solid lines. The dotted lines represent values after KClO4 was discontinued. The two patients without underlying thyroid disease (o) did not receive any additional antithyroid drug therapy, while patients with underlying thyroid disease (●) required 5-20 mg/methimazole daily to maintain the euthyroid state.

AMIODARONE-ASSOCIATED HYPOTHYROIDISM (AAH)

Although the diagnosis of hypothyroidism was often not suspected clinically, the serum TSH concentration was markedly elevated in all hypothyroid patients from West Tuscany and Worcester. The serum total and free T_4 concentrations were low or low-normal in these patients, especially when compared with levels observed in euthyroid amiodarone-treated patients, in whom the serum T_4 concentration tended to be elevated or in the high-normal range. To a lesser extent, serum total and free T_3 values were also reduced in AAH patients, but considerable overlap with values observed in the control groups was found. The serum concentration of rT_3 was significantly reduced compared to euthyroid amiodarone-treated patients, but not compared to

control subjects. Thus, the laboratory diagnosis of hypothyroidism in patients chronically treated with amiodarone is readily made when the serum TSH concentration is elevated and the serum total and free T_4 concentrations are low or in the low-normal range. The serum thyroglobulin was elevated in most of these (10/13) hypothyroid patients in whom it was measured. This observation is consistent with the increased thyroidal release of thyroglobulin during TSH stimulation. Thyroid microsomal antibodies were positive in 40% and 33% of patients with hypothyroidism from West Tuscany and Worcester, respectively, suggesting the presence of an underlying autoimmune thyroiditis. This disorder is known to be frequently associated with iodine-induced hypothyroidism (33). The etiology of AAH in the absence of autoimmune thyroiditis is unknown, but the large quantity of iodide released from the metabolism of amiodarone is the most likely cause. In normal subjects, the administration of large amounts of iodine induces a transient decrease in thyroid hormone synthesis, but no adaptation mechanism subsequently leads to normalization of thyroid hormonogenesis (16). Possibly patients who develop AAH have an intrinsic subtle defect resulting in failure to adapt to excess of iodine.

THERAPY OF AAH

AAH is usually mild and often is limited to biochemical evidence of thyroid underfunction with no overt clinical manifestations. However, severe myxedema may also occur (34) and a patient who died of probable myxedema coma has recently been reported (35). The occurrence of overt hypothyroidism during amiodarone treatment requires discontinuation of the drug. When this is not possible, thyroid hormone replacement therapy is indicated under careful monitoring of serum thyroid hormone and TSH concentrations. The question of whether preference should be given to thyroxine or triiodothyronine remains to be clarified (35). Spontaneous remission of AAH after withdrawal of amiodarone is usually observed, but hypothyroidism may persist for several months. To overcome the inconvenience of this relatively prolonged hypothyroid state, a course of thyroid hormone therapy may be considered. Since the development of AAH appears to be related to the intrathyroidal iodide excess, we considered the possibility of restoring euthyroidism by the administration of $KClO_4$. This would act by depleting excess intrathyroidal iodide, thus removing its inhibitory effects on thyroid hormone synthesis and release. So far,in four patients with AAH have been treated with 1 g of $KClO_4$ daily after withdrawal of amiodarone. A prompt rise in both serum T_4 and T_3 concentrations associated with a fall in serum TSH was observed in all cases within a few days. Further administration of $KClO_4$ resulted in a reversible decrease in serum thyroid hormone concentrations. Further details are given in a separate report by Martino et al. (36) published elsewhere in this volume. These data indicate that short-term administration of $KClO_4$ may be used in AAH to shorten the period of thyroid insufficiency.

CONCLUSIONS

In conclusion, amiodarone-induced thyroid dysfunction is most likely due to the excess iodide released during metabolism of this iodine-rich drug. Hyperthyroidism occurs more frequently in areas of moderate iodine deficiency, while hypothyroidism is more frequently found in areas of iodine sufficiency.

The diagnosis of AAT is best confirmed by elevations in the serum total and free T_3 concentrations, since elevated serum total and free T_4 concentrations are not infrequently found in clinically euthyroid subjects chronically treated with amiodarone. Similarly, TSH unresponsiveness to TRH may

also be encountered in association with euthyroidism, possibly in relation to a weak T_3 agonistic effect of amiodarone on the pituitary. Conventional methods of treatment are relatively ineffective or not feasible in AAT patients, although spontaneous remission after withdrawal of the drug occurs, especially in patients with no underlying thyroid disease. In patients with goiter, successful control of hyperthyroidism is achieved by combined therapy with MMI and $KClO_4$.

AAH is usually mild and its recognition is mainly based on laboratory rather than clinical grounds. An elevated serum TSH concentration and an inappropriately low-normal serum T_4 concentration are the best indicators for the diagnosis of this condition. AAH also may remit spontaneously after discontinuation of amiodarone therapy. Recovery may be substantially accelerated by short-term treatment with $KClO_4$.

In view of the relatively high incidence of amiodarone-induced thyroid dysfunction, thyroid function should be carefully monitored in patients receiving chronic amiodarone therapy, especially in those patients with goiter or Hashimoto's thyroiditis.

REFERENCES

1. Nademanee K, Singh BN, Hendrickson J, et al. Ann Int Med 98: 577, 1983.
2. Marcus FI, Fontaine GH, Frank R, et al. Am Heart J 101: 480, 1981.
3. Burger A, Dinichert D, Nicod P, et al. J Clin Invest 58: 255, 1976.
4. Jonckheer MH, Block P, Broeckaert I, et al. Clin Endocrinol (Oxf) 9: 27, 1978.
5. Melmed S, Nademanee K, Reed AW, et al. J Clin Endocrinol Metab 53: 997, 1981.
6. Martino E, Safran M, Aghini-Lombardi F, et al. Ann Intern Med 101: 28, 1984.
7. Lambert MJ, Burger AG, Galeazzi RL, et al. J Clin Endocrinol Metab 55: 1058, 1982.
8. Sogol PB, Hershmann JM, Reed AW, et al. Endocrinology 113: 1464, 1983.
9. Kannan R, Tidwell D, Ookhtens M, et al. Clin Res 31: 24A, 1983.
10. Aanderud S, Sundsfjord J, and Aarbakke J. Endocrinology 115: 1605, 1984.
11. Chopra IJ, Huang T, Hurd RE, et al. Endocrinology 114: 2039, 1984.
12. Franklin JA, Davis JR, Gammage MD, et al. Clin Endocrinol (Oxf) 22: 257, 1985.
13. Jaggarao NSV, Sheldon J, Grundy EN, et al. Post Grad Med J 58: 693, 1982.
14. Jonckheer MH. Acta Cardiol 3: 199, 1981.
15. Savoie JC, Massin JP, Thomopoulos P, et al. J Clin Endocrinol Metab 41: 685, 1975.
16. Fradkin JE and Wolff J. Medicine (Baltimore) 62: 1, 1983.
17. Amioco JA, Richardson V, Alpert V, et al. Arch Int Med 144: 487, 1984.
18. Posner J, Sobel RJ, and Glick S. Isr J Med Sc 20: 113, 1984.
19. Leger AF, Massin JP, and Maurent MF. Eur J Clin Invest 14: 449, 1984.
20. Puletti M, Erba SM, Borgia C, et al. Am J Cardiol 17: 399, 1984.
21. Borowski GD, Garofano CD, Rose LI, et al. Am J Med 78: 443, 1984.
22. Burger AG, Lambert MJ, and Cullen M. In A Albertini and R Ekins (eds), Free Hormones in Blood, Elseviere Biomedical Press, Amsterdam, 1982, p 303.
23. Krenning EP, Docter R, Visser TJ, et al. J Endocrinol Invest 6: 59, 1983.
24. Staubli M and Studer H. Klin Wochenschr 63: 168, 1985.

25. Safran M, Fang SL, Martino E, et al. Clin Res 33: 313A, 1985.
26. Pinchera A, Martino E, Pacchiarotti A, et al. In O Von, H Eber, and W Langsteger (eds), Freie Schilddrusenhormone in der Klinischen Diagnostik, Deu Arzte-Verlag, Koln, 1985, p 147.
27. Mariotti S, Martino E, Cupini C, et al. New Engl J Med 307: 410, 1982.
28. Martino E, Aghini-Lombardi F, Lippi F, et al. J Nucl Med 26: 1402, 1985.
29. Fenzi GF, Macchia E, Aghini-Lombardi F, et al. Program 9th Internat Thyroid Congress, Sao Paulo, 1985.
30. Aghini-Lombardi F, Martino E, Fenzi GF, et al. In R Hall and J Koebberling (eds), Thyroid Disorders Associated with Iodine Deficiency and Excess, Raven Press, New York, in press.
31. Wimpfheimer C, Staubli M, Schadelin J, et al. Br Med J 284: 1835, 1982.
32. Martino E, Baschieri L, Aghini-Lombardi F, et al. Ann Endocrinol 45: 15, 1984.
33. Vagenakis AG, Wang G, Burger A, et al. New Engl J Med 287: 523, 1972. 1972.
34. Hawthorne GC, Campbell NPS, Geddes JS, et al. Arch Int Med 145: 1016, 1985.
35. Mazonson PD, Williams ML, Cantley LK, et al. Am J Med 77: 751, 1984.
36. Martino E, Mariotti S, Morabito S, et al. Program 9th International Thyroid Congress, Sao Paulo, 1985.

ISOLATION AND CHARACTERIZATION OF THE GENE ENCODING THE β-SUBUNIT OF RAT THYROTROPIN

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Thyrotropin (TSH) is a member of the glycoprotein hormone family which includes pituitary lutropin (LH) and follitropin and placental chorionic gonadotropin. As with the other glycoprotein hormones, TSH is composed of two nonconvalently linked subunits, α and TSHβ. The α subunit is common to all of the hormones and the β subunit is distinct for each hormone (1,2). Separate mRNAs which are encoded by separate genes have been identified for the TSH subunits (3-8). In the rat, there appears to be a single TSHβ mRNA (0.7 Kb) which is consistent with data from Chin et al. (7) that indicates a single rat TSHβ gene. On the other hand, Kourides and co-workers have suggested the possibility of two TSHβ genes in the mouse with only one being expressed (9).

The synthesis and secretion of TSH by the thyrotropes are suppressed by thyroid hormones (2). The mechanisms by which thyroid hormones induce a coordinate regulation of TSH subunit gene expression is not well understood. In euthyroid and hypothyroid animals and thyrotopic tumors, thyroid hormones cause a decrease in TSH with a preferential decrease in the TSHβ subunit mRNA (10-14). Thyroid hormones also rapidly decrease the rate of transcription of the TSH subunit genes with a greater effect noted for the TSHβ subunit (15).

In order to investigate the mechanisms by which thyroid hormones regulate TSH subunit biosynthesis, we are studying the effect of thyroid hormones on the expression of these genes. In this study, we report the identification of a single rat TSHβ gene, its overall structure, and partial nucleotide sequence.

METHODS AND RESULTS

Identification of Rat Genomic DNA Fragments Encompassing the TSHβ Gene

The rat TSHβ gene was isolated from a bacteriophage λ Charon 4A rat liver DNA library. TSHβ-gene containing phage clones were selected by filter hybridization (16) using a nick-translated (17) mouse TSHβ cDNA probe (10). Restriction-enzyme-digested bacteriophage λ Charon 4A DNA containing the rat TSHβ gene was resolved and processed according to the method of

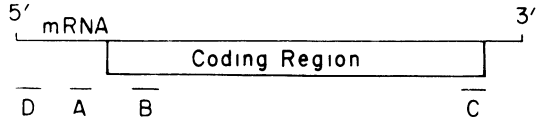

Fig. 1. Synthetic oligodeoxyribonucleotide probes were made to correspond to different regions of the rat TSHβ subunit mRNA. The indicated probes were made to correspond to 27 nucleotides in the 5' untranslated region, 32 to 6 bases upstream from the mRNA translational start site (probe A); 25 nucleotides in the leader sequence, codons -12 to -5 (probe B); 21 nucleotides in the 3'-coding region, codons +112 to +118 (probe C); and 27 nucleotides in the 5' untranslated region, 70 to 43 bases upstream from the translational start site (probe D).

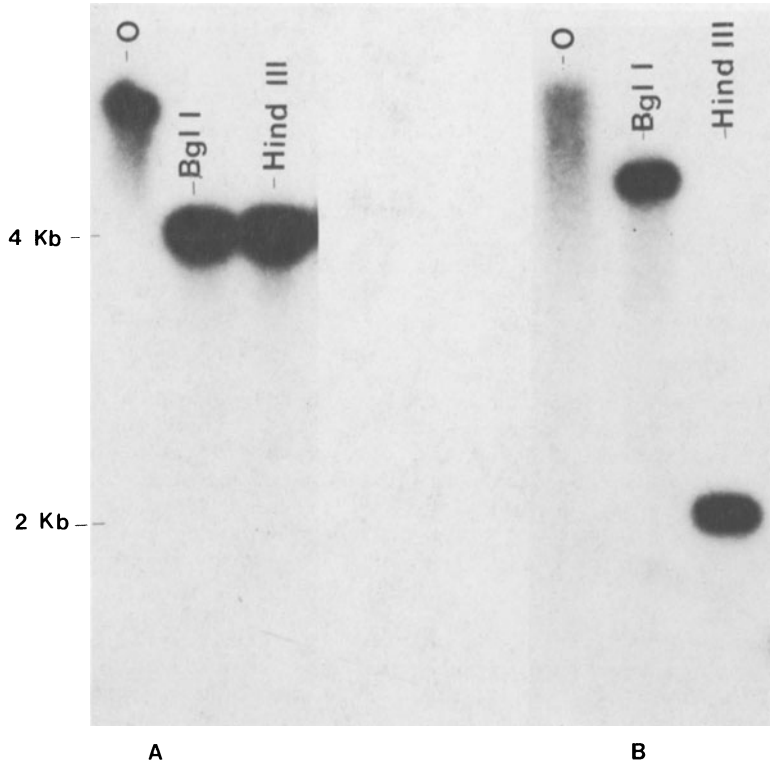

Fig. 2. Bacteriophage λ Charon 4A-containing rat TSHβ gene (1 μg DNA) was digested with the indicated restriction enzymes. The DNA was resolved by electrophoresis and transferred to nitrocellulose. The DNA was then hybridized to probe A (2A) or to probe B (2B). Both probes hybridized to the undigested DNA. Probe A hybridized to a 4.0 Kb DNA fragment and probe B to a 2.0 Kb fragment generated by the restriction enzyme Hind III.

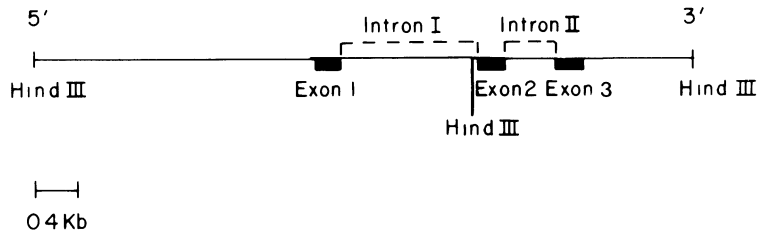

Fig. 3. Structure of the rat TSHβ gene. The rat TSHβ gene is 2.5 Kb and consists of 3 exons and 2 introns. The translational start site (ATG) is within exon 2 and the transcriptional start site is 28 nucleotides upstream within exon 1. Furthermore, a TATAAAA region is located 30 nucleotides upstream from the transcriptional start site.

Southern (18). Hybridization with the (^{32}P)-end labeled synthetic deoxyribonucleotides that are complementary to specific regions of the rat TSHβ mRNA (13) (Fig. 1) resolved 4.0 Kb and 2.0 Kb Hind III DNA fragments encoding the 5' untranslated region and the coding region, respectively (Fig. 2). The Hind III-generated DNA fragments were subcloned into the plasmid vector pBR322 and subsequently selected by colony hybridization (19) with a nick-translated rat TSHβ cDNA probe (7).

Structure of the Rat TSH β Gene

The 4.0 Kb and 2.0 Kb Hind III fragments were further digested with restriction enzymes and subcloned into the M13 cloning vector (20,21). The sequencing of the subcloned fragments was carried out using the dideoxynucleotide method with M13 DNA primers (New England Biolabs, Beverly, MA). The rat TSHβ gene consists of 3 exons and 2 introns. The junction of the exons and introns was defined by comparison of the gene sequences with the known rat TSHβ cDNA sequence (6,7). Intron 1 is located 1 nucleotide upstream from the translational start site and intron 2 is located between codons +33 and +34 (Fig. 3)

Identification of the Site of Initiation of Transcription

Specific primed cDNA synthesis was used to determine the transcriptional initiation site of the rat TSHβ gene (22). The size of the synthesized DNA corresponding to the 5'-end of the TSHβ mRNA was determined by electrophoresis with known molecular size markers. The authentic rat TSHβ mRNA cDNA transcript was identified as the band increased in thyroidectomized relative to normal rat pituitary RNA. The transcriptional initiation site is located 28 nucleotides upstream from the translational start site (data not shown).

DISCUSSION

The structure of the single rat TSHβ gene that we have isolated and characterized is similar to that of the other glycoprotein hormone β subunit genes. The 2.5 Kb gene consists of 3 exons and 2 introns, the locations of which are similar but not identical to those of the rat LHβ gene (22) and human LHβ and CGβ genes (23). However, while the sizes of the 3 exons and intron 2 are similar, intron 1 of the rat TSHβ gene is much larger (>1 Kb compared to 0.3 Kb). The 5'-untranslated region of the rat TSHβ gene

like that of the rat LHβ gene, is short with the transcriptional start site (exon 1) located 28 nucleotides upstream from the translational start site (exon 2). The presence of TATAAA region 30 nucleotides upstream in the 5'-flanking region is consistent with the promoter location described for other gene sequences.

Recently, Samuels et al. (24) reported that thyroid hormone stimulation of growth hormone gene expression is mediated through the 5'-flanking region of the gene in the rat. The complete characterization of the rat TSHβ gene will enable us to begin studies in the mechanism of regulation of its expression and of the structural regions (notably in the 5'-flanking region) that are important for the functional response of this gene to thyroid hormones and other stimuli.

REFERENCES

1. Pierce JG. Endocrinology 89: 1331, 1971.
2. Pierce JG and Parsons TF. Annu Rev Biochem 50: 465, 1981.
3. Chin WW, Habener JF, Kieffer, JD, et al. Biol Chem 253: 7985, 1978.
4. Vamvakopoulos NC and Kourides IA. Proc Natl Acad Sci USA 76: 3809, 1979.
5. Kourides IA, Baker PE, Gurr JA, et al. Proc Natl Acad Sci USA 81: 517, 1984.
6. Croyle ML and Maurer RA. DNA 3: 231, 1984.
7. Chin WW, Muccini JA, and Shin L. Biochem Biophys Res Commun 128: 1152, 1985.
8. Gurr JA, Catterall JF, and Kourides IA. Proc Natl Acad Sci USA 80: 2122, 1983.
9. Kourides IA, Gurr JA, and Wolf O. In, Recent Progress in Hormone Research, Vol. 40, 1984, p 102.
10. Chin WW, Shupnik MA, Ross DS, et al. Endocrinology 116: 873, 1985.
11. Gurr JA and Kourides IA. Endocrinology 115: 830, 1984.
12. Ross DS, Downing MF, Chin WW, et al. Endocrinology 112: 2050, 1983.
13. Carr FE and Chin WW. Endocrinology, 1985 (in press).
14. Gurr JA and Kourides IA. J Biol Chem 258: 10208, 1983.
15. Shupnik MA, Chin WW, Habener JF, et al. J Biol Chem 260: 2900, 1985.
16. Benton WD and Davis RW. Science (Wash, D.C.) 196: 186, 1977.
17. Rigby PWJ, Dieckman DM, Rhodes C, et al. J Mol Biol 113: 237, 1977.
18. Southern EM. J Mol Biol 98: 503, 1975.
19. Grunstein M and Hogness D. Proc Natl Acad Sci USA 72: 3961, 1979.
20. Messing J and Vieira J. Gene 19: 269, 1982.
21. Hu N and Messing J. Gene 17: 171, 1982.
22. Jameson JL, Chin WW, Hollenberg AN, et al. J Biol Chem 259: 15474, 1984.
23. Talmadge K, Vamvakopoulos NC, and Fiddes JC. Nature 307: 37, 1984.
24. Samuels HH, Casanova J, Copp RP, et al. Clin Res 33: 601A, 1985.

ISOLATION OF A GENE ENCODING HUMAN THYROTROPIN BETA SUBUNIT

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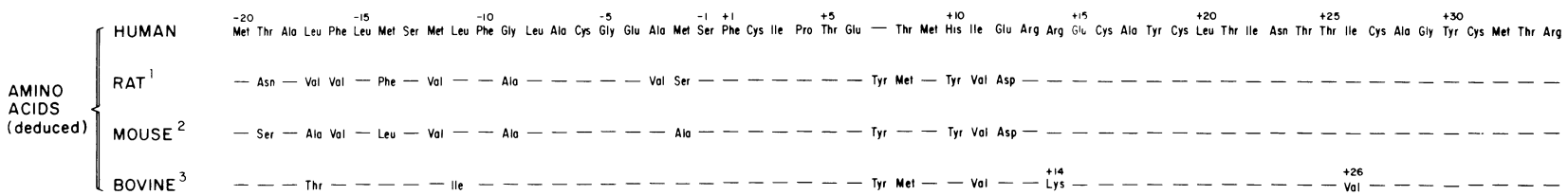
Thyrotropin (TSH) is a member of the family of glycoprotein hormones which also includes the pituitary gonadotropins, lueteinizing hormone and follicle-stimulating hormone, and the placental chorionic gonadrotropin. Each of these hormones is composed of dissimilar, non-convalently interacting, glycosylated subunits called α and β . Within a species, the α subunits are identical, and the β subunit determines the biologic and immunologic specificity of the complete hormone (1).

In the human, there is a single α gene, about 10 Kb in length (2,3). In contrast, the CG-β/LH-β genes are small, about 1.5 Kb in length, and CG genes are multiple (4,5). The CG-β/LH-β genes are arranged together in a block of genomic DNA of $>$ 30 Kb on a single chromosome (6,7), and it has been suggested that the seven CG-β genes arose by duplication of the single LH-β gene (4,8). Although we have cloned mouse TSH-β complementary DNA (9) and studied the structure of the mouse TSH-β gene (10) and Maurer and his colleagues have cloned bovine TSH-β complementary DNA (11), the human TSH-gene had not been studied. Thus, we decided to screen a human genomic library with a combination of mouse and bovine TSH-β cDNA probes.

The human genomic library used was made by partial EcoRi digestion of the DNA (a gift of Dr. Arthur Bank, Columbia University), which was cloned in Charon 4A lambda. Screening of $\sim$ 300,000 plaques yielded three hybridizing phages. After purification of the phage, similar restriction fragments were found in all three phage DNAs. Restriction fragments from two of these phages were subcloned in the plasmid vector pBR322 to allow more detailed mapping and DNA sequencing. A 0.91 KbEcoRi-BamHI human TSH-β gene fragment had its nucleotide sequence partially determined. It was found to contain an exon coding for 34 amino acids of the amino terminus of human TSH-β plus a 20 amino acid apparent signal peptide. Comparison of the amino acid sequence of human TSH-β deduced from the nucleotide sequence showed essentially a perfect match with the published amino acid sequence (1,12,13) (Fig. 1). Moreover, there is $>$ 80% amino acid homology of human with rat (14), mouse, and bovine TSH-β in this amino-terminal region.

We have found the mouse TSH-β gene to be $\sim$ 5 Kb in length and to contain 3 introns. To date we have found that the two 3'-ward exons of the human TSH-β gene match the two 3'-ward exons of the mouse TSH-β gene in size and position. Only two introns have been located in the human TSH-β



1 from M L Croyle and R A Maurer (1984) DNA 3 231-236
2 from J A Gurr, J F Catterall and I A Kourides (1983) Pro Natl Acad Sci USA 80 2122-2126
3 from R A Maurer, M L Croyle and J E Donelson (1984) J Biol Chem 259 5024-5027

Fig. 1. Partial amino acid sequences of the various TSH-β subunits as deduced from the nucleotide sequences.

gene. The location of the 3'-ward intron within the amino acid coding region of the mouse and human TSH-β genes is conserved as compared with those in the human CG-β and LH-β genes (5,8). The position of the more 5'-ward intron of the human TSH-β gene is somewhat different from that found in the human CG-β and LH-β genes.

A comparison of the structure of the cloned human TSH-β gene, isolated from our genomic library, with Southern blots of total genomic DNA is consistent with the presence of only one human TSH-β gene. Similarly, the other genes for the glycoprotein hormone subunits with the exception of hCG-β have also been single.

We had previously demonstrated that the α and β subunits of TSH were located on two different mouse chromosomes, 4 and 3 respectively (15). Mouse LH-β has been assigned to chromosome 7 (15,16). In order to map human TSH-β and to compare the chromosomal locations of α, LH-β, and TSH-β in man and mouse, we used our 0.91 Kb human TSH-β gene fragment as a probe for the analysis of Southern blots of DNA extracted from 35 rodent-human somatic cell hybrids (17). Southern blots were prepared from EcoRi digested DNA, parental cell lines, and hybrid cells. A single 2.3 Kb fragment was detected in the human parental cell lines and 22 of the 35 hybrids. Analysis of the cell panel showed concordant segregation of this fragment with chromosome 1 (17).

Subregional localization of the human TSH-β gene to 1p22 was undertaken using a panel of hybrids containing different segments of human chromosome 1, but not the intact chromosome (17).

Using our hybrid cells, we also have independently confirmed the previous assignment by Naylor et al. (16) of the α subunit gene to human chromosome 6. We agree, additionally, with the assignment of the human CG-β/LH-β gene cluster to chromosome 19.

A conserved group of genes exists on the proximal region of the short arm of human chromosome 1 and mouse chromosome 3. In addition to TSH-β, the nerve growth factor-β gene and the proto-oncogene N-ras comprise this group of genes (18-21). TSH biosynthesis is primarily regulated by the suppressive action of thyroid hormones on the synthesis of TSH-β (22). Moreover, thyroid hormones increase the synthesis of nerve growth factor (23-25). Therefore, further molecular analysis of these genes may shed light on the structure necessary for thyroid hormone regulation.

REFERENCES

1. Pierce JG and Parsons TF. Annu Rev Biochem 50: 465, 1981.
2. Fiddes JC and Goodman HM. J Mol Appl Genet 1: 3, 1981.
3. Boothby M, Ruddon RW, Anderson C, et al. J Biol Chem 256: 5121, 1981.
4. Talmadge K, Boorstein WR, and Fiddes JC. DNA 2: 281, 1983.
5. Policastro P, Ovitt CE, Hoshina M, et al. J Biol Chem 258: 11492, 1983.
6. Boorstein WR, Vamvakopoulos NC, and Fiddes JC. Nature 300: 419, 1982.
7. Whitfield GK and Kourides IA. Endocrinology 117: 231, 1985.
8. Talmadge K, Vamvakopoulos NC, and Fiddes JC. Nature 307: 37, 1984.
9. Gurr JA, Catterall JF, and Kourides IA. Proc Natl Acad Sci USA 80: 2122, 1983.
10. Kourides IA, Gurr JA, and Wolf O. Recent Prog Horm Res 40: 79, 1984.
11. Maurer RA, Croyle ML, and Donelson JE. J Biol Chem 159: 5024, 1984.
12. Shome B and Parlow AF. Endocrinology 92: A-60 (Abstract), 1973.
13. Sairam MR and Li C-H. Can J Biochem 55: 755, 1977.
14. Croyle ML and Maurer RA. DNA 3: 231, 1984.

15. Kourides IA, Barker PE, Gurr JA, et al. Proc Natl Acad Sci USA 81: 517, 1984.
16. Naylor SL, Chin WW, Goodman HM, et al. Somat Cell Genet 9: 757, 1983.
17. Dracopoli NC, Rettig WJ, Old LJ, et al. Science (submitted).
18. Francke U, de Martinville B, Coussens L, et al. Science 222: 1248, 1983.
19. Ryan J, Hart CP, and Ruddle FH. Nucleic Acids Res 12: 6063, 1984.
20. Muenke M, Lindgren V, de Martinville B, et al. Somat Cell Mol Genet 10: 589, 1984.
21. Human Gene Mapping 7. Cytogenet Cell Genet 37: 1, 1984.
22. Gurr JA and Kourides IA. J Biol Chem 258: 10208, 1983.
23. Walker P, Weichsel Jr ME, Fisher DA, et al. Science 204: 427, 1979.
24. Walker P, Weichsel Jr ME, Hoath SB, et al. Endocrinology 109: 582, 1981.
25. Walker P, Weil ML, Weichsel Jr ME, et al. Life Sciences 28: 1777, 1981.

AUTORADIOGRAPHICAL PORTRAYAL OF DISTRIBUTION PATTERNS OF TSH RECEPTORS IN HUMAN THYROID GLAND TISSUES

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INTRODUCTION

The TSH receptor has a central role in the physiological and pathophysiological control of thyroid function. For example, Graves' disease results from a stimulation of cell metabolism by immunoglobulins binding to the TSH receptor. Recent biochemical studies have been able to establish the subunit structure of the TSH receptor (1,2). However, little is known about the concentration and distribution patterns of TSH receptors in different thyroid tissues. Therefore, we have investigated the possibility of localizing the receptor in tissue sections using ^{125}I-labeled TSH and autoradiography.

MATERIAL AND METHODS

Cryostatic specimens (-80°C) of human thyroid tissue (normal tissue, n=1; autonomous adenoma, n=1; Hurthle cell adenoma, n=1; nodular goiter, n=2) were examined (three sections from each tissue). Biologically active TSH (Henning Co., Berlin), labeled with ^{125}Iodine (^{125}I-TSH), was dissolved in 1 ml buffer (10 mM Tris-HCl, pH 7.5; 50 mM NaCl and 1 mg BSA/ml). In order to remove any free ^{125}Iodine, the solution was dialyzed against this buffer for 12 h at 4°C immediately prior to use. The cryostatic specimens of thyroid tissue were fixed in acetone at 4°C for 10 min, dried at 4°C for 15 min, and rehydrated with PBS (pH 7.4). They were incubated with 110 µl ^{125}I-TSH solution (407 Bq resp. 3 nU ^{125}I-TSH/100 µl). The incubation was carried out for three hours at 37°C in a humid atmosphere. The material under investigation was bathed in PBS (four times, five minutes each time). Specimens were treated twice (five minutes each time) with a chromalaune gelatin solution (0.5 g gelatin at 37° C in 100 ml double distilled water; 0.05 g KCr $(SO_4)_2$ x 12 H_2O added after cooling to room temperature). The stripping film procedure was then applied for autoradiography (3). The film exposure time was three weeks. Supplementary incubations were carried out to asess the specificity of ^{125}I-TSH binding to TSH receptors in the tissues: a) thyroid tissues (guinea pig, hog, human) with 1) ^{125}Iodine-labeled hCG (^{125}I-hCG), (222 Bq; 2.2 Bq; 0.02 Bq), 2) free ^{125}Iodine (222 Bq; 2.2 Bq; 0.02 Bq), 3) ^{125}I-TSH with non-labeled TSH

Table 1. Autoradiography in Human Thyroid Tissue

Normal thyroid tissue	Nontoxic I	Nodular goiter II	Autonomous adenoma	Hurthle cell adenoma
3.4 ± 0.25	3.7 ± 0.26	region 1: 1.8 ± 0.15 region 2: 1.3 ± 0.05	1.5 ± 0.2	1.6 ± 0.15

Specific reflection (R) in volts, defined as R = reflection of follicular epithelium - reflection of background, of TSH receptor autoradiography applied to tissues with various human thyroid gland diseases. Mean ± SD, n = 20 for all points. ^{125}I-TSH binding to normal thyroid tissue and nontoxic nodular goiter I differed from ^{125}I-TSH binding of nontoxic nodular goiter II, autonomous and Hurthle cell adenoma. Regionally variable ^{125}I-TSH binding was found in nontoxic nodular goiter II.

(Sigma, 3 mU, 1 mU, 0.3 mU/µl buffer) (film exposure time was six weeks); b) human liver tissue and guinea pig epididymal fat pad tissue (EFP) with 1) ^{125}I-hCG, 2) free ^{125}Iodine, and 3) ^{125}I-TSH in concentrations as described under a). The film exposure time was six weeks.

Reflection photometry (MPV III Microscopic photometer, Leitz Co.) was used (wave length 624 nm) for quantitation of the autoradiographs (4). The

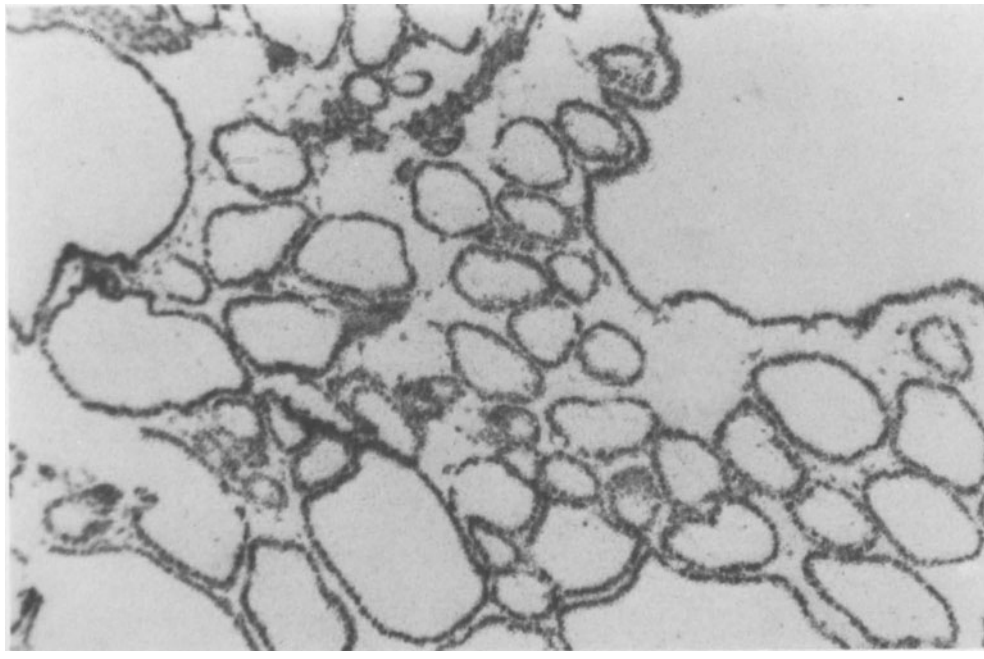

Fig. 1. ^{125}I-TSH receptor autoradiography of a follicular adenoma with 3 nU ^{125}I-TSH. Homogeneous ^{125}I-TSH labeling of follicular epithelium.

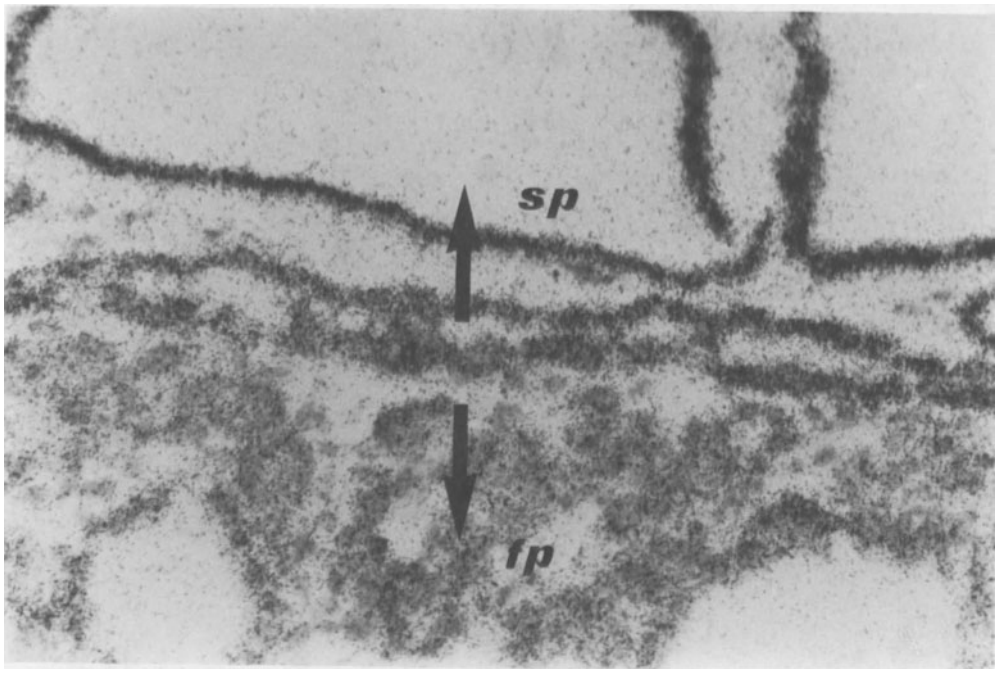

Fig. 2. ^{125}I-TSH autoradiography of TSH receptors in nontoxic nodular goiter. Significant decrease of ^{125}I-TSH binding (R = 1.6) in a region with focal nodular hyperplasia (fp) as compared to the surrounding parenchyma (sp), (R = 3.7).

reflection was directly related to the number of silver grains per area investigated and measured in volts (V). Reflections of different regions of follicle epithelium and background were each measured 20 times. Specific reflection (R) was calculated (R = reflection of follicular epithelium - reflection of background).

RESULTS AND DISCUSSION

Table 1 shows autoradiography applied to different types of human thyroid tissue. ^{125}I-TSH binding to autonomous adenoma, Hurthle cell adenoma, and nontoxic nodular goiter (II) differed from normal thyroid tissue and from a second case of nontoxic nodular goiter (I). Nontoxic nodular goiter II displayed different R's depending on the region examined. Fig. 1 shows ^{125}I-TSH receptor autoradiography in a case of follicular adenoma. Fig. 2 demonstrates autoradiographic localization of ^{125}I-TSH in a case of nontoxic nodular goiter with non-uniform binding of the labelled hormone.

An R of 2.5 was recorded in EFP cells containing TSH receptors. ^{125}I-TSH binding to thyroid tissue (three different species) and EFP was inhibited in a dose-dependent manner by unlabeled TSH. The liver, colloid, and thyroid interstitium of the various specimens examined showed no specific ^{125}I-TSH labeling (R=0). None of the investigated thyroid tissues depicted any significant ^{125}I-hCG or ^{125}Iodine binding of epithelium, interstitium, or colloid (R=0).

The results suggest that the technique described can detect specific ^{125}I-TSH binding to tissues containing TSH receptors. The different R's,

recorded from tissues with various thyroid diseases, could be explained by the variation of TSH receptor density, TSH receptor affinity, or TSH receptor-occupying antibodies. It would seem, therefore, that ^{125}I-TSH receptor autoradiography is suitable for portrayal of regionally variable ^{125}I-TSH binding in intact thyroid gland tissue. Possibly, this technique could be helpful in determining TSH receptor distribution patterns in tissues with functional heterogenicity, e.g., nontoxic and toxic nodular goiter.

REFERENCES

1. Rees Smith B, Rickards CR, Davies Jones E, et al. J Endocrinol Invest 8: 175, 1985.
2. Kajita Y, Rickards CR, Buckland PR, et al. Biochem J 227: 413, 1985.
3. Schmiegelow P, Lindner J, and Puschmann M. Akt Dermatol 9: 62, 1983.
4. Schultze B. In Handbuch der Allgemeinen Pathologie II/5, Springer, Berlin-Heidelberg-New York, 1968, p 466.

BOVINE THYROTROPIN BINDING SITES IN NUCLEI AND NUCLEAR MATRIX OF PORCINE THYROCYTES*

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INTRODUCTION

Thyrotropin (TSH) stimulates its thyroid target cells by interacting with specific receptors on the membrane (1). TSH binding sites have been purified and characterized in human and animal thyroid tissue using crude broken cell preparations, purified membranes, or whole cells (1,2). Our immunocytochemical studies, at light microscopical level, documented TSH binding sites intracellularly in porcine thyrocytes, particularly at the nuclear membrane and within the nucleus (2,3).

The aim of the present study was to verify the TSH binding sites at the nuclear membrane (NM) and within the nucleus (N) at ultrastructural level and to characterize these binding sites by radioreceptor assays.

IMMUNOCYTOCHEMICAL STUDY OF TSH BINDING SITES

In this study, a protein A-gold (pAg) complex (gold particle size: 10 nm) is used for the visualization of bTSH binding sites in porcine thyrocytes. Porcine thyroid fragments were fixed prior to or after incubation in a hormone-free medium (Leibovitz-15) or in a medium with bTSH (100 mU; Thytropar, USV, Ontario). Three incubation sequences were used for both incubated groups: 1) 60 min at 4°C, 2) 60 min at 4°C and subsequently at 37°C for 5 min, or 3) for 20 h. The fixed tissue was prepared for immunoelectronmicroscopical examination according to the method described by Van Putten et al. (4), using anti-bTSH (UCB-Bioproducts S.A., Brussels, Belgium) as first antibody and pAg complex (prepared according to the method of Slot & Geuze <5>) as a second step reagent. Fragments which were incubated for 5 min at 37°C with bTSH show groups of gold particles in droplets, cisternae of the rough endoplasmatic reticulum, at the nuclear membrane, and within the nuclei (Fig. 1).

BIOCHEMICAL STUDY OF TSH BINDING SITES

N and NM of porcine thyroids were isolated according to the (slightly modified) method of Buttyan et al. (6). Morphological analysis at struc-

*Supported by MRC grant MA-7908.

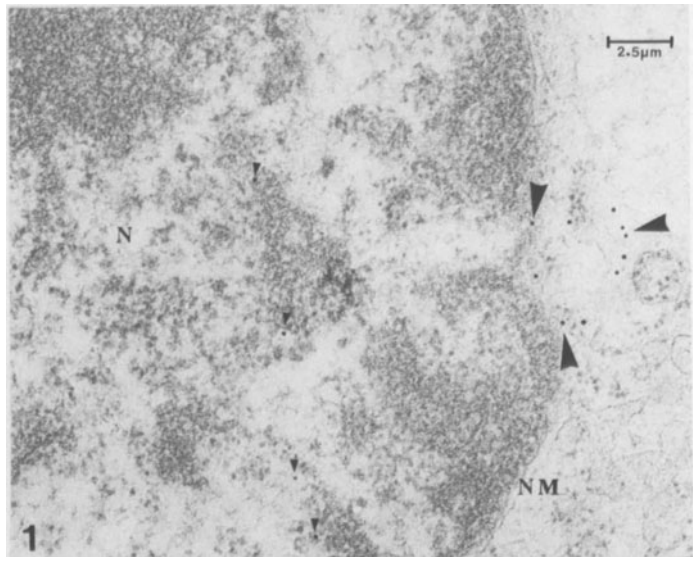

Fig. 1. Electron micrograph of a thyrocyte from a fragment, which was incubated with bTSH for 60 min at 4°C and sugsequently for 5 min at 37°C. Gold particles are found at the nuclear membrane (NM,▼) and within the nucleus (N, ▼). Immunoreaction.

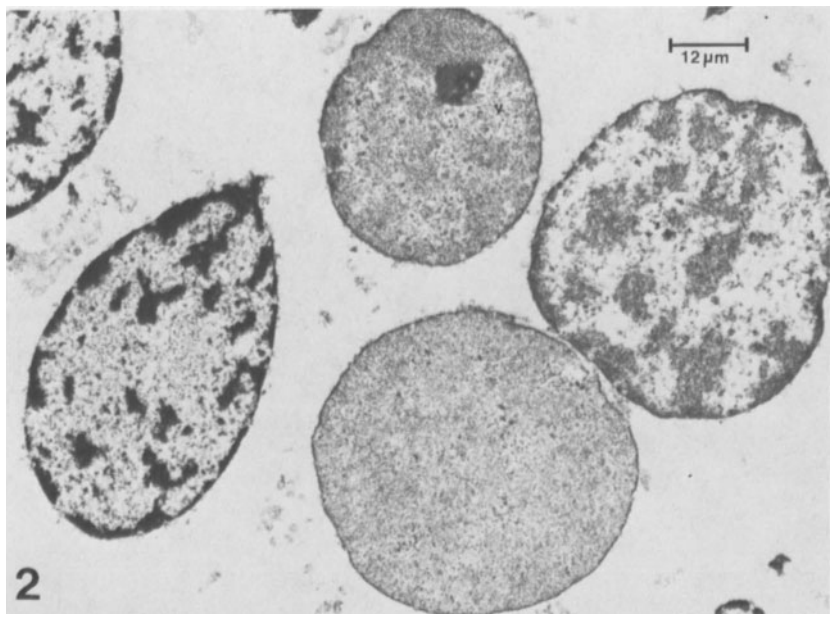

Fig. 2. Electron micrograph of a nuclei fraction.

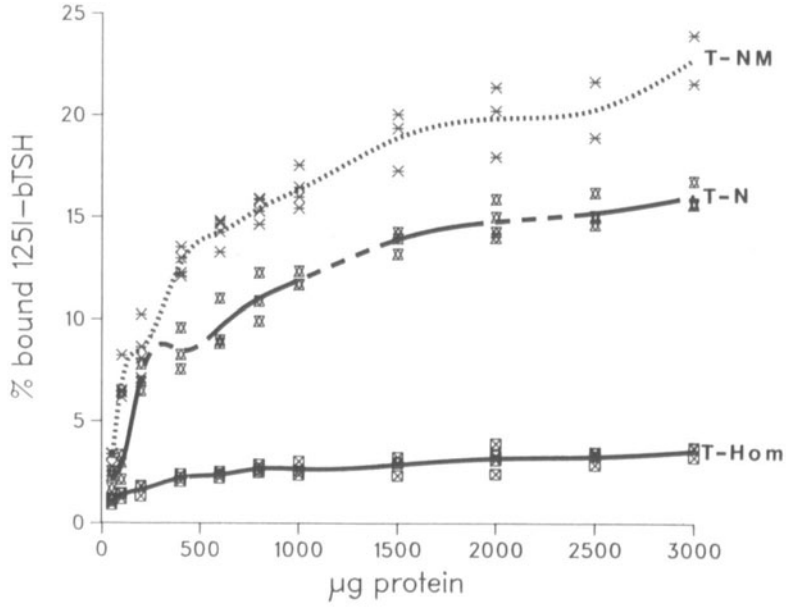

Fig. 3. Binding of ^{125}I-bTSH as a function of protein concentrations of thyroid homogenate (T-Hom), nuclei (T-N), and nuclear matrix (T-NM). Homogenate, nuclei, and nuclear matrix (protein concentrations 50-3000 µg) in 0.5 ml N-buffer (10 mM Tris-HCl, 2.5 mM KCl, 2.5 mM $MgCl_2$, 0.5 M sucrose; pH 7.5) were incubated with 1.38 ng of ^{125}I-bTSH for 1 hr at 37°C in a waterbath shaker at 100 oscillations/min. After incubation, 1.0 ml of ice-cold Dulbecco's phosphate buffered saline was added to each tube, the tubes were centrifuged in a Beckman Microfuge B for 5 min, and the washed pellets counted in a γ-counter. Each point is the mean of three observations.

tural and ultrastructural level shows a relatively pure fraction of intact nuclei (3.7 ± 0.8 mg protein/gr tissue; Fig. 2) and nuclear matrix (1.1 ± 0.3 mg protein/gr tissue). 5'Nucleotidase (measured according to Arkesteijn <7>) was detectable in the N fraction, but was 25%-50% lower than in the homogenate fraction; phosphodiesterase I activity (measured according to Touster et al. <8>) was not detectable. Radioreceptor assays (experimental procedures described elsewhere, manuscript in preparation) show that N and NM of thyrocytes can bind ^{125}I-bTSH, dependent on the amount of protein and incubation time (Figs. 3,4). At an incubation time of about 20 min at 37°C, a number of TSH binding sites of both fractions are approaching saturation. The same binding pattern has been found for TSH receptors in human thyroid membranes (9), suggesting that the decrease in binding after 15-20 min of incubation might be due to the shedding of receptors and/or releasing of receptors in the medium. Non-labeled bTSH (0.001-1 mU) shows a dose-wise inhibition of ^{125}I-bTSH binding to N, as well as to NM (Fig. 5). The binding specificity of ^{125}I-bTSH to N and NM is assessed by the lack of displacement observed in the presence of human chorionic gonadotropin or insulin (up to 1 unit). Thyroid homogenate and nuclei isolated from porcine heart tissue give little and/or non-specific binding of bTSH (Figs.

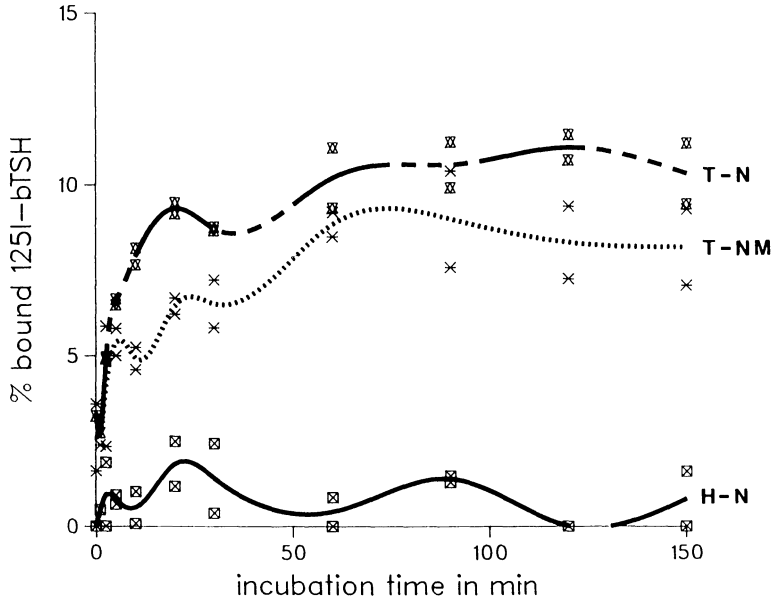

Fig. 4. Binding of ^{125}I-bTSH as a function of time. Heart nuclei (H-N), thyroid nuclei (T-N), and nuclear matrix (T-NM) were incubated with 1.38 ng of ^{125}I-bTSH for from 1 min to 150 min at 37°C. H-N and T-N: 3000 µg/tube; T-NM: 1100 µg/tube. Assay conditions were the same as in Fig. 3. The specific binding at each point is calculated as the total binding minus the non-specific binding (determined by adding an excess of unlabeled bTSH: 200 mU/tube), which averages 3-5% of the total radioactivity for T-N and T-NM.

3,4). Scatchard analyses of N and NM binding reveal one class of bTSH binding sites (Fig. 6). The binding affinities are similar (0.4 nm^{-1}), but the number of available TSH binding sites varies: R_{nuclei}:1.0 pmol/mg of protein; $R_{nuclear\ matrix}$: 1.7 pmol/mg of protein. These results are in agreement with studies on bovine luteal tissue, which indicate that several intracellular organelles, including the nucleus, contain gonadotropin binding sites (10,11).

Both morphological and biochemical approaches agree that porcine thyrocytes contain saturable and specific bTSH binding sites in their nuclei and that these sites are predominantly in the nuclear matrix. This study provided the rationale for our present investigations of the potential role of nuclear receptors in mediating some of the physiologic actions of TSH.

ACKNOWLEDGMENT

We thank Dr. L.J.A. van Putten for her support in carrying out the immuno-electronmicroscopical study.

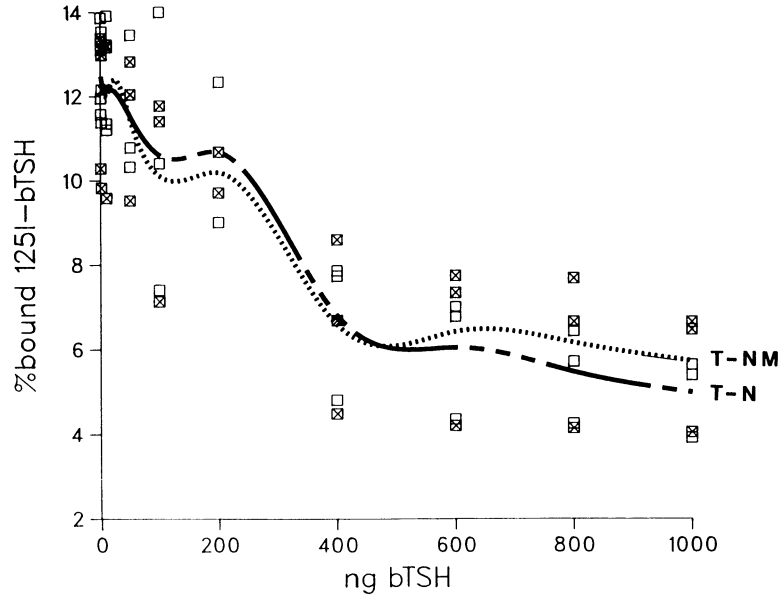

Fig. 5. Displacement of total ^{125}I-bTSH binding. T-nuclei (2000 µg protein/tube) and T-nuclear matrix (800 µg protein/tube) were incubated with 1.38 ng ^{125}I-bTSH for 1 hr at 37°C with different concentrations of unlabeled bTSH (0.001 - 1 mU). Assay conditions were the same as in Fig. 3.

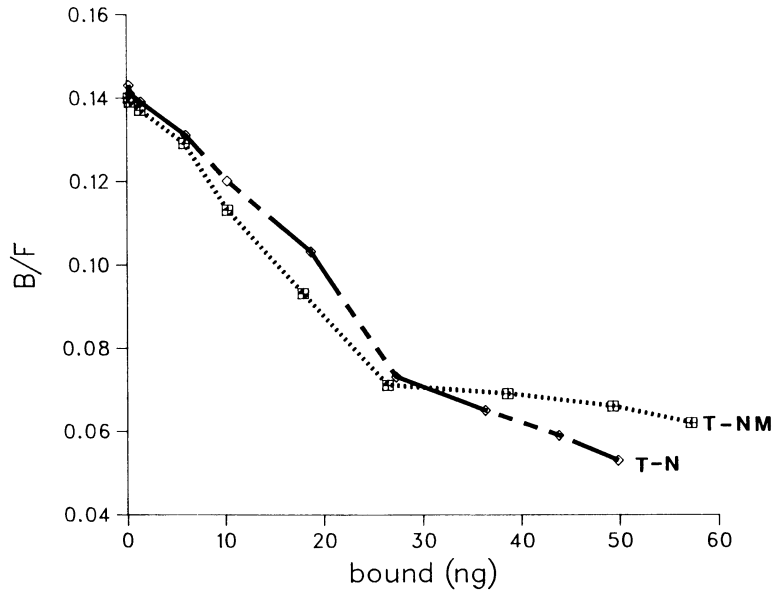

Fig. 6. Scatchard plot of the data in Fig. 5.

REFERENCES

1. Manley SWW, Knight A, and Adams DD. Springer Semin Immunopathol 5: 413, 1983.
2. Farid NR, Fahraeus-van Ree GE, and Briones-Urbina R. In PG Walfish, JR Wall, and Volpe (eds), Autoimmunity and the Thyroid, Academic Press, New York, 1985, p 249.
3. Fahraeus-van Ree GE and Farid NR. Clin Res 31: 679, 1983.
4. Van Putten LJA, Van Oordt PGWJ, Terlou M, et al. Cell Tissue Res 231: 185, 1983.
5. Slot JW and Geuze HJ. J Cell Biol 90: 533, 1981.
6. Buttyan R, Olsson C, Sheard B, et al. J Biol Chem 258: 14366, 1983.
7. Arkesteijn CLM. J Clin Chem Clin Biochem 14: 155, 1976.
8. Touster O, Anderson NN, Dulaney JT, et al. J Cell Biol 47: 604, 1970.
9. De Bruin TWA and Van der Heide D. Acta Endocrinol 99: 522, 1982.
10. Rao ChV, Mitra S, and Carman Jr FR. J Biol Chem 256: 2628, 1981.
11. Chegini N, Rao ChV, and Carman Jr FR. Exp Cell Res 151: 466, 1984.

FLOW CYTOMETRIC ANALYSIS OF TSH BINDING TO ITS RECEPTORS

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It is generally accepted that benign thyroid nodules and differentiated thyroid carcinomas have TSH receptors, and some of these tumors are stimulated by endogenous TSH. In these cases, thyroid hormone suppressive therapy is widely used to control tumor growth (1,2).

TSH receptor has been measured by Radioreceptor assay (3) or Adenylate cyclase activation method (4). Recently, flow cytometry has become widely used in cell biology because of its rapidity and objectivity. We studied flow cytometric analysis of receptor-binding of TSH to human thyroid tissues. TSH receptor might also be measured indirectly by this method.

MATERIALS AND METHOD

Human thyroid tissues were obtained at surgery. Fifteen thyroid tissues were used; one adenomatous goiter, five papillary carcinomas, two follicular carcinoma, one medullary carcinoma, two malignant lymphomas of the thyroid, one small cell carcinoma of the thyroid, and three specimens from patients with Graves' disease.

The tissues were immediately cooled at 4°C after resection. An indirect immunofluorescence method was used. The tissue was minced with scissors, and taken to pieces with pinsette in chilled PBS. Single cell suspension was prepared by filtering the solution through 60 μm Nylon mesh. The first antibody, 200 μl of anti-hTSH (Rabbit; UCB Bioproducts, lot No. I550/001) which was 10-fold diluted, was added to a pellet of 10^6-10^8 cells. After incubation for 30 min at 4°C, the cells were rinsed twice in chilled PBS. The second antibody, 200 μl of Fluorescein Isothiocyanate (FITC)-conjugated anti-Rabbit IgG (Goat; Miles-Yeda, lot No. E069) which was 10-fold diluted, was added to the cell pellet. After incubation for 30 min at 4°C, the cells were rinsed twice in chilled PBS. Methanol (2 ml of 100%) was added in the pellet, which was then left overnight at 4°C in a darkroom in order to fix the cells and stabilize the fluorescence of FITC (5). After rinsing with distilled water, 2 ml of 1 N HCl was added to the cell pellet and incubated at 60°C for 5 min to remove nonspecific binding of FITC (5). After being rapidly cooled in ice, the cells were rinsed in PBS.

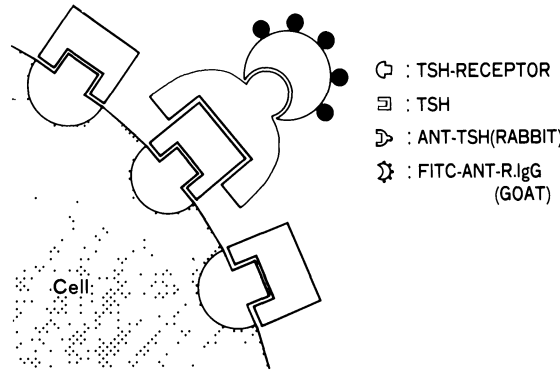

Fig. 1. Scheme of indirect membrane immunofluorescence method.

The instrument used was a CS-20 Flow Cytometer (Showa Denko Ltd., Japan). The instrument setting was constant and the flow speed was 200-600 cells/sec in each assay.

Fig. 1 shows a scheme of the indirect membrane immunofluorescence method. FITC of the second antibody is stimulated by the laser beam of the flow cytometer, and emits fluorescence. The amount of receptor-bound TSH should correlate with the fluorescence intensity of FITC.

When FITC is used, it is necessary to test the specificity of the fluorescence because of its nonspecific binding to proteins. Autofluorescence (AF), fluorescence intensity of a specimen which was incubated only with the second antibody, was measured in each assay as an indicator of nonspecific fluorescence intensity.

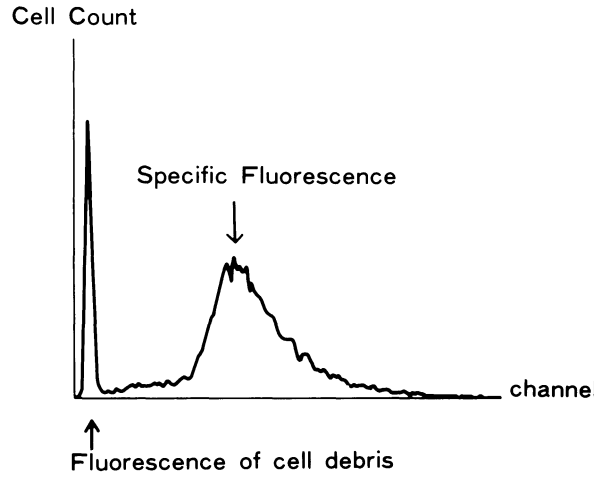

Fig. 2. A flow cytometric data of follicular carcinoma.

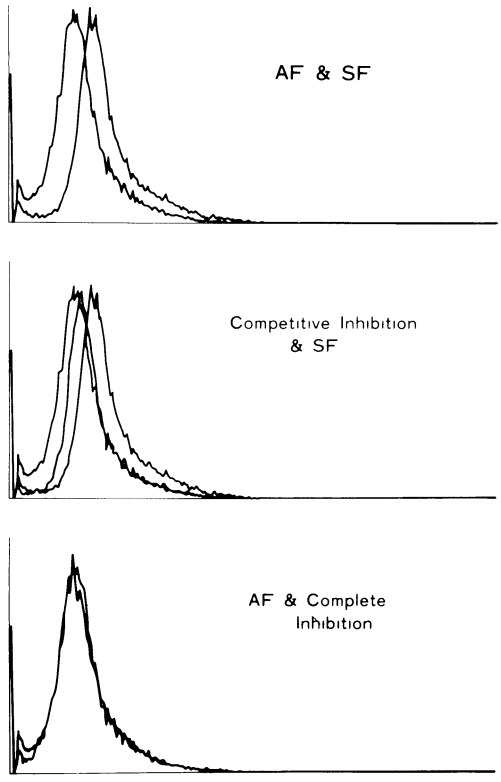

Fig. 3. Competitive fluorescence inhibition test. The specimen was a follicular carcinoma.

RESULTS

Flow cytometric data from a follicular carcinoma is shown in Fig. 2; the vertical axis is the cell count, and the horizontal axis is the channel number for the fluorescence intensity range. The wedge-shaped wave at the center shows specific fluorescence (SF). The mean fluorescence intensity (MFI) of SF is expressed by mean channel $\pm$ S.D., as the wave of SF can

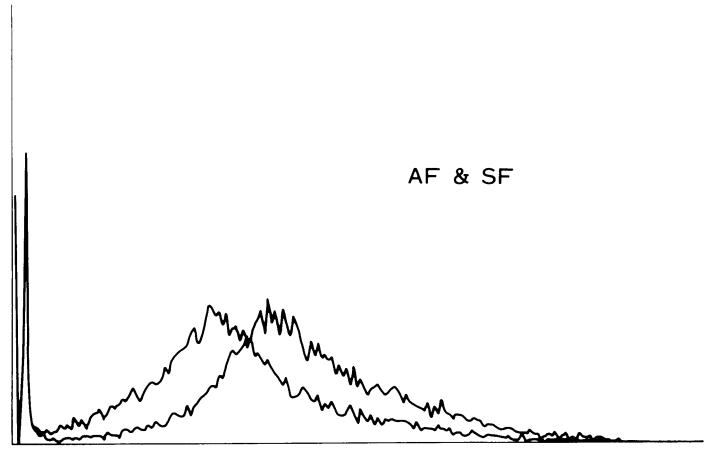

Fig. 4. Papillary carcinoma 56 y. M. MFI of AF and SF were 57.7 $\pm$ 19.2 ch. and 76.5 $\pm$ 20.4 ch.

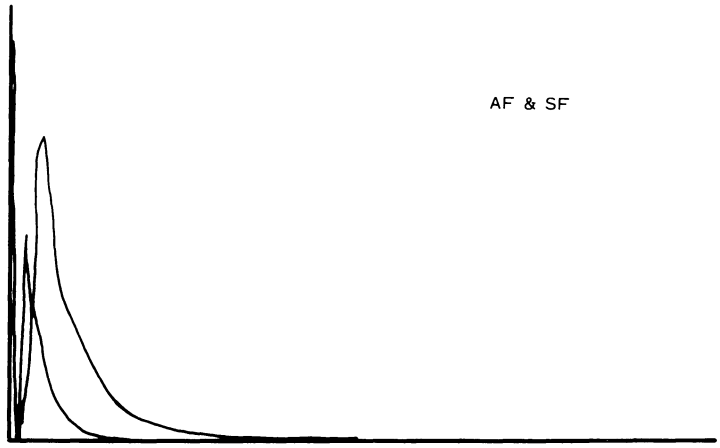

Fig. 5. Small cell carcinoma of the thyroid 71 y. F. MFI of AF and SF were 7.4 ± 1.8 ch. and 14.2 ± 1.9 ch.

be regarded as a normal distribution curve. The sharp wave at the left side shows fluorescence of cell debris.

Competitive inhibition of fluorescence, using anti-Rabbit IgG not conjugated with FITC (Miles-Yeda Ltd. lot No. S140) as a competitor with the second antibody, was carried out to demonstrate the specificity of this assay. The results using follicular cancer cells are shown in Fig. 3. The patient's preoperative serum TSH level was 2.4 μU/ml (0.5-5.5). The top of the figure is the complex of AF (lt. peak) and SF (rt. peak); peak channels (ch.) were 33 ch. and 41 ch., and MFI were 35.9 ± 11.5 ch. (mean ± S.D.) and 48.4 ± 8.9 ch., respectively. A significant difference was observed between AF and SF. The results of competitive inhibition are shown in the middle of the figure; the peak of SF (rt. peak) was shifted to the left as the content of the competitor was increased. At the bottom is seen the complex of AF and the result of complete inhibition by competitor of the second antibody;

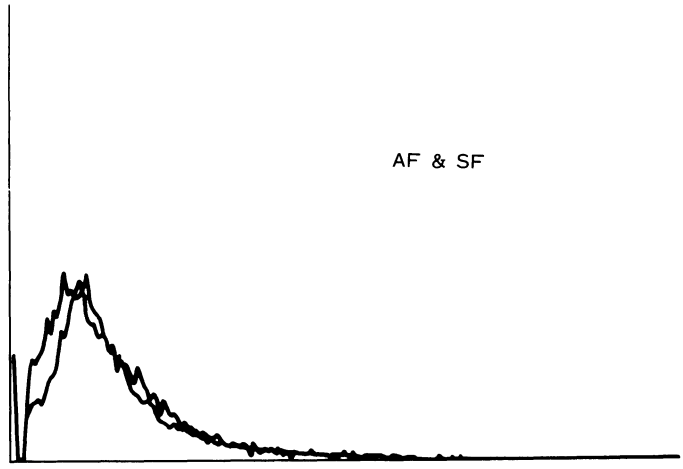

Fig. 6. Medullary carcinoma 68 y. M. MFI of AF and SF were 23.8 ± 13.4 ch. and 24.7 ± 12.5 ch.

peak ch. was the same 33 ch., and MFI were 35.9 ± 11.5 ch. and 36.4 ± 11.5 ch., respectively. The same result was gained with an experiment using adenomatous goiter cells.

Data from one of five papillary cancer cases is shown in Fig. 4. Left peak is AF, and the right peak is SF.

Data from a case of small cell carcinoma of the thyroid, which is difficult to differentiate pathologically from malignant lymphoma, is shown in Fig. 5. A difference between AF and SF can be recognized though the waves are close to the peak of cell debris because of the weakness of fluorescence intensity. There was no significant fluorescent peak in two cases of malignant lymphoma.

Data from a case of medullary cancer is shown in Fig. 6. MFI of AF and SF were 23.8 ± 13.4 ch. and 24.7 ± 12.5 ch. There was little difference between the two.

One of three cases of Graves' disease is shown in Fig. 7. The patient was in a euthyroid state after drug therapy, and her TSH level was within normal limits at operation. At the top of the figure is the complex of AF and SF. The middle figure is a complex of SF (lt. peak) and the result after preincubation with TSH, 10 mU/ml, at 4°C for 30 min before cells were treated with the immunofluorescence method (rt. peak). The bottom figure represents the complex of AF, SF, and TSH pretreatment, from the left peak, in order. Fluorescence intensity of follicular cells of Graves' disease was enhanced by pretreatment with 10 mU/ml of TSH.

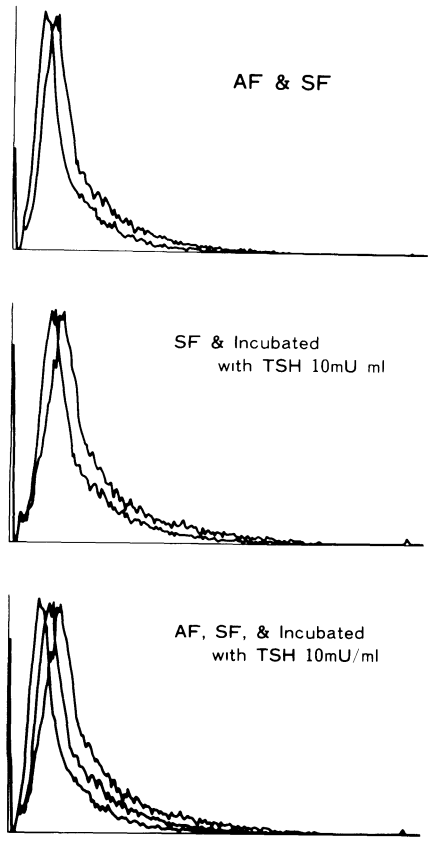

Fig. 7. Graves' disease 29 y. F. MFI of AF, SF, and TSH pretreatment were 24.0 ± 14.3 ch., 29.7 ± 15.8 ch., and 36.5 ± 20.1 ch., respectively.

CONCLUSION

The characteristics of this assay are as follows: 1) Experimental operation is easily done without use of radioisotopes. 2) The amount of receptor-bound TSH per cell, which could not be measured by another method, can be measured rapidly and objectively. 3) Analysis of TSH receptors can be done per cell unit because of flow cytometric measurement.

This method will be available for TSH receptor analysis of Graves' disease and thyroid neoplasms.

REFERENCES

1. Werner SC and Ingbar SH. In, The Thyroid, Harper and Row Publ., Hagerstown, 1978, p 525.
2. Cady B and Sedgwick CE. Ann Surg 5: 541, 1976.
3. Ichikawa Y, Saito E, Abe Y, et al. J Clin Endocrinol Metab 41: 395, 1976.
4. Field JB, Bloom G, Chou MCY, et al. J Clin Endocrinol Metab 47: 1052, 1978.
5. Fukuda M, Tsuchihashi Y, Takamatsu T, et al. Histochemistry 65: 269, 1980.

GANGLIOSIDE AND PHOSPHOLIPID CONTENT IN NORMAL AND PATHOLOGICAL HUMAN THYROID

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INTRODUCTION

The first step in the action of TSH is the binding of the hormone to a specific receptor in the thyroid plasma membrane (1). Following this event, there is an activation of adenylate cyclase, resulting in increased intracellular cyclic AMP levels (2,3). Cyclic AMP, in turn, interacts with an intracellular target and mediates many of the effects of TSH on the thyroid gland (4). Kohn et al. (5) have recently suggested that gangliosides play an important role in the interaction of TSH with thyroid plasma membrane receptor. Evidence to support this view comes from the demonstration of specific TSH-ganglioside interactions (6), the presence of gangliosides in biologically active systems and their absence in biologically inactive systems (7), and from the reconstitution of a cyclase stimulatory activity by resynthesis of higher order gangliosides in thyroid membranes where a receptor defect has been correlated with a deficiency in higher order gangliosides (8,9).

All this information comes essentially from experiments with bovine and rat thyroid, whereas there are only few data (10-12) on the role of gangliosides in human thyroids. In order to clarify this point, we have begun to study the ganglioside pattern of normal and pathological human thyroid tissue. Since, in addition to the gangliosides, phospholipids were also shown to be components and modulators of the TSH-receptor in the plasma membranes (13), we also analyzed the phospholipid fractions in the same tissues.

In this paper we compare the ganglioside and the phospholipid content of normal human thyroids to those of five nontoxic diffuse goiters, one congenital hypofunctional goiter, two toxic adenomas, and three thyroid cancers (one follicular, one papillary, and one medullary carcinoma).

MATERIALS AND METHODS

Tissues

Thyroid tissue adjacent to cold nodules with no evidence of macroscopic or microscopic abnormalities were used as normal tissue.

Pathological thyroid tissue was obtained in the operating room from patients undergoing surgery for thyroid disease. Diagnosis of thyroid disease was made by usual clinical and laboratory criteria and confirmed by histopathological findings on the tissues after surgery. Serum T_4, T_3, TSH, FT_4, and FT_3 were measured by radioimmunoassay using Becton Dickinson kits, and serum Tg by Sorin kit. Thyroid tissues were frozen in liquid nitrogen and stored at -80°C immediately after collection until used.

Ganglioside Analysis

Thyroid plasma membranes were prepared as described by Tate et al. (14). Gangliosides were extracted from sonicated membrane preparations (Braun Inc., Model Labsonic, 1510 cell disrupter; 3 min at 300 watt).

The procedure utilized was that of Yu and Leeden (15), and the final purified fraction was obtained using the method described by Williams and McCluer (16) utilizing a Sep Pak™ cartridge.

The purified fractions were assayed for total gangliosides by the resorcinol method using N-acetyl-neuraminic acid (Sigma Chemical Company, USA) as standards (17). Determination of the ganglioside pattern was performed on a purified fraction by thin layer chromatography. The purified fractions were previously dried under a stream of nitrogen and dissolved in methanol. For chromatography, silica gel 60 (HP-TLC) plates (E. Merck, Darmstadt, Germany) were used in a solvent chloroform:methanol:KCl 0.03 M (120:70:16) (9). The gangliosides were visualized with resorcinol reagent (ganglioside vis. Supelco, Bellefonte, USA) identified by comparison with standard gangliosides and quantitated densitometrically at 570 nm using a Camag TLC-HPTLC scanner (Camag, Muttenz, Switzerland).

Phospholipid Analysis

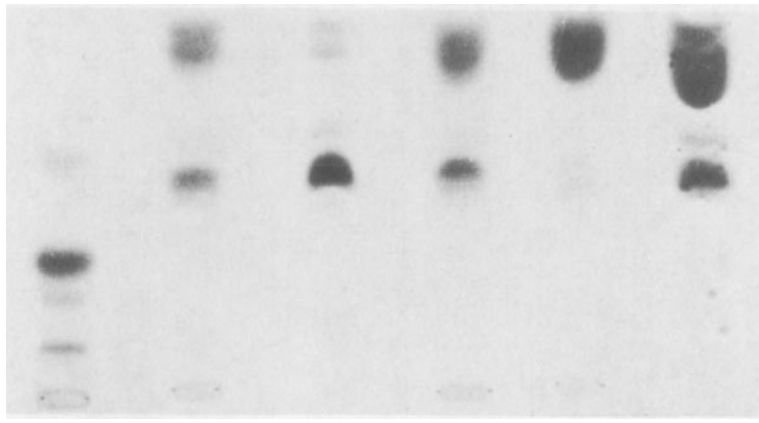
Phospholipid analysis was performed by thin layer chromatography as previously described (18). The phospholipids were located by exposing the plates to iodine vapor. The spots were scraped from the plates and the phosphate content measured.

RESULTS AND DISCUSSION

As shown in Table 1, the total ganglioside content in normal and pathological human thyroid plasma membranes ranged from 65 to 183 nmol/sialic

Table 1. Lipid-bound Sialic Acid Concentration in Human Thyroids

Tissue	Lipid-bound sialic acid (nmol/gr fresh tissue)
Normal (n.5)	75 ± 10
Nontoxic diffuse goiter (n.5)	95 ± 12
Hypofunctional congenital goiter (n.1)	147
Toxic adenomas (n.2)	127 ± 10
Cancers (n.3)	113 ± 50



a b c d e f

Fig. 1. Thin layer chromatography of human thyroid gangliosides. (a) mixed brain ganglioside; (b) normal thyroid; (c) medullary carcinoma; (d) toxic adenoma; (e) follicular carcinoma; (f) papillary carcinoma.

acid/gm fresh tissue. The lowest content was found in normal tissue whereas, the highest, with a large scattering, in the neoplastic tissues. The ganglioside content in the thyroids used in our study was lower than that found by Bouchon et al. (12) in thyroid tissue. This discrepancy could be easily explained by the different experimental conditions. In our study, gangliosides were extracted from the plasma membranes, whereas in Bouchon's study the gangliosides were extracted from the whole homogenates. The analysis of gangliosides, based on TLC migration, showed that human thyroid plasma membranes were rich in gangliosides co-migrating with authentic GM3, GM1, GD3, GD1a, GD1b, and GT1b (Figs. 1,2 and Table 2). In normal tissue, the major ganglioside was GM3 (53%), GD3 was the second major ganglioside

Table 2. Relative Distribution (%) of Gangliosides in Normal and Pathological Human Thyroids

Tissue	Gangliosides (%)					
	GT1	GD1b	GD1a	GD3	GM1	GM3
Normal	3.4	2.3	2.8	33.0	5.0	53.0
Nontoxic diffuse goiter	3.0	3.7	2.9	34.0	4.0	52.0
Toxic adenoma	3.1	2.6	2.5	32.0	5.8	54.0
Hypofunctional congenital goiter	3.2	3.2	3.8	7.0	28.0	53.7
Follicular carcinoma	0.5	1.0	2.5	7.3	6.7	82.0
Papillary carcinoma	1.9	3.7	3.4	20.3	7.1	64.0
Medullary carcinoma	2.9	5.0	9.0	52.0	11.0	19.7

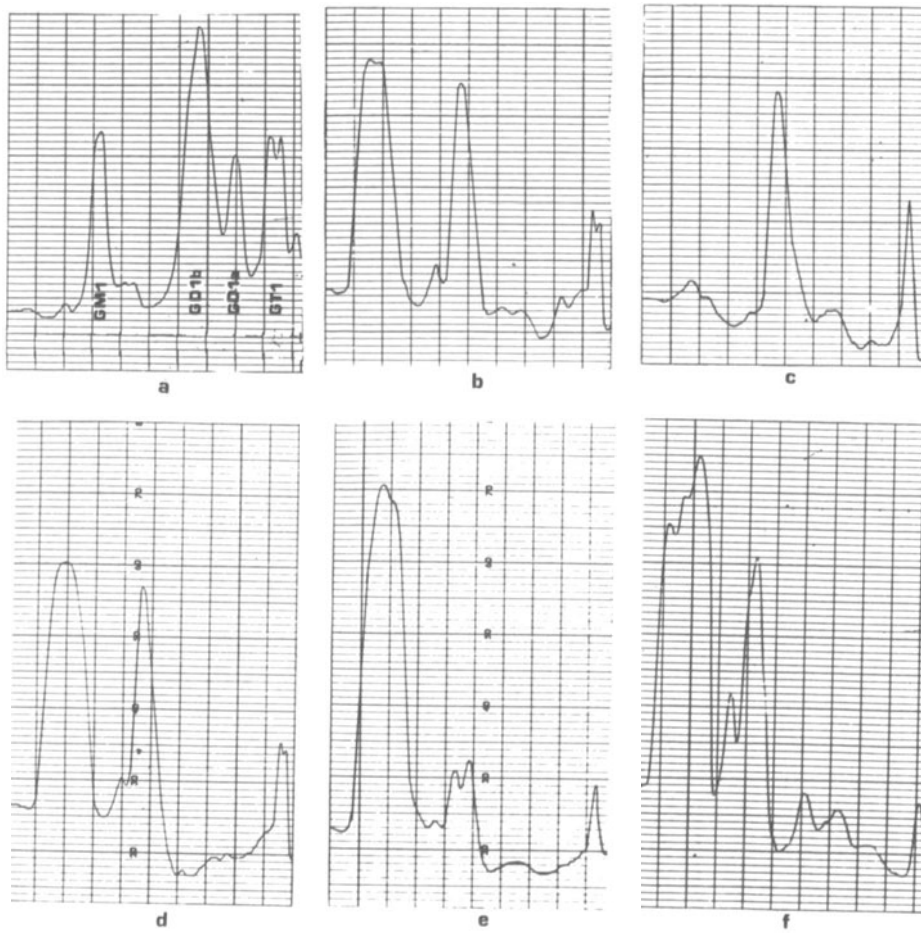


Fig. 2. Scans of the resorcinol-stained thin layer chromatography plates of human thyroid gangliosides. (a) mixed brain gangliosides; (b) normal thyroid; (c) medullary carcinoma; (d) toxic adenoma; (e) follicular carcinoma; (f) papillary carcinoma.

(33%), whereas all the other gangliosides (GM1, GD1a, GD1b, and GT1) represented 13.5% of the total. As shown in Fig. 2, GM3 appears as a doublet as previously described by Bouchon et al. (12). No qualitative or quantitative differences were observed comparing the ganglioside pattern of normal thyroids to those of nontoxic diffuse goiter and of toxic adenoma (Fig. 1, Table 2).

On the other hand, in the hypofunctional goiter, the ganglioside pattern showed abnormalities compared to that of normal tissue. In this tissue, GM3 and GM1 represented about 80% of the total and GD3 represented only 7% of the total (Table 2, Fig. 3). In a follicular carcinoma, GM3 was at the highest level (82%), GM1 and GD3 represented only 14% of the total, whereas all the other higher order gangliosides were at the lowest level if compared to the same ganglioside in the other pathological thyroids (Table 2).

Similar abnormalities in ganglioside pattern have been reported in a rat thyroid tumor with a TSH-receptor defect (19). In this tumor, the TSH-receptor deficiency was correlated with the alteration in the plasma membrane content of higher order gangliosides and with a deficiency in the

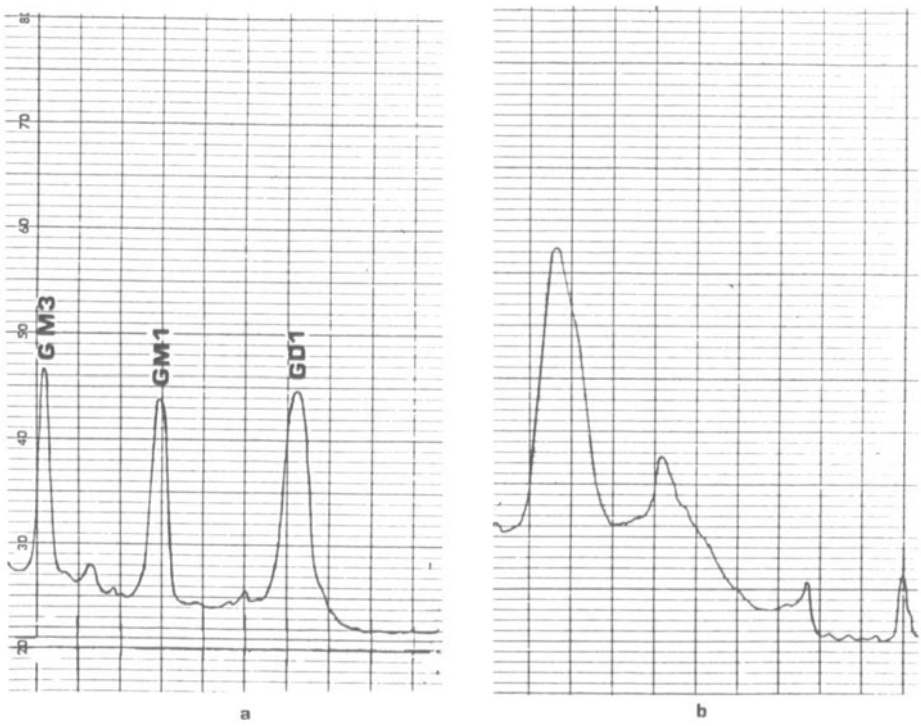


Fig. 3. Scans of the resorcinol-stained thin layer chromatography plates of congenital hypofunctional goiter gangliosides. (a) ganglioside standards as indicated; (b) congenital hypofunctional goiter gangliosides.

enzymes responsible for the synthesis of the higher order gangliosides implicated as a potential component of the TSH-receptor; GD1b, GT1, and GM1 (7). Abnormalities in the ganglioside patterns were observed in different carcinomas. In a medullary carcinoma, the major ganglioside was GD3 (52%), whereas GM3 represented only 19% of the total (Table 2). In a papillary carcinoma, the ganglioside pattern was qualitatively similar to that of normal tissue; quantitatively, however, there were some differences. GM3 was higher (65%) than in normal tissue and GD3 was lower (21%) (Table 2).

These data indiate that in various states of thyroid function, there are some abnormalities in the ganglioside pattern. Since, in addition to the gangliosides, phospholipids were also shown to be components or modulators of TSH-receptor in the plasma membranes (4,13), we analyzed phospholipid fractions in the same normal and pathological thyroid plasma membranes. The analysis of the phospholipid fractions in the same normal and pathological thyroid plasma membranes did not show significant differences in the phospholipid patterns as reported in Table 3.

CONCLUSIONS

Our data indicate that, in state of altered thyroid function, ganglioside but not phospholipid patterns are modified. However, at present, it is not clear whether a correlation between functional modification of the thyroid and ganglioside composition exists. This area is now under investigation.

Table 3. Relative Distribution (%) of Phospholipids in Normal and Pathological Human Thyroids

Tissue	Phospholipids (%)			
	SP	LE	PI	PE
Normal	15	37	15	33
Nontoxic diffuse goiter	16	41	14	27
Hypofunctional congenital goiter	4	50	14	27
Toxic adenoma	17	37	18	31
Follicular carcinoma	12	38	19	21
Papillary carcinoma	4	49	10	36
Medullary carcinoma	8	52	11	28

REFERENCES

1. Pastan I, Roth J, and Macchia V. Proc Nat Acad Sci 56: 1802, 1966.
2. Pastan I and Katzen R. Biochem Biophys Res Commun 29: 792, 1967.
3. Macchia V and Pastan I. J Biol Chem 242: 1864, 1967.
4. Macchia V, Tamburrini O, and Pastan I. Endocrinology 86: 787, 1970.
5. Aloj SM, Lee G, Consiglio E, et al. J Biol Chem 254: 9030, 1979.
6. Mullin BR, Fishman PM, Lee G, et al. Proc Nat Acad Sci 73: 842, 1976.
7. Meldolesi MF, Fishman PM, Aloj SM, et al. Proc Nat Acad Sci 73: 4060, 1976.
8. Meldolesi MF and Laccetti P. In, Proc of the Sixth International Congress of Endocrinology, Melbourne, 1980, p 211.
9. Laccetti P, Grollman EF, Aloj SM, et al. Biochem Biophys Res Commun 110: 772, 1983.
10. Lee G, Grollman EF, Aloj SM, et al. Biochem Biophys Res Commun 77: 139, 1977.
11. Sawada K, Iwamori M, Hara Y, et al. In, Proc of the Sixth International Symposium on Glicoconjugates, Tokyo, 1981, p 101.
12. Bouchon B, Portokalian J, and Bornet H. Biochem Inter 10: 531, 1985.
13. Macchia V and Wolff J. FEBS Letters 10: 219, 1970.

CHARACTERIZATION OF THE OPTIMAL THYROTROPIN (TSH) INDUCED CYCLIC AMP ACCUMULATION IN THE CLONED HUMAN THYROID CELL LINE (GEJ)

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ABSTRACT

Cloned human thyroid epithelial cells, which we previously selected for their capacity to bind ^{125}I-TSH to their TSH receptors, can produce cAMP in a dose-dependent manner, when stimulated by either TSH or GD IgG.

This cell line provides an effective tool which is both easily available and highly efficient and which, in addition, circumvents the need for human thyroid tissue, thyroid membranes, or receptor preparations. Moreover, this material excludes the possibility that species specificities reduce the chances of measuring a positive response.

INTRODUCTION

The existence of circulating thyroid-stimulating antibodies (TSAb) in Graves' disease (GD) is now widely recognized (1), TSAb being considered as the cause of GD hyperthyroidism (2). The different assay procedures used for their detection can be separated into those directly assessing stimulation by the antibodies (3) and those reflecting the antibody-induced modulation of the membrane receptor for thyrotropin (TSH) (4). In the first group of assays, the measurement of cyclic AMP (cAMP) in extracts of human thyroid slides (5), or thyroid cell cultures (6) after incubation with test material, is commonly used because of its high degree of specificity and sensitivity.

In previous studies, we developed a monoclonal human thyroid cell line (7) by fusing fresh normal human thyroid cells with a human lymphoblastoid cell line. The resultant cell line, called GEJ, was selected for its expression of TSH receptors, and has characteristics of normal human thyroid cells. In this work, we investigated the characteristics of TSH-induced cAMP production by GEJ cells and determined whether this cloned human thyroid cell line could be a useful and convenient alternative for detecting TSAbs.

MATERIALS AND METHODS

GEJ was obtained by fusing the human GM1500 6TGA-12 cell line with normal human thyroid cells. Before assay, GEJ cells were washed twice in

Hanks' Balanced Salt Solution (HBSS) and left to rest during 2 hrs at room temperature (RT) in RPMI 1640 (Gibco, Paisley, Scotland) without serum.

Test stimulators. TSH used in this study and referred to as E-TSH (Endo, Organon, France) was crude extract from porcine and bovine pituitaries. Its assigned biological activity was 1 IU per 2.3 mg. Immediately before bioassay, lyophilized E-TSH preparations were reconstituted in 0.8 ml distilled water and appropriate dilutions prepared in modified hypotonic HBSS (8) containing 40 mM Hepes, 4 mM 3 isobutyl-1 methyl-Xanthine (IsoBX), and 0.4% bovine serum albumin (BSA). Certain experiments used IgG purified fractions of serum from GD patients; these fractions were obtained after serum dialysis on a GF05 column (IBF, France) containing (Tris 25 mM) NaCl (35 mM) pH = 8.8. Then, they were purified by passage through DEAE-Trisacryl (IBF, France) and dialyzed through a GF05 column in hypotonic HBSS (8). With this method, 85% of the IgG were recovered with a purity purity of 95.98%. IgG were also prepared from normal individuals, these preparations having been previously shown to be free of residual stimulatory activity (9).

Assay of cAMP production. 150 µl of E-TSH or control or experimental IgG (2 mg/ml) were added to triplicate of GEJ cells. Incubations were perperformed at 37°C in an atmosphere of 10% CO_2 in air. Their durations and the numbers of GEJ cells used are further specified. After the incubation period, the medium was removed and 300 µl of 10% trichloracetic acid (TCA) were added for 30 min at RT. The TCA-cell mixtures were then centrifuged and the supernatants used for intracellular cAMP assay after they were extracted three times with 2 ml of water-saturated ether; after evaporation, the residues were resuspended in 0.3 ml of 0.05 M acetate buffer pH = 6.2. No acetylation of samples or standard was performed, their cAMP contents being measured by mean of a radioimmunoassay with ^{125}I-cAMP antibodies (Pasteur Production, Paris, France). Results are expressed as mean picomoles (pmoles) of cAMP for each set of triplicate cells.

RESULTS

Characteristics of cAMP Production by GEJ Cells after Stimulation by E-TSH

The accumulation of intracellular cAMP in 25,000 GEJ cells in response to 30 min incubation with increasing doses of E-TSH is shown in Fig. 1. A typical S shape curve response is obtained. Basal cAMP production ($\simeq$ 5 pmoles) is observed in GEJ cells similarly incubated in only hypotonic HBSS supplemented with Hepes, IsoBX, and BSA. GEJ cells were consistently responsive to more than 25 µu/ml E-TSH, a plateau being reached with the dose of 500 µu/ml.

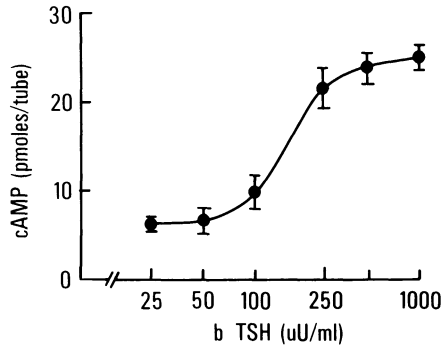

Fig. 1. Intracellular cAMP accumulation by GEJ cells after stimulation by various doses of b. TSH.

Table 1. Characteristics of Intracellular cAMP Accumulation by GEJ Cells After Stimulation by 1,000 μu/ml E-TSH: One Typical Experiment

	GEJ cells (10^3)	Time of incubation (min)	cAMP (pmoles/tube)
1a	25	0	5.5
	25	5	7.2
	25	15	15.1
	25	30	22.0
	25	45	25.0
	25	60	17.0
	25	90	13.0
	25	120	6.5
1b	12.5	30	11
	25	30	25
	50	30	46

Kinetic analysis of response to 10^3 μu/ml E-TSH added to 25,000 GEJ cells shows that maximum stimulation of intracellular cAMP accumulation occurs at 30-45 min followed by a decline (Table 1 a). An analysis of response to 10^3 μu/ml E-TSH during 30 min with increasing GEJ cell concentrations indiated that 12,500 to 50,000 GEJ cells per tube gave dose-related cAMP production (Table 1 b).

Table 2. Prevalence of TSAb in Hyperthyroid GD

Subjects	% increase from baseline cAMP
1*	70
2	500
3	200
4	540
5	250
6*	130
7	340
8	200
9	240
10	500

*Patients on antithyroid drugs.

Interaction of IgG Fractions with GEJ Cells

IgG fractions from eight untreated hyperthyroid GD patients caused significantly greater cAMP levels when compared to control IgG ($p<0.05$ by Student's t test). Overall, the percent increase of intracellular cAMP for the GD group ranged from 200 to 540% above baseline control IgG. However, 50% of the positive samples caused stimulation equal to 200% above baseline (Table 2).

DISCUSSION

The experiments we describe here demonstrate that E-TSH induces a dose-dependent production of intracellular cAMP by the human thyroid epithelial cell we cloned (7). Similarly, GD IgG, which stimulates thyroid cell via TSH receptors and induces thyroid hyperactivity (10), also stimulates GEJ to produce cAMP. The GEJ cells allow the detection of TSAb found in the sera of most GD patients. These results indicate that GEJ cells possess the adenylate cyclase system which allows them to behave as normal thyroid cells. These results could be anticipated from our previous experiments, since all the GEJ cell characteristics that we investigated, either in terms of TSH binding or of thyroid function (thyroid hormones and thyroglobulin productions), were similar to those of normally functioning thyroid cells.

It is clear that this cell has multiple advantages. Apart from its high availability and consistently extreme convenience, experiments can be conducted using defined material at any time. Moreover, their human origin excludes the possibility that species specificity could reduce the chance of measuring a positive response (11). In contrast to the results reported by Vitti et al. (12) who demonstrated that the use of the cloned rat thyroid cell line detected more TSAb positive GD patients, GEJ cells do not do so. When comparing the TSI indexes obtained with GD IgG preparations tested on either GEJ or human thyroid epithelial cell culture (13), no discrepancies were found. More precisely, TSAb negative ot TSAb positive IgG preparations remain respectively negative or positive regardless of the nature of the human thyroid cell population utilized. This could be explained by the fact that these authors compared cAMP production by human thyroid membrane preparations to those obtained with the rat FRTL-5 thyroid cells. It could be postulated that TSH receptors could be modified during the preparation of the membranes and, thus, allow detection of higher amounts of TSAb.

REFERENCES

1. Strakosh CD, Wenzel BE, Row VV, et al. N Eng J Med 307: 1499, 1982.
2. Burke G. Acta Endocrinol (Copenh) 66: 558, 1971.
3. Atkinson S and Kendall-Taylor P. J Clin Endocrinol Metab 53: 1263, 1981.
4. Smith BR and Hall R. FEBS Lett 42: 301, 1974.
5. Orgiazzi J, Williams DE, and Chopra IJ. J Clin Endocrinol Metab 42: 341, 1976.
6. Rapoport B, Greenspan, FS, Filetti S, et al. J Clin Endocrinol Metab 58: 332, 1984.
7. Karsenty G, Michel-Bechet M, and Charreire J. Proc Natl Acad Sci USA 82: 2120, 1985.
8. Davies TF, Platzer M, Schwarz A, et al. J Clin Endocrinol Metab 57: 1021, 1983.
9. Charreire J, Karsenty G, Lepage, et al. In ED Albert (ed), Histocompatibility Testing, Springer-Verlag, Berlin-Heidelberg, 1984, p 657.

10. Hinds WE, Takai NA, Rapoport B, et al. J Clin Endocrinol Metab 52: 1204, 1981.
11. Rapoport B, Takai NA, and Filetti S. J Clin Endocrinol Metab 54: 1059, 1982.
12. Vitti P, Rotella CM, Valente WA, et al. J Clin Endocrinol Metab 57: 782, 1983.
13. Charreire J, Karsenty G, Bouchard P, et al. Clin Exp Immunol 57: 633, 1984.

CLINICAL EXPERIENCE WITH A SENSITIVE IMMUNORADIOMETRIC ASSAY FOR SERUM THYROTROPIN (TSH): DIAGNOSTIC AND THERAPEUTIC IMPLICATIONS FOR HYPERTHYROID AND THYROID CANCER PATIENTS

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A few patients with subtle signs or symptoms of mild hyperthyroidism, concurrent with consistently normal serum thyroid hormone levels, prompted us to adapt highly sensitive TSH measurements (1-4) to routine clinical use. TSH results in over 300 patients were found to be highly consistent with the clinical impressions and of value in several clinical situations. This account will focus on specific clinical applications, while a more complete report will be published separately (5).

SUBJECTS AND METHODS

The patients were evaluated in the Stanford University Thyroid Clinic by standard clinical criteria: history, physical examination, and conventional thyroid function tests (6).

TSH-Assay: We modified, as described elsewhere (5), a commercial, solid-phase, sequential immunoradiometric assay (IRMA) which uses in the first step a β-subunit specific, monoclonal anti-TSH and, in the second step, another monoclonal, ^{125}I-labeled, TSH antibody directed against the α-subunit (Tandem R-TSH, Hybritech, San Diego, CA) (7). The detection limit of the modified assay was <0.1·μU/ml and the smallest TSH concentration that can be precisely measured was <0.4 μU/ml. Values below 0.3 μU/ml were less well reproducible and stated as <0.3. The normal range for TSH was 0.4-3.7 μU/ml as determined in healthy individuals and in clinic patients with miscellaneous non-thyroid disorders (excluding severe systemic illnesses).

RESULTS AND DISCUSSION

Basal TSH in Lieu of TRH-stimulation Studies in the Diagnosis of Borderline Hyperthyroidism

The assay differentiated well between serum TSH levels in euthyroidism and hyperthyroidism, i.e., 97% of 29 patients with a diagnosis of hyperthyroidism (Graves' disease, multinodular goiter, toxic nodule of unknown etiology) had suppressed TSH of <0.3 μU/ml. Also, there was a strong correlation between basal TSH and TSH measured 20-30 min post i.v. bolus of 400 μg TRH; n=25, y=0.10 + 0.21, r=0.85.

Table 1. Serum TSH in Equivocal Hyperthyroidism

Patient/ date	Clinical status/impression	TSH(μU/ml) 0.4-3.7*	FT_4(ng/dl) 0.8-2.3*	T_3(ng/dl) 70-200*
		Biochemically Hyperthyroid		
M.B.	Goiter, asymptom., "slightly toxic"	0.3	1.7	155
H.B.	Graves', "appears hyperthyroid"	<0.3	1.5	180
W.A. 8/84	Minimal Graves' ophthalmopathy,	<0.3	1.8	200
(12/84	Overtly hyperthyroid	<0.3	-	450)
		Biochemically Borderline Hyperthyroid		
A.L.	Multinodular goiter, previous heart symptoms, "euthyroid"	0.4	1.3	135
O.M.	"Borderline Graves'"	0.4	2.4	200
H.E.	"Borderline Graves'"	0.4	2.5	140
		Biochemically Euthyroid		
W.R. 9/84	Graves' ophthalmopathy, "euthyroid"	1.8	1.4	160
6/85		0.7	1.2	120
H.M.	Goiter, "euthyroid"	1.7	1.9	145
M.M. 5/84	Hot Nodule, -	1.9	1.5	150

*Normal range

Table 2. Serum TSH Measured During PTU Treatment

Patient	Date	TSH (μU/ml)	FT_4 (ng/dl)	T_3 (ng/dl)
H.D.		0.3	1.8	200
A.M.		<0.3	1.7	100
H.M.	12/10/84	<0.3	3.6	280*
	12/21/84	<0.3	2.7	120
	1/22/85	<0.3	1.1	110
	3/22/85	1.7	1.2	-
A.D.	9/84	<0.3	1.1	185
	6/85	1.7	1.1	185
C.B.	6/84	1.85	1.6	135
	4/85	1.9	1.6	140

*Start PTU

As illustrated in Table 1, in patients with an equivocal diagnosis of hyperthyroidism, i.e., a clinical suspicion but normal or, at most, borderline high free T_4 (FT_4) and T_3, a subnormal TSH result can help establish the diagnosis and enable the clinician to either treat the patient earlier, or monitor him closely. For instance, of the three patients listed on top of Table 1, in whom a suppressed TSH confirmed early stages of hyperthyroidism, at least one (W.A.) became overtly hyperthyroid within the ensuing four months. Also, in patients who, by all clinical and biochemical criteria, are still euthyroid, a significant fall in TSH can indicate progression of disease (W.R.).

Management of Patients Undergoing Antithyroid Drug Therapy

At early stages of therapy, or in patients poorly controlled by propylthiouracil (PTU), we observed generally suppressed TSH levels of <0.3 μU/ml (Table 2). Conversely, in patients responding well to therapy, the usual course of events was that serum FT_4 and T_3 dropped first and stabilized somewhere within the normal range (individual threshold), while serum TSH concentrations tended to reach normal, measurable levels only after another few months (H.M., A.D., Table 2). Consequently, it would appear that this resumption of normal pituitary TSH secretion can serve as a marker for successful treatment and might be useful in making further treatment decisions.

Sequential Sensitive TSH Measurements During Remission Following Antithyroid Drug or ^{133}I Therapy. Prediction/detection of Recurrences Before They Become Clinically Overt, or Before Other Thyroid Indices Become Abnormal

As illustrated in Table 3, in patients in remission, serum TSH levels changed significantly over a period of a few months, often in the absence of any major changes in serum thyroid hormone levels (S.A. and D.M.). We stipulate that patients in whom serum TSH falls drastically (D.M. and L.M.), or in whom TSH remains subnormal (H.M.), are most likely to suffer a relapse, while rising TSH levels (S.A., R.R., H.E.) would appear to be a strong indicator for a good prognosis, at least near term.

Table 3. Serum TSH in Graves' Patients During Remission Post PTU or ^{131}I

Patient	Date	TSH (μU/ml)	FT_4 (ng/dl)	T_3 (ng/dl)	Rx
D.M.	8/84	6.0	1.4	200	Post PTU
	9/84	3.1	1.7	200	
	10/84	0.8	2.0	200	
L.M.	10/84	0.5	1.5	135	Post PTU
	5/85	<0.3	2.0	165	
S.A.	5/84	0.3	1.5	140	Post PTU
	6/84	0.5	1.5	110	
	11/84	2.7	1.5	120	
	3/85	1.3	1.3	100	
R.R.	6/84	<0.3	1.3	-	Post ^{131}I, PTU
	9/84	0.5	1.1	145	
H.E.	7/84	0.4	2.5	140	Post ^{131}I
	11/84	0.7	1.9	120	
	1/85	0.5	2.2	-	
	4/85	1.1	1.9	90	
	6/85	1.3	1.9	105	

Optimization of Thyroxine Suppression Therapy in Thyroid Cancer Patients

Sensitive TSH measurements in 35 patients with a history of papillary and/or follicular thyroid cancer, who had undergone thyroidectomy and had been taking thyroxine for extended periods, revealed that 77% had indeed suppressed TSH of <0.3 μU/ml, as is recommended in order to minimize the risk of metastases. The remaining 23%, however, had measurable TSH levels ranging from 0.5 - 4.0 μU/ml (Fig. 1) suggesting that their T_4 uptake was inadequate. A large proportion (43%) of those with suppressed TSH had borderline high FT_4 levels. It is unclear whether in all cases thyroid hormone concentrations can be lowered to normal levels while TSH secretion is kept subnormal. Nonetheless, routine sensitive TSH assays are of value in the management of thyroid cancer patients to ensure suppression of TSH without subjecting the patients to unnecessary high doses of T_4.

Precise Adjustment of Thyroxine-replacement Therapy in Hypothyroid Patients

We found, in good agreement with earlier data by Wehmann et al. (8), that a large percentage (45%, n=36) of patients who have been taking thyroxine for six weeks to several years, and who presented with normal FT_4 and T_3 levels, had subnormal TSH levels of <0.3 μU/ml, indicative of a certain degree of overtreatment (Fig. 1). Six of these patients had subtle signs of hyperthyroidism (rest tachycardia, quadriceps weakness, onycholysis) and there the subnormal TSH results confirmed the clinical suspicion of excessive T_4 therapy; however, the majority of these patients were totally asymptomatic. Thus, in patients on thyroid hormone replacement, normal serum thyroid hormone levels do not guarantee physiological euthyroidism, but sensitive TSH measurements help the physician to adjust replacement dosage to simultaneously normalize serum thyroid hormones and

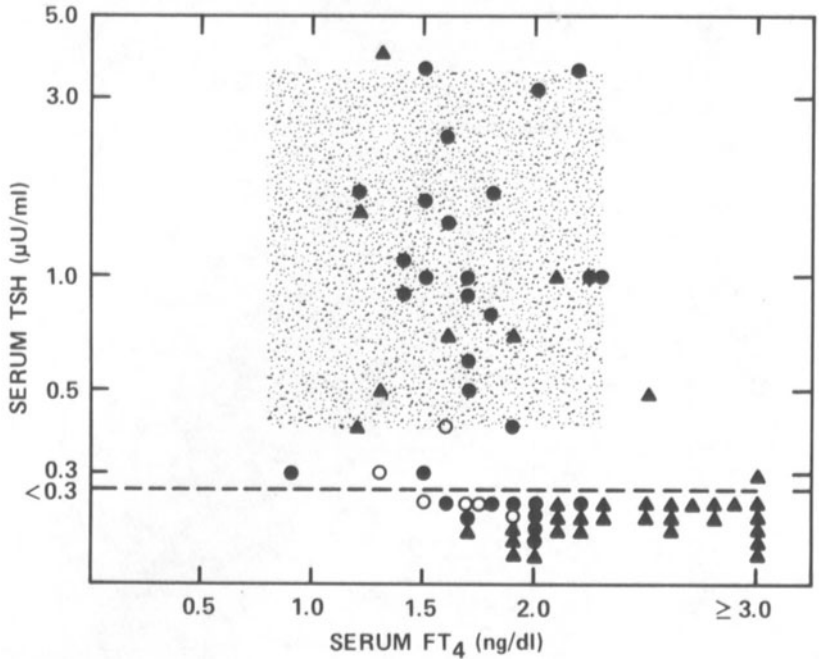

Fig. 1. Relationship between serum TSH and FT_4 in patients taking Synthroid. Hypothyroid patients on T_4 replacement with normal FT_3: asymptomatic (n=30, ●) or with mild symptoms (n=6, ○). Thyroid cancer patients post thyroidectomy on T_4 (n=35, ▲). Shaded area denotes the normal range.

TSH. It is difficult to dispute that optimal adjustment of the treatment dose over many years may have beneficial effects on the cardiovascular system or bone metabolism, even though some patients may refuse to reduce their T_4 dose or complain of symptoms when on less medication.

SUMMARY

Sufficiently sensitive TSH measurements are of clinical value in diagnosis and management of many hyperthyroid patients, in particular those with equivocal disease, those on PTU, or those presumably in remission after therapy, and help optimize T_4 replacement for hypothyroid patients, or T_4 replacement/suppression therapy for thyroid cancer patients.

REFERENCES

1. Wehmann RE, Rubenstein HA, and Nisula BC. Endocr Res Commun 6: 249, 1979.
2. Spencer CA and Nicoloff JT. Clin Chim Acta 108: 415, 1980.
3. Pekary AE, Hershman JM, and Parlow AF. J Clin Endocrinol Metab 41: 676, 1975.
4. Nisula BC and Louvet JP. J Clin Endocrinol Metab 46: 729, 1978.
5. Bayer MF, Kriss JP, and McDougall IR. J Nucl Med (in press).
6. Bayer MF and McDougall IR. Clin Chem 26: 1186, 1980.
7. Pekary AE and Hershman JM. Clin Chem 30: 1213, 1984.
8. Wehmann RE, Rubenstein HA, Pugeat MM, et al. Southern Med J 76: 969, 1983.

A HIGHLY SENSITIVE, NON-ISOTOPIC ASSAY FOR HUMAN TSH WITH EXTENDED CLINICAL UTILITY

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Serum thyrotropin (TSH) is widely regarded as the best indicator of thyroid function. The ideal clinical TSH assay would combine features of a short incubation time with precise measurement over a range encompassing hyperthyroid and hypothyroid values. Currently, most commercial assays have sensitivity limitations and, therefore, are relegated solely to the confirmation of primary hypothyroidism (1). The ultrasensitive research assays have displayed excellent delineation between euthyroid and hyperthyroid individuals but also possess time and range limitations (2-4). We report the development of a sensitive and precise immunoenzymometric assay (IEMA or EIA) for TSH with an extended clinical range. In a controlled clinical study, the EIA was compared to an ultrasensitive TSH RIA (UST) developed by Spencer and Nicoloff (2).

METHODS

HTSH-EIA: The EIA employs two distinct, TSH-specific monoclonal antibodies bound to a quarter-inch polystyrene bead and a third monoclonal antibody (directed against the α-subunit of TSH) coupled to horseradish peroxidase. Briefly, 100 µl of standards, controls, or specimens are added to the appropriate well of a plastic reaction tray. One anti-TSH-coated bead is then added to each well and the reaction tray is floated in a 37°C waterbath and the beads washed 3X with water using Abbott's Pentawash system. 200 µl of anti-TSH: peroxidase conjugate is added to each washed bead and the tray placed back into the 37°C waterbath for one hour. Following this second incubation, the beads are washed 3X and transferred to plastic reaction tubes. 300 µl of o-phenylenediamine (OPD) substrate solution containing H_2O_2 is added to each bead and the reaction allowed to proceed for 30 minutes at room temp. The reaction is stopped with 1 ml of 1N H_2SO_4 and the absorbance read at 492 nm on the Abbott Quantum® or appropriate spectrophotometer. The color intensity is directly proportional to the concentration of TSH.

UST-RIA: The UST is an RIA for TSH utilizing a double antibody precipitating technique. The assay employed in this study is a modification of the assay published previously (2).

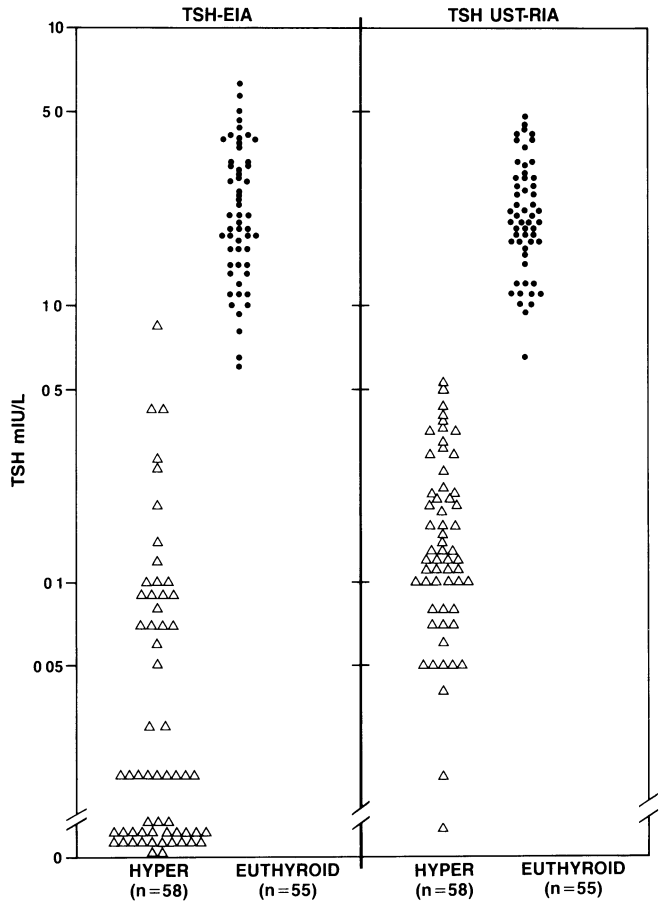

Fig. 1. Comparison of the distribution of hyperthyroxinemic and euthyroid populations between the EIA and UST. The assays were performed on the same samples (hyper and euthyroid). Hyperthyroxinemic (△) and euthyroid (●) specimens were previously characterized by the UST and other thyroid function tests and clinical assessments. See methods for assay protocol.

RESULTS AND DISCUSSION

Both the EIA and the UST displayed good delineation between the values from 55 normal and 58 hyperthyroxinemic subjects (Fig. 1). The mean ± S.D. for the euthyroids was 2.46 ± 1.33 mIU/L for the EIA and 2.28 ± 1.02 mIU/L for the UST. For the hyperthyroxinemic subjects the mean ± S.D. was 0.07 ± 0.14 mIU/L for the EIA and 0.16 ± 0.11 mIU/L for the UST. Comparison between the EIA and the UST revealed good correlation ($r = 0.966$, slope = 1.16, $y = -0.16$) for the concentration range 0-8 mIU/L. The correlation was found to be 0.82 in the range 0-3 mIU/L. The ability of the EIA to distinguish hyper from euthyroid states is further evident from a larger clinical study involving 315 euthyroid and 139 hyperthyroid subjects. The central 95% of the euthyroid population was found to display TSH values between 0.45 and 6.20 mIU/L, while greater than 95% of the hyperthyroxinemic patients had TSH levels less than 0.35 mIU/L. Hypothyroid subjects possessed TSH levels greater than 12 mIU/L (Fig. 2).

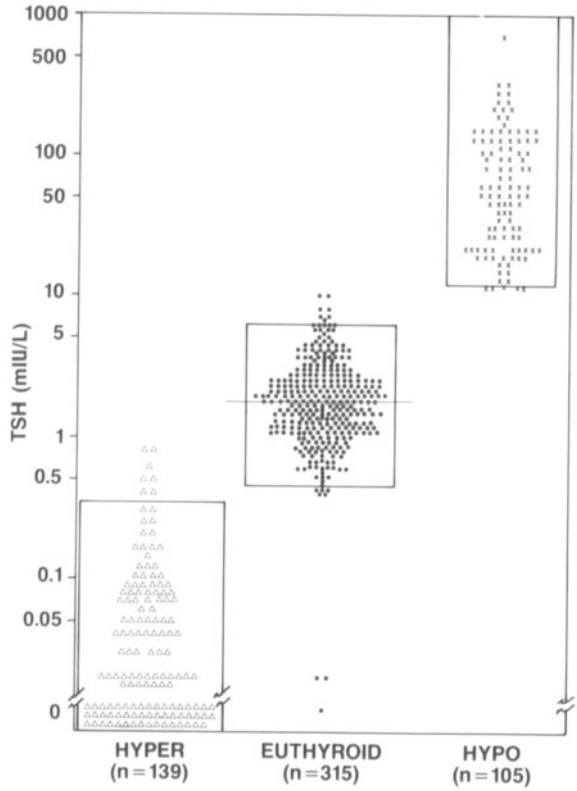

Fig. 2. Composite distribution of hyperthyroxinemic, euthyroid, and hypothyroid populations from 4 clinical sites utilizing TSH-EIA. The boxes represent the 95% limits of the population distribution. The log mean of the euthyroid concentrations is designated by the line through the euthyroid box. The log mean is 1.8 mIU/L (linear mean 2.2 mIU/L).

EIA sensitivity was determined to be 0.05 $\pm$ 0.02 mIU/L by the method of Rodbard (5) and by dilution of normal human serum samples. This was reflected by the excellent precision at the low end of the dose response curve. The within and between assay variability (C.V.) at 1.5 mIU/L was 3-6% and 4-7% respectively.

Additional clinical studies, using the EIA, revealed that 21% of 165 patients on thyroid hormone replacement therapy displayed subnormal TSH levels with normal Free T_4 (data not shown). This suggests that a basal TSH measurement, utilizing a highly sensitive assay, may be more appropriate than a Free T_4 measurement when establishing replacement dosage and will avoid chronic mild chemical thyrotoxicosis.

TSH assays with high sensitivity will also be important in monitoring suppression therapy patients. It was observed in 32 goiter patients on supression therapy that the response to TRH challenge was proportional to basal TSH when the initial TSH levels were greater than 0.1 mIU/L. However,

3 patients out of 15 with undetectable basal TSH (i.e., less than 0.05 mIU/L) showed a response to TRH, suggesting that these undetectable levels of TSH may still be biologically active. This also suggests that assay sensitivities below 0.01 mIU/L may be required to replace the TRH challenge test in assessing the adequace of suppression in these patients. Alternatively, measurement of the TSH response to TRH with an ultrasensitive TSH assay should significantly enhance management of these patients.

Thus, the clinical data indicates that the HTSH-EIA can be useful as a primary thyroid screening test and as a monitor for evaluating hyperthyroxinemic and suppressed patient states.

REFERENCES

1. Burger HG and Patel YC. Med J Australia 2: 293, 1972.
2. Spencer CA and Nicoloff JT. Clin Chim Acta 108: 415, 1980.
3. Wehmann RE, Rubenstein HA, Pugeat MM, et al. Southern Med J 76: 969, 1983.
4. Pekary AE, Hershman JM, and Parlow AF. J Clin Endocrinol Metab 41: 676, 1975.
5. Rodbard D. Anal Biochem 90: 1, 1978.

MEASUREMENT OF CIRCULATING THYROTROPIN CONCENTRATIONS BY AN IMMUNOCHEMILUMINOMETRIC ASSAY

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INTRODUCTION

We have developed an immunochemiluminometric assay (ICMA) which has a sensitivity far greater than that of existing immunoassays and which discriminates clearly between euthyroid and hyperthyroid basal TSH levels (1). As part of the clinical validation of this assay, we have measured basal TSH levels in normal and hyperthyroid subjects over a twenty-four hour period. We have also measured the variation in basal TSH levels in a variety of clinically euthyroid patients with acute and chronic non-thyroidal disease.

PATIENTS AND METHODS

Five normal euthyroid, six hyperthyroid, and three euthyroid subjects with multinodular goiter were sampled hourly over 24 hours. In addition, serum samples were obtained at 11.00 hours from four groups of 20 euthyroid patients with chronic liver disease (CLD; mean age 54; age range 17-72; 15 male, 5 female), chronic renal disease (CRD; mean age 42; age range 18-73; 10 male, 10 female), acute myocardial infarction (MI; mean age 63; age range 36-79; 15 male, 5 female), and acute cerebrovascular accident (CVA; mean age 68; age range 52-86; 10 male, 10 female). The control group consisted of 20 healthy, euthyroid subjects (mean age 32; age range 19-57; 8 male, 12 female). Concentrations of fT_3 and fT_4 were measured using Amerlex kits (Amersham International, Cardiff UK) and TSH concentrations were measured as described previously (1).

RESULTS

Circadian Rhythm Studies

As shown in Figs. 1-4, all five control subjects demonstrated a normal circadian variation in TSH levels. Three hyperthyroid patients (2a, 2b, and 3b) showed a normal secretory pattern but basal TSH levels were suppressed. In patients with euthyroid multinodular goiter, one subject (4a) showed suppressed TSH levels throughout the period of study, one subject (4b) had a normal pattern of secretion with suppressed TSH levels during the day, and

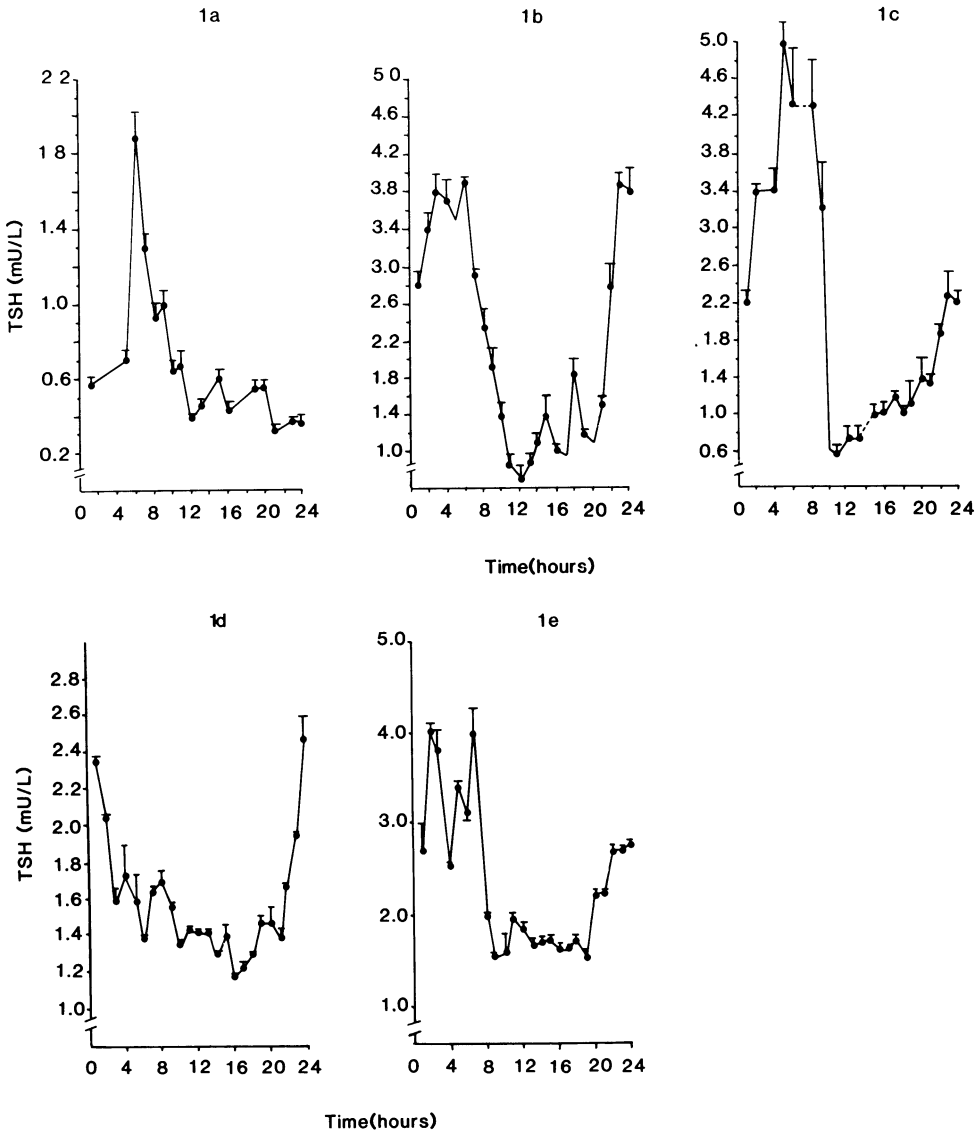

Fig. 1. TSH concentration measured every hour for 24 hours in normal subjects. Each point is plotted as mean ± ISD.

one subject (4c) showed normal basal TSH levels with a normal circadian pattern of secretion.

Acute and Chronic Non-thyroidal Illness

As shown in Table 1, fT_3 and fT_4 were significantly lower in all patient groups than in normals, the most profound decrease occurring in those with CRD. In contrast, TSH concentrations in patients with CRD, CLD, and MI were not significantly different from normal. TSH levels in patients

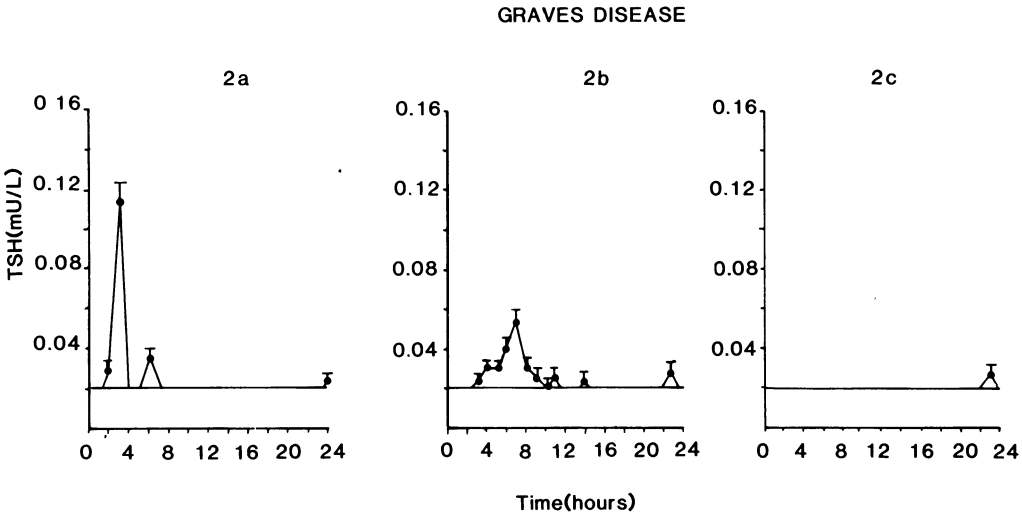

Fig. 2. TSH concentration measured every hour for 24 hours in patients with Graves' disease. Each point is plotted as mean ± ISD.

with CVA were marginally reduced ($p<0.05$) and albumin concentrations were significantly lower in CRD, CLD, and CVA.

DISCUSSION

Using this ultrasensitive ICMA for TSH, normal subjects showed the expected physiological variation in basal TSH levels. Interestingly, some hyperthyroid individuals showed a circadian rhythm which indicates that other factors may still exert a controlling influence on the thyrotroph

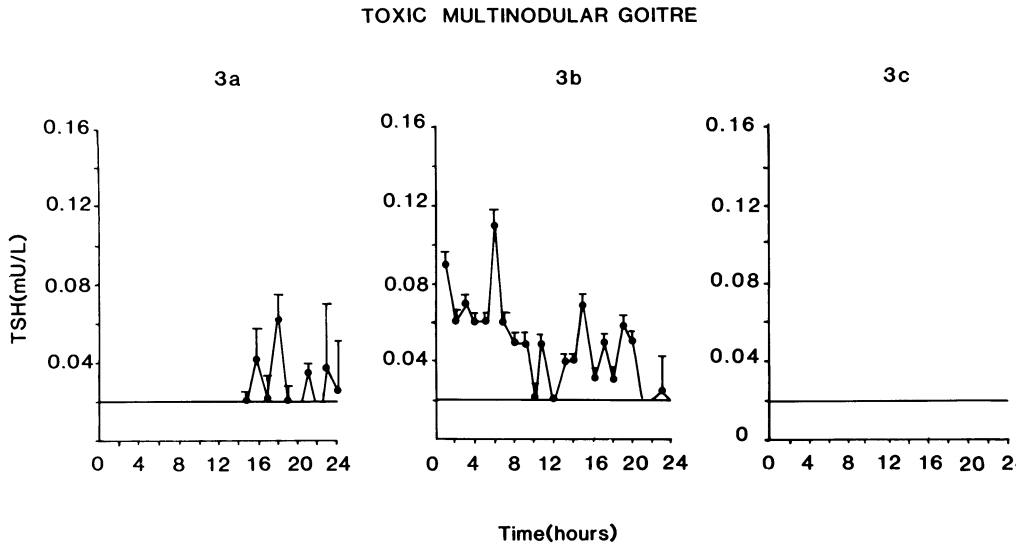

Fig. 3. TSH concentration measured every hour for 24 hours in patients with toxic multinodular goiter. Each point is plotted as mean ± ISD.

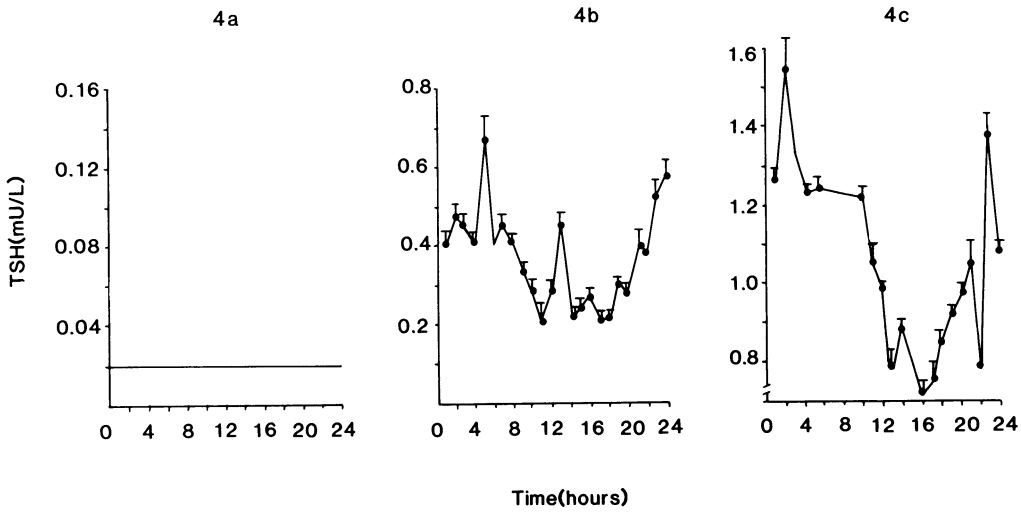

Fig. 4. TSH concentration measured every hour for 24 hours in patients with euthyroid multinodular goiter. Each point is plotted as mean ± ISD.

Table 1. TSH, fT_4, fT_3 and Albumin Concentrations in Normal Subjects and Clinically Euthyroid Patients with Non-thyroidal Illness

	TSH (mU/l)	fT_4 (pM)	fT_3 (pM)	Albumin (g/L)
Normal	1.58 ± 0.81	17.4 ± 2.2	7.6 ± 1.0	48 ± 3
	(0.60 - 3.30)	(12 - 21)	(5.7 - 9.0)	(42 - 54)
CRD	1.63 ± 0.95	8.7 ± 3.2***	3.2 ± 1.1***	41 ± 8***
	(0.54 - 4.35)	(1.0 - 15)	(0.9 - 4.6)	(24 - 53)
CLD	1.70 ± 0.79	13.0 ± 3.1***	3.5 ± 1.9***	30 ± 8***
	(0.38 - 3.21)	(8.0 - 18)	(1.2 - 7.9)	(29 - 57)
MI	1.51 ± 1.12	15.2 ± 2.4**	4.3 ± 1.0***	52 ± 5
	(0.54 - 4.91)	(10 - 20)	(1.8 - 6.3)	(44 - 61)
CVA	0.98 ± 0.58*	15.7 ± 3.0*	3.8 ± 0.8***	45 ± 6**
	(0.41 - 2.68)	(12 - 22)	(2.2 - 5.2)	(30 - 53)

Results are mean ± 2 SD (n = 20), range in parentheses. *$p < 0.05$; **$p<0.01$; ***$p<0.005$. Mann Whitney Test.

even in the face of elevated circulating levels of thyroid hormones. Patients with euthyroid multinodular goiters showed a gradation from normal to hyperthyroid basal levels of TSH which is compatible with the spectrum of autonomy of thyroid function known to exist in such subjects. It is clear that this assay highlights the potential importance of sensitive TSH measurements in identifying patients with some degree of autonomy in the presence of normal thyroid hormone levels.

All individual TSH levels measured in patients with acute and chronic non-thyroidal illness fell within our previously established normal range of 0.4 to 4.0 mU/l. However, the group mean concentration in CVA patients was significantly reduced which may reflect a degree of central suppression of TSH release as suggested by other studies in severely ill patients (2). The assessment of thyroid status in patients with non-thyroidal illness using free hormone assays can be unsatisfactory. While these studies confirm that free T_3 may be genuinely reduced in such cases, there is the additional problem of artifactual reduction caused by altered serum binding protein concentrations. This problem is most apparent in the fT_4 measurement where the greatest reduction coincides with lowest albumin levels in patients with CRD and CLD. In studies using equilibrium dialysis to measure fT_4, normal levels have been found in chronically ill patients (3). Our data support the view that a sensitive TSH assay may prove useful as a first line test of thyroid function particularly where established thyroid function tests may provide misleading information.

REFERENCES

1. Weeks I, Sturgess M, Siddle K, et al. Clin Endocrinol 20: 489, 1984.
2. Wehmann RE, Gregerman RI, Burns WH, et al. N Engl J Med 312: 546, 1985.
3. Chopra IJ, Van Herle AJ, Teco GNC, et al. J Clin Endocrinol Metab 51: 135, 1980.

EVALUATION OF PATIENTS ON THYROXINE (T_4) REPLACEMENT WITH A HIGHLY SENSITIVE COMMERCIAL TSH IMMUNORADIOMETRIC ASSAY (TSH-IRMA)

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Current strategies for the optimal management of patients requiring thyroid hormone replacement or suppression therapy are limited by the non-specificity and insensitivity of clinical symptoms and by the impracticality of frequent or extensive laboratory evaluation of thyroid status (e.g., TSH testing, radioiodine uptake). The recent introduction of highly sensitive TSH assays which distinguish suppressed from normal TSH levels offers a practical means for applying more precise assessment of basal TSH secretion. This study was conducted to illustrate the potential for under- or over-medication of patients receiving T_4 replacement or T_4 suppression when managed conventionally and to suggest a practical strategy for improved management employing sensitive TSH measurement.

METHODS

Patients receiving L-thyroxine as replacement therapy for primary hypothyroidism (32) or as suppression therapy for euthyroid goitrous or nodular thyroid disease (12) met the following criteria:

a) Continuous treatment with T_4 for at least three months prior to study,
b) Clinical assessment by the treating physician as euthyroid on the basis of current symptoms and physical examination,
c) Credible history of compliance to therapy,
d) Absence of concurrent illness or drug therapy known to alter thyroid hormone binding proteins or T_4-to-T_3 conversion, and
e) Normal T_3-Resin-Uptake or equivalent assay.

High sensitivity serum TSH concentrations were determined by the Boots-Calltech Diagnostics (Slough, U.K.) Sucrosep™ TSH-IRMA assay. The sensitivity (0.08 μU/ml) and approximate range of normal values (0.2 - 5.45 μU/ml) quoted by the manufacturer were consistent with those determined in this laboratory, 0.08 μU/ml and 0.3 - 5.0 μU/ml, respectively. Serum T_4, serum T_3, and T_3-Uptake determinations were obtained by the Tetrabead, T_3 Riabead and Triobead assay kits (Abbott Laboratories, North Chicago), respectively. Normal ranges for the above assays were: T_4, 5-13 μg/dl; T_3, 80-220 ng/dl; T_3 Uptake, 23-32%.

Table 1. TSH-IRMA Levels in Normal Subjects and Untreated Primary Hypothyroidism and Hyperthyroidism

Patient Group	n	Mean	Stand. Dev.	Range
1° Hypothyroidism	13	96.2	106.3	12.2 - 346
Normal subjects	30	1.6	0.98	0.31 - 4.7
Hyperthyroidism	20	0.01*	0.02*	0 - 0.07*

*All values were below the limit of sensitivity.

RESULTS

TSH-IRMA Levels in Normal, Hypo- and Hyperthyroid Subjects

A normal range of 0.3 - 5.0 μU/ml was established from 30 normal subjects (Table 1). Patients with untreated primary hypothyroidism exhibited TSH-IRMA levels greater than 5 μU/ml. Untreated clinically hyperthyroid patients all exhibited TSH-IRMA levels below the limit of detectability. A TSH-IRMA level of 0.1 μU/ml, conservatively approximating the sensitivity of the assay, was defined as the range of suppressed basal TSH concentrations.

Serum T_4 and T_3 Concentrations in T_4 Replacement Therapy

As illustrated in Fig. 1, a high proportion of patients (7/32) exhibited elevated TSH-IRMA levels. In patients whose T_4 levels were in the lower-normal range, 5-9 μg/dl, an elevated TSH-IRMA level was common (4/11). The TSH-IRMA levels of patients whose T_4 levels were in the upper-normal range, 9-13 μg/dl, were highly variable, ranging from frankly elevated (3/15) to undetectable (4/15). In contrast, the TSH-IRMA levels of patients whose serum T_3 levels were in the lower-normal range were highly variable,

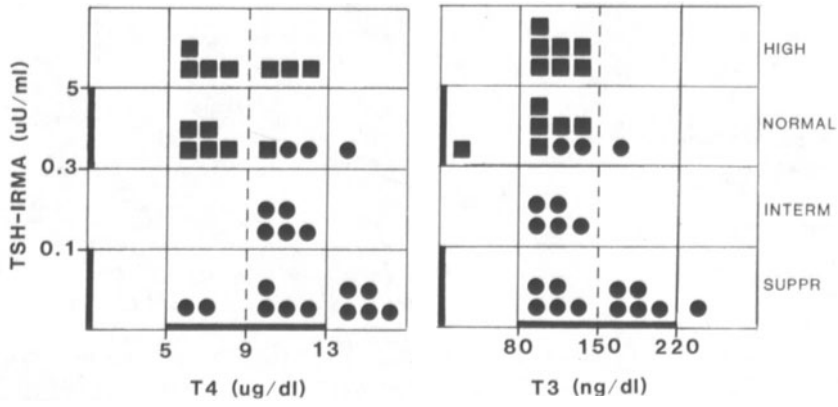

Fig. 1. Serum T_4, T_3, and TSH-IRMA concentrations in T_4-replaced patients. Squares indicate values greater than 0.5 μU/ml; circles indicate values equal to or less than 0.5 μU/ml.

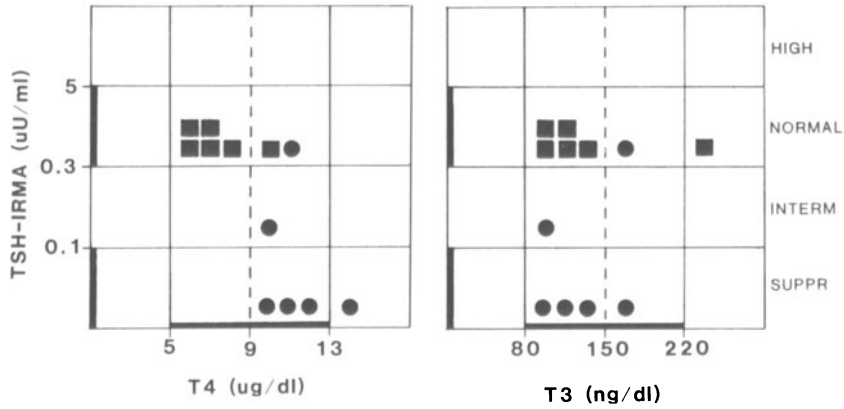

Fig. 2. Serum T_4, T_3, and TSH-IRMA concentrations in T_4-suppressed patients. Squares indicate values greater than 0.5 μU/ml; circles indicate values equal to or less than 0.5 μU/ml.

whereas those with upper-normal T_3 levels typically exhibited undetectable TSH-IRMA levels (5/6).

Serum T_4 and T_3 Concentrations in T_4 Suppression Therapy

In patients on T_4 suppression therapy the relationships of TSH-IRMA levels to the concurrent levels of T_4 and T_3 were similar to those observed in the T_4-replaced patients (Fig. 2). The principal difference was the absence of elevated TSH-IRMA levels, predictable by the definition of the patient group.

DISCUSSION

The high proportion of elevated and "normal" TSH levels observed among clinically euthyroid, T_4-replaced patients with normal T_4 levels emphasizes the limited efficacy of clinical symptoms and serum T_4 levels as measures of adequate T_4 replacement. Further, maintenance of the serum T_4 level in the upper-normal range, typically accompanied by normal T_3 levels (1,2) may be compatible with euthyroidism but is no assurance of complete substitution of endogenous hormone nor of complete suppression of basal TSH. These data and others' (3,4) establish that conventional TSH assays with a typical sensitivity of 0.5 μU/ml cannot discriminate between suppressed basal TSH levels and many normal TSH levels. The highly sensitive TSH-IRMA, however, allows the clinician to determine that not only is the patient's TSH production reduced below the hypothyroid range, but also reduced to below that level (0.3 - 5.0 μU/ml) which is "normal", by definition, only for healthy, untreated individuals.

Overmedication with T_4 constitutes an additional management problem since mild thyrotoxicosis is often difficult to detect with certainty. Clinical symptoms are commonly nonspecific and insensitive. Blunted TSH responses to TRH administration and suppressed radioiodine uptakes may reflect optimal T_4 replacement, as well as overmedication. Recognizing that a fully suppressed basal TSH level by any assay cannot distinguish complete T_4 replacement from excessive T_4 administration, we offer for consideration the recommendation that TSH-IRMA levels of patients receiving T_4 replacement be maintained in the range 0.1 - 0.3 μU/ml. This practice would assure not

only that frank hypothyroidism is precluded, but also that basal TSH secretion which is "normal" only for untreated, normal individuals is not permitted to sustain goiter growth. At the same time, the undefined but possible risks attendant to long-term, subclinical hyperthyroidism would be verifiably precluded by the substantially but incompletely suppressed TSH-IRMA levels.

Further studies are indicated to confirm that basal TSH levels in the range of 0.1 - 0.3 μU/ml are not sufficient to foster appreciably the growth of goiters, nodules, or occult malignancy. Other studies are indicated to document that TSH-IRMA levels within this range are attainable without excessively frequent blood sampling and titration of dosage.

REFERENCES

1. Maeda M, Kuzuya N, Masuyama Y, et al. J Clin Endocrinol Metab 43: 10, 1976.
2. Pearce CJ and Himsworth RL. Br Med J 288: 693, 1984.
3. Alexander WD, Kerr DJ, and Ferguson MM. Lancet ii: 647, 1984.
4. Seth J, Kellett HA, Caldwell G, et al. Br Med J 289: 1334, 1984.

STUDIES OF CALMODULIN-LIKE PORTION IN THE TSH RECEPTOR

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SUMMARY

Calmodulin (CaM) antagonists, such as W-7, W-5 chlorpromazine, and haloperidol, inhibited dose-dependently ^{125}I-bTSH binding to its receptor. This inhibitory effect by CaM antagonist was diminished by the addition of EDTA. Not only anti-CaM antibody but also CaM inhibited dose-dependently ^{125}I-bTSH binding to its receptor. These results may indicate the presence of a CaM-like structure in the membrane receptor for TSH.

Four sera out of 300 patients with Graves' disease had increased CaM binding activities as compared to normal control subjects. The binding could be demonstrated as the autoantibody to CaM by double antibody method, polyethyleneglycol method, and also gel-filtration. Three antibodies were monoclonal and one was polyclonal immunoglobulins (IgG and/or IgA).

Although the incidence of the autoantibody to CaM in Graves' disease is low and the pathologiccal significance of the autoantibody to CaM remains obscure, the existence of this antibody in the serum of patients may suggest the production of antibody to a CaM-like structure in the TSH receptor.

INTRODUCTION

Calmodulin (CaM), a low molecular intracellular Ca^{2+} binding protein, has been recognized as a regulatory factor of various cellular mechanisms. Recently, considerable evidence has been presented that CaM is implicated in relation to the hormone receptor, as a regulatory factor of hormonal action. To date, our research has examined the effects of CaM antagonists on the TSH receptor assay and CaM binding immunoglobulin in sera from Graves' disease patients. The obtained results showed that a CaM-like structure may exist in the membrane receptor for TSH. We also found CaM binding immunoglobulin in sera from patients with Graves' disease.

MATRIALS AND METHODS

Highly purified bovine CaM was obtained as previously described (1). We used ^{125}I-bTSH and solubilized porcine thyroid receptor from a

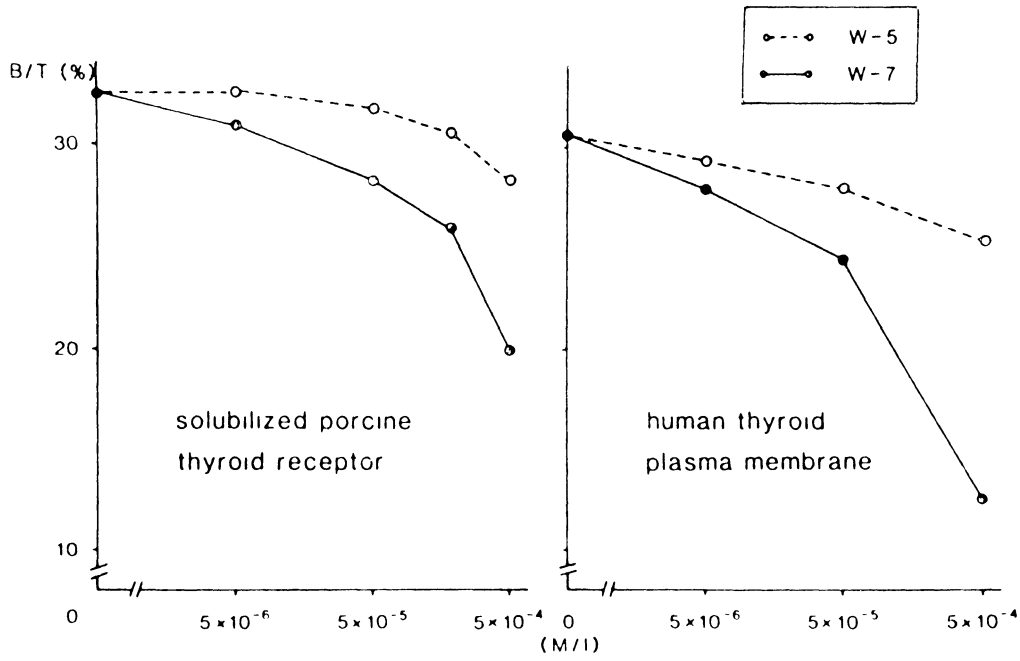

Fig. 1. Effects of W-7 and W-5 on the binding of ^{125}I-bTSH to thyroid receptor.

commercially available kit (R.S.R. Ltd., U.K.). ^{125}I-CaM and anti-CaM was obtained from Amersham (U.K.). We examined 30 sera from patients with Graves' disease with LATS activity, 270 sera from patients with Graves' disease without LATS activity, 30 sera from patients with Hashimoto's disease, 8 sera from subacute thyroiditis patients, 3 sera from silent thyroiditis patients, 5 sera from thyroid cancer patients, and 27 sera from normal volunteers. TSH radioreceptor assay was carried out using human thyroid plasma membrane (2) and solubilized porcine thyroid receptor (3). Detection of autoantibody to ^{125}I-CaM was examined by PEG precipitation and double antibody method, as previously described (4).

RESULTS

Effects of W-7 and W-5 on the binding of ^{125}I-bTSH to the thyroid receptor are shown in Fig. 1. The binding of ^{125}I-bTSH to human thyroid plasma membrane was dose-dependently inhibited by the addition of W-7. However, such a remarkable inhibition was not observed after the addition of W-5. Similar results were obtained using solubilized porcine thyroid receptor. Similar inhibition was also observed by other CaM antagonists such as chlorpromazine and haloperidol (data not shown).

When normal human serum was removed from the assay mixture, the inhibition of ^{125}I-bTSH binding was diminished. By adding calcium ion to the assay mixture, almost in the same concentration as human serum, the inhibitory effect by W-7 was again resumed. However, the inhibition of ^{125}I-bTSH binding was also diminished by the addition of EDTA (data not shown). Preincubation of human thyroid plasma membrane with anti-CaM antibody dose-dependently resulted in the decreased binding of ^{125}I-bTSH to plasma membrane, whereas other antibodies did not show such inhibition. Similar results are obtained using solubilized porcine thyroid receptor (Fig. 2).

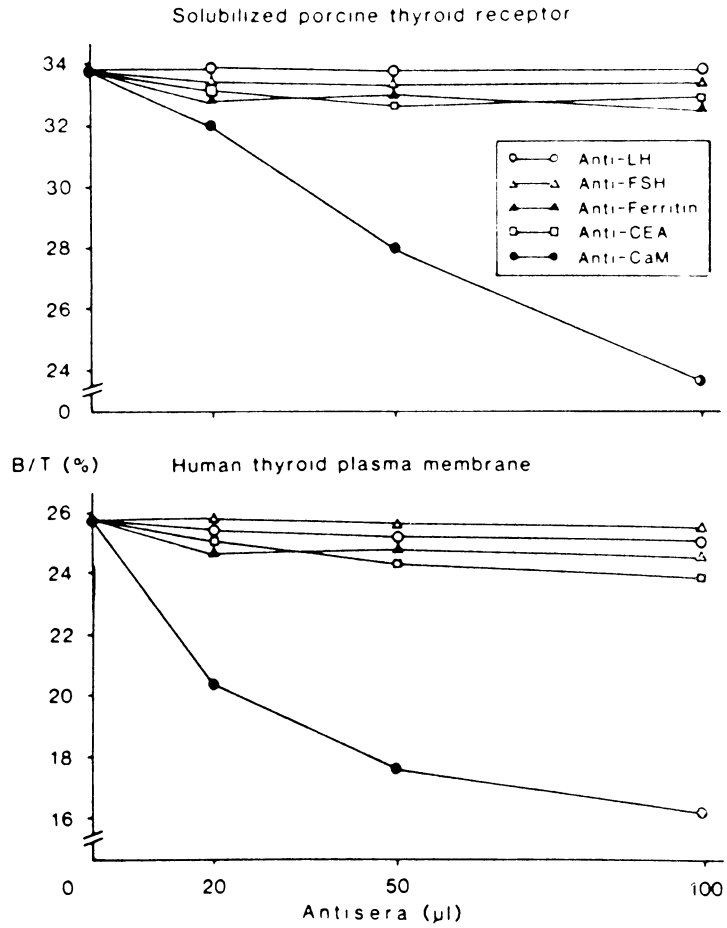

Fig. 2. Effects of various antisera on the binding of ^{125}I-bTSH to thyroid receptor.

A large amount of CaM inhibited ^{125}I-bTSH binding to its receptor, although no direct binding of ^{125}I-bTSH to CaM was observed (Fig. 3).

The binding of ^{125}I-CaM to sera from various thyroidal diseases is shown in Fig. 4. One out of 30 LATS positive sera from Graves' disease, and three out of 270 sera without LATS activity showed increased binding activity. However, none of the sera from other thyroidal diseases showed any increased binding activity. The same results were obtained by using double antibody and Protein A adsorbent (data not shown). Binding of ^{125}I-CaM with patient's serum increased proportionally to the amounts of serum. Also, binding of ^{125}I-CaM to patient's serum was dose-dependently inhibited by the addition of unlabeled CaM over the range of $10^{-6} \sim 10$ μg/tube (data not shown).

Using the double antibody method, three patient's sera (K.K., T.U., and L.A.) were precipitated by antiserum for IgG and IgA; only one patient's serum (M.A.) for IgG. The light chain type of CaM binding immunoglobulins were kappa (T.U., M.S., and L.A.), and both kappa and lambda (K. K.) (Table 1).

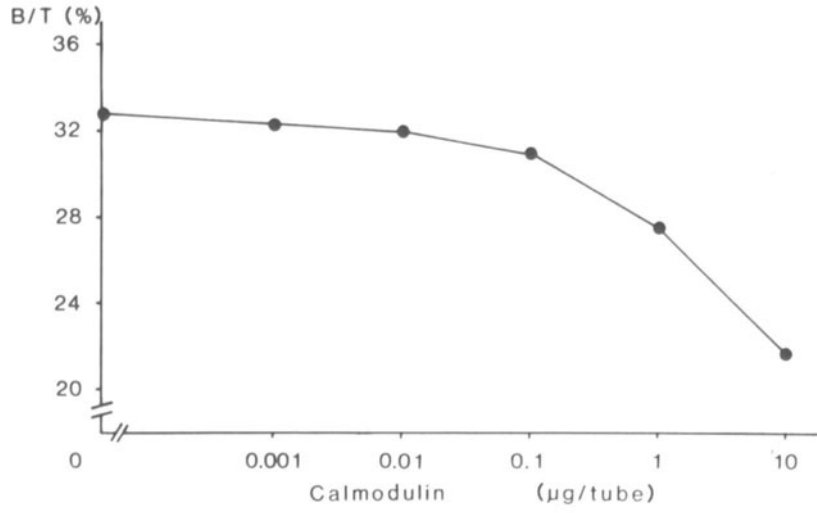

Fig. 3. Effects of CaM on the binding of ^{125}I-bTSH to solubilized porcine thyroid receptor.

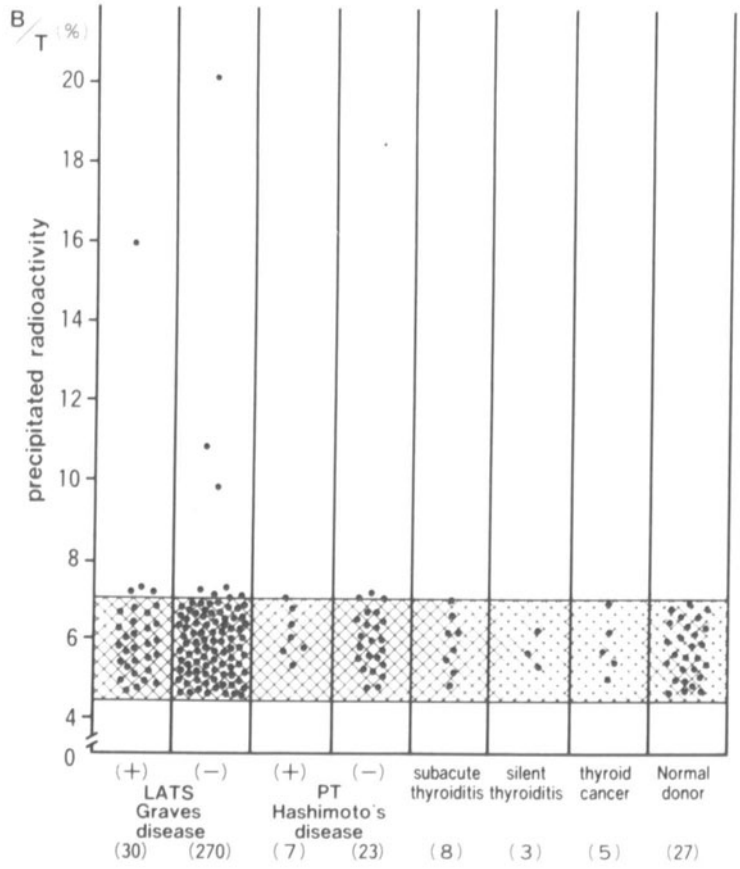

Fig. 4. Binding of ^{125}I-CaM to sera of various thyroidal diseases.

Table 1. Specific Binding of ^{125}I-calmodulin with Immunoglobulin Class and Light Chain Type of Patient's Sera

Rabbit serum	B/T (%)				
	Normal serum	K.K.	T.U.	M.S.	L.A.
Anti-IgG	3.1	6.7	8.5	7.7	7.3
Anti-IgA	4.0	8.9	8.2	3.9	7.0
Anti-IgM	3.3	2.9	3.9	3.5	3.8
Anti-κ*	4.3	14.2	8.2	8.8	8.2
Anti-λ*	4.2	7.7	3.8	4.0	3.8

*Sheep antiserum against rabbit gamma-globulin was added.

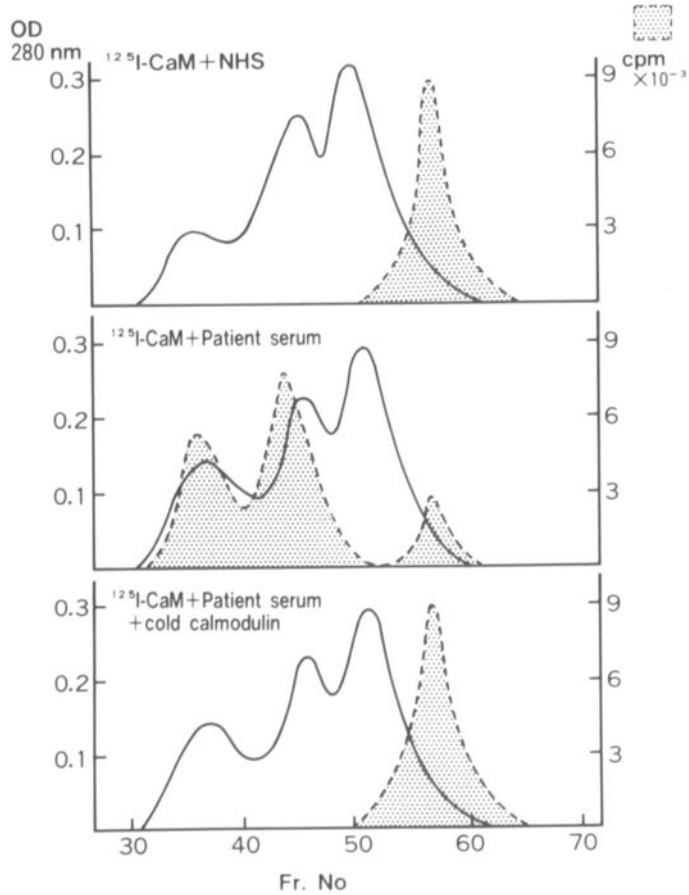

Fig. 5. Gel-filtration of ^{125}I-CaM containing test serum on Sephacryl S-300.

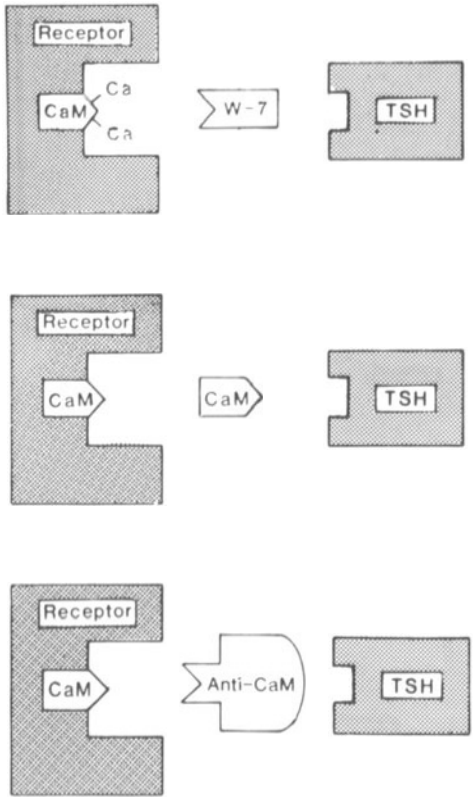

Fig. 6. Possible schematic structure of the TSH receptor.

The elution pattern of ^{125}I-CaM with patient's serum (case K.K.) on a Sephacryl S-300 is shown in Fig. 5. CaM has a M.W. of about 16,700. Thus, the eluted radioactivity was found behind the third peak (albumin fraction). When ^{125}I-CaM was preincubated with the patient's serum and then eluted, the radioactivity was found behind the void volume and before the second peak. When preincubated with unlabeled CaM (10 μg/tube), however, this radioactivity disappeared completely.

DISCUSSION

In the present study, we examined the effects of CaM antagonists on the binding of ^{125}I-bTSH to its receptor in order to investigate the participation of calcium ion and CaM. W-7 inhibited ^{125}I-bTSH binding to its receptor. This phenomenon required calcium ion. These results may suggest the involvement of CaM on the binding of ^{125}I-bTSH to its receptor. The incubation of thyroid plasma membranes with anti-CaM antibody resulted in decreased binding of ^{125}I-bTSH to membranes. From the resulting evidence we postulated a CaM-like structure in the membrane receptor for TSH as shown in Fig. 6.

In addition to the hypothesis described above, we examined the binding immunoglobulin for CaM in sera from patients with various thyroidal diseases in order to elucidate the involvement of a CaM-like structure in relation to the autoimmune mechanism. Four sera out of 300 from patients with Graves' disease had CaM binding immunoglobulins but there were no CaM binding immunoglobulins in sera from other thyroidal diseases. These sera bound to CaM dose-dependently and the binding was inhibited by the addition of unlabeled CaM. CaM binding immunoglobulins were confirmed to be IgG and/or IgA.

When ^{125}I-CaM was mixed with patient's serum, an immune complex formation was demonstrated by gel-filtration on Sephacryl S-300. The radioactivity before the second peak (IgG fraction) is thought to be an immune complex between ^{125}I-CaM and IgA or IgG. The radioactivity behind the void volume is thought to be an aggregation of these immune complexes.

CaM antibody activity correlates with neither TSH receptor antibody activity (determined by Smith's kit), nor with antithyroidal antibodies (microsome and thyroglobulin). The significance of autoantibodies to CaM is unknown, but it may have some relation to a CaM-like portion in the TSH receptor.

REFERENCES

1. Hidaka H, Yamaki T, Naka M, et al. Mol Pharmacol 17: 66, 1980.
2. Rees Smith B and Hall R. Methods Enzymol 74: Pt. C, 405, 1981.
3. Kajita Y, Nakajima Y, Ishida M, et al. Endocrinol Japon 31: 369, 1984.
4. Kajita Y, Nakajima Y, Ishida M, et al. Acta Endocrinol 104: 423, 1983.

TSH SECRETORY REGULATION: NEW EVIDENCE THAT TRIIODOTHYRONINE (T_3) and THYROXINE (T_4) CAN INHIBIT TRH SECRETION BOTH IN VIVO AND IN VITRO*

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ABSTRACT

The potential influence of thyroid hormones *in vivo* upon TRH secretion was examined using rat hypothalami *in vitro* derived from hyperthyroid (50 μg L-T_4/100 g.B.W. x 4 days) and hypothyroid (4 weeks post total thyroidectomy) rats. Ouabain-activated TRH secretion from hypothalami derived from hyperthyroid animals was lowered in 14 ± 3.7%, significant at $p<0.02$, and TRH secretion stimulated by ouabain was augmented 36 ± 10% from hypothyroid hypothalami. Similarly, when L-triiodothyronine (T_3) (0.1 μg/100 g.B.W.) was injected intraventricularly, serum TSH was lowered significantly without a concomitant elevation in peripheral T_3 concentrations. In contrast, administration of the identical quantity of T_3 i.p. had no suppressive effect upon TSH release. It is concluded that thyroid hormones regulate TSH secretion, at least in part, by inhibition of hypothalamic TRH secretion directly.

INTRODUCTION

Abundant evidence has supported the view that thyroid hormones (T_4 and T_3) regulate TSH secretion exclusively by opposing TRH actions at the level of pituitary thyrotroph cell (1-3). However, recently we have demonstrated that T_3 can inhibit ouabain-stimulated TRH secretion from rat hypothalami *in vitro* (4). Further evidence that thyroid hormones can inhibit TRH secretion both *in vitro* and *in vivo* forms the substance of this report.

MATERIALS AND METHODS

Male Sprague-Dawley rats, weighing approximately 200 g., were used. Experimental hyperthyroidism was induced by giving L-T_4 (50 μg/100 b.B.W.) i.p. for four days. Experimental hypothyroidism was produced by total thyroidectomy and animals were used four weeks postoperatively.

Rat hypothalamic incubations were performed *in vitro* after dissection into eight thin pieces and incubation in Krebs-Ringer bicarbonate (KRB) buffer, 10 mM glucose, 0.25% BSA, equilibrated with 95% O_2, 5% CO_2 at pH

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7.5. Tissues were preincubated for 90 minutes prior to two 1 hour experimental periods. Media were harvested separately and stored at -30°C for TRH determinations by RIA (5).

Medium TRH concentrations were expressed as percent of control (TRH hr 2/TRH hr 1 x 100).

Intraventricular stainless steel cannulae (#26) were established using a stereotaxic apparatus and ketamine anesthesia. After a 1 week postoperative period, animals received either isotonic saline or T_3 (0.1 µg/100 g.B.W.) IVT or i.p. Samples (0.5 ml) for TSH RIA were secured from the subclavian vein immediately before and 60 minutes after T_3 or vehicle was administered. Plasma TSH was determined by our specific RIA (6) and T_3 by RIA was quantitated using an immunoassay kit from Corning. Results were analyzed by paired Student's t test.

Ouabain, T_3, and T_4 were purchased from Sigma Chemical Co., St. Louis, MO, USA.

EXPERIMENTAL RESULTS

Inhibition of ouabain-induced TRH secretion *in vitro* after short-term *in vivo* T_4 administration is shown in Fig. 1. After animals received 50 µg T_4 per 100 g.B.W. for 4 days, ouabain-activated TRH release was inhibited 14 ± 3.7% (n=6), significant at $p<0.02$ compared to TRH secretion from euthyroid-derived hypothalami (n=6).

Augmentation of ouabain-stimulated TRH secretion by experimental hypothyroidism is displayed in Fig. 2. TRH released from hypothyroid hyopthalami (right panel) was enhanced 36 ± 10% over TRH secretion from euthyroid controls (n=11), significant $p<0.01$.

The inhibiting action of L-T_3 when administered centrally (IVT) is shown in Fig. 3 (left section). Plasma TSH was lowered from 215 ± 32 µU/ml to 144 ± 25 µU/ml (n=11), $p<0.005$. This effect could not be attribute to a rise in peripheral T_3 concentrations (right section). In contrast, when the identical amount of T_3 was administered peripherally (i.p.), TSH concentrations were not lowered significantly (194 ± 33 vs. 160 ± 25 µU/ml) even though peripheral T_3 concentrations increased slightly (54 ± 3 ng/dl to 73 ± 6 ng/dl, $p<0.005$).

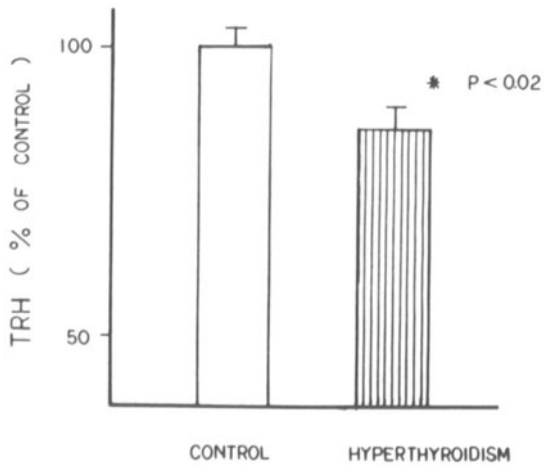

Fig. 1. Effect of T_4 treatment *in vivo* upon TRH secretion from rat hypothalami *in vitro*. TRH release from euthyroid controls is expressed as 100%. Vertical bars represent 1 SEM (N=6). Ouabain-activated TRH secretion from hypothalami from hyperthyroid rats was lowered 14 ± 3.7%. significant statistically at $p<0.02$.

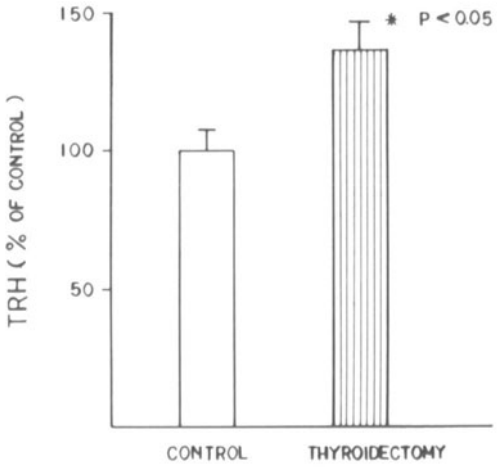

Fig. 2. Effect of thyroidectomy upon TRH secretion from rat hypothalami in vitro. TRH release from euthyroid controls is expressed as 100%. Vertical bars represent 1 SEM (N=11). Ouabain-activated TRH secretion from hypothalami from hypothyroid rats was augmented 36 ± 10%, significant at $p<0.05$.

DISCUSSION

Three lines of new evidence herein provide support for the view that thyroid hormones may regulate TSH secretion, at least in part, by inhibiting TRH secretion per se, and extend our previous demonstration of T_3 inhibition in vitro of ouabain-activated TRH release (4). Not only can TRH secretion be augmented by experimental hypothyroidism and inhibited, conversely, by hyperthyroidism, T_3 given in IVT (lateral ventricle) can lower TSH in vivo in dosage schedules that fail to affect TSH when administered peripherally (i.p.). Although the evidence from the latter studies concerning TRH secretory events is indirect, we consider it unlikely that centrally administered T_3 acted on the thyrotroph cell per se, since identical amounts administered i.p., associated with increments in circulating T_3 concentrations (Fig. 3) not seen following the intraventricular route, failed to reduce circulating TSH concentrations.

Our present observations are supported by the earlier studies of Cook (7), who reported that systemically ineffective quantitites of L-T_4 administered IVT to rats could suppress TSH secretion, as inferred from reductions

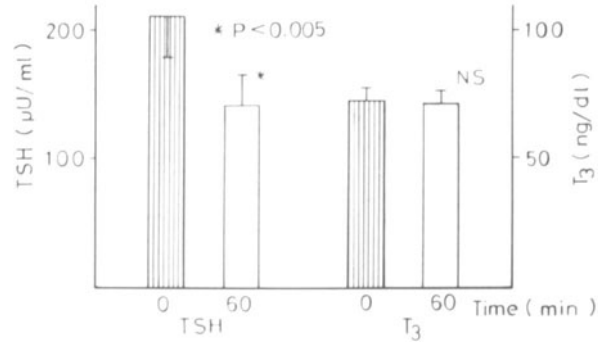

Fig. 3. Effect of intraventricular administration of T_3 on plasma TSH and T_3 concentrations. Values are expressed as the mean ± SEM (N=11) and analyzed by paired Student's t test. NS indicates not significant.

in thyroidal ^{131}I release rates. Moreover, recently, injections of T_3 (2 ng) directly into hypothalamus were reported by Belchetz, et al., to rapidly inhibit TSH secretion, whereas direct pituitary injection of identical T_3 quantities did not lower TSH (8). The failure of T_3 to block KCl-stimulated TRH in vitro, in the studies of Tapia-Arancibia, et al. (9), we infer reflected inadequate preincubation time with T_3.

Further studies are in progress to explore in greater detail the physiological implications of this new regulatory concept of modulation of TRH secretion by thyroid hormones.

REFERENCES

1. Vale W, Burgus R, and Guillemin R. Neuroendocrinology 3: 34, 1968.
2. Schrey MP and Larsen PR. Endocrinology 108: 1690, 1981.
3. Hinkle PM, Perrone MH, and Schonbrunn A. Endocrinology 108: 199, 1981.
4. Iriuchijima T, Rogers D, and Wilber JF. Clin Res 32: 866a, 1984.
5. Montoya E, et al. Endocrinology 96: 1413, 1975.
6. Wilber J and Utiger R. Endocrinology 81: 145, 1967.
7. Cook DM, Kendall JW, and Nichols EM. In K Fellinger and R Hofer (eds), Further Advances in Thyroid Research, Verlag der Wiener Medizinischen Akcademie, Vienna, 1971, pp 1165-1171.
8. Belchetz PE, et al. Endocrinology 76: 439, 1978.
9. Tapia-Arancibia L, Arancibia S, and Astier H. Neurosci Lett 45: 47, 1984.

EFFECT OF TRH ON 5'-DEIODINASES IN RAT PITUITARY GLANDS

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INTRODUCTION

It is well known that the activity of 5'-deiodinase in the pituitary increases in thyroidectomized rats (1-4). However, the cause of this increase in the activity of 5'-deiodinase has not been elucidated. Either the direct effect of reduced thyroid hormones or the enhancement of the sensitivity to TRH was considered as the causative factor. Recently, it was reported that TRH increased the activity of 5'-deiodinase of cultured GH4C1 cells (5). Hypothalamic factors, including TRH, were also reported to regulate 5'-deiodinase activity in the pituitary (6). The modulation of 5'-deiodinase activity by trophic hormone per se was exemplified in the 5'-deiodinase of thyroid gland. It is increased by TSH, while that in liver and kidney was not influenced at all (7). From this evidence, it is conceivable that TRH stimulates the increase of 5'-deiodinase in pituitary *in vivo*. To determine the effect of TRH on pituitary T_4 5'-deiodinase *in vivo*, the present experiments were carried out.

MATERIALS AND METHODS

Experiment 1. Male Wistar rats, weighing 180-200 g, were injected intraperitoneally with a dose of 100 μg TRH/200 g BW twice a day for three days. The same volume of saline was injected into control rats. On the fourth day, pituitaries were harvested.

Experiment 2. Male Wistar-Imamichi rats at the age of 4 weeks (60-70 g BW) were surgically thyroidectomized by the supplier. Effect of thyroidectomy was examined by serum levels of the T_4, T_3, and TSH measured by RIA. On the 15th day after thyroidectomy, a dose of 800 μg TRH/200 g BW was injected ip twice a day for 3 days. On the 18th day, pituitaries were harvested.

Experiment 3. On the 15th day after thyroidectomy, a single dose of 1 mg TRH/150 g BW was injected ip and pituitaries were harvested at the 24th hour after the injection.

All through these three experiments, the body weight of the TRH-injected group was not different from that of the control group.

Table 1. Km and Vmax of High Km 5'-deiodinase in Pituitary After Chronic Treatment with TRH in Normal Rats

	Km (μM)	Vmax (pmol/mg protein·min)
Control	0.93 ± 0.077	0.993 ± 0.182
TRH	1.14 ± 0.148	0.328 ± 0.031*

Mean ± SD; *$p < 0.01$

Measurement of T_4 5'-deiodinase. Pituitaries were homogenized in 50 mM Tris buffer, pH 7.4, containing 5 mM dithiothreitol (DIT). The homogenate was centrifuged at 1500 x g for 30 min. The particulate fraction obtained by the centrifugation at 100,000 x g for 1 hr was suspended in 50 mM phosphate buffer, pH 6.5, containing 5 mM DIT to make 20-fold dilution of the pituitary weight. After preincubation of the suspension at 37°C for 5 min, graded doses of T_4 (0.613 - 9.8 μM) were added and further incubated at 37°C for 20 min. Reaction was terminated by the addition of 2 aliquots of 95% ethanol (7). After centrifugation at 1500 x g for 30 min, T_3 was measured by RIA kit. Low Km enzyme was measured by Visser's method (8). The buffer containing 0.32 M sucrose, 10 mM HEPES, and 10 mM DIT, pH 7.0, was used to homogenate pituitaries. The precipitate obtained by different centrifugation between 3500 and 100,000 x g was suspended in 0.2 M phosphate buffer, pH 7.0, containing 20 mM DIT.

RESULTS

Experiment 1. Two kinds of T_4 5'-deiodinase were observed in normal rats' pituitary (Fig. 1), low Km (3.7 - 80 nM) and high Km (0.9 - 2 μM). After chronic treatment with TRH as described in the materials and methods, Km and Vmax of low Km enzyme were not changed by TRH. On the contrary,

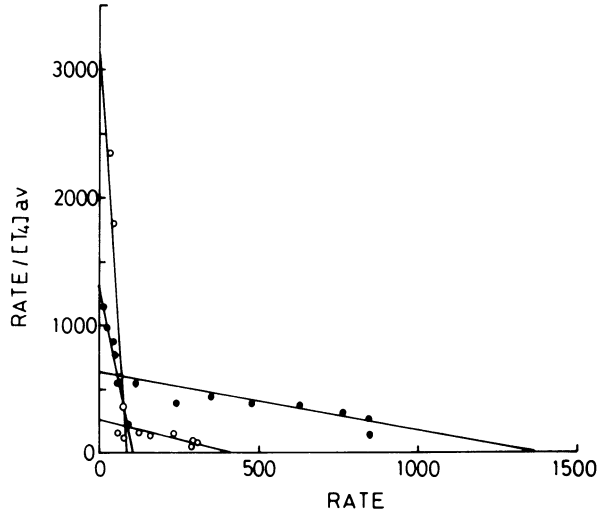

Fig. 1. Eadie-Hofstee plot of 5'-deiodinase in normal rat pituitaries.

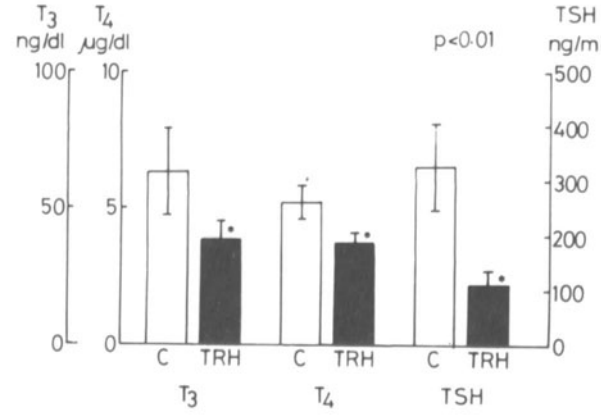

Fig. 2. Serum concentration of T_3, T_4, and TSH after chronic treatment with TRH. See experiment 1.

Vmax of high Km enzyme was decreased by TRH (Table 1). Km of high Km enzyme was not changed by TRH. Serum concentrations of T_3, T_4, and TSH were significantly decreased by the treatment with TRH (Fig. 2).

Experiment 2. In thyroidectomized rats, the chronic treatment with TRH increased Vmax of low Km enzyme, but the extent was slightly short of statistical significance (Fig. 3). Km of low Km enzyme was not changed by

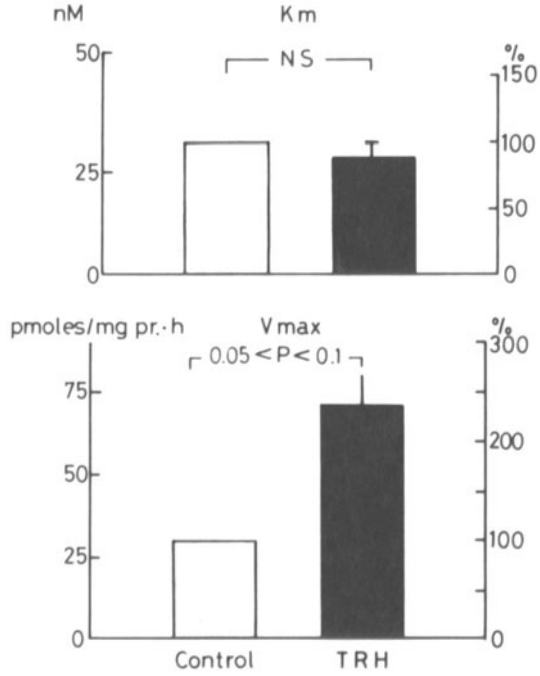

Fig. 3. Km and Vmax of pituitary 5'-deiodinase in thyroidectomized rats after chronic treatment with TRH. See experiment 2.

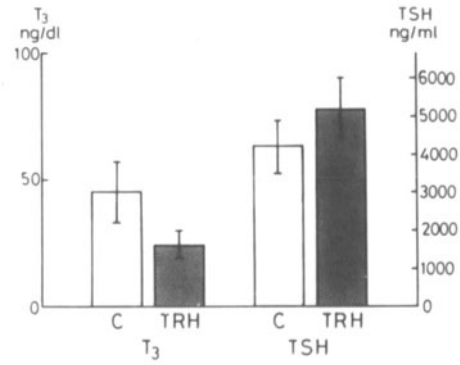

Fig. 4. Serum T_3 and TSH in thyroidectomized rats after chronic treatment with TRH. See experiment 2.

TRH. High Km enzyme was not detected in the pituitaries of thyroidectomized rats. Serum T_3 was slightly decreased and serum TSH was slightly increased by TRH, but both of them did not exhibit statistic significance (Fig. 4).

Experiment 3. The Vmax of low Km 5'-deiodinase in pituitaries was significantly increased by a single dose of TRH at 24 hours after the injection (Fig. 5). The Km of that was not changed by TRH. Serum T_3, T_4, and TSH were not changed by a single injection of TRH.

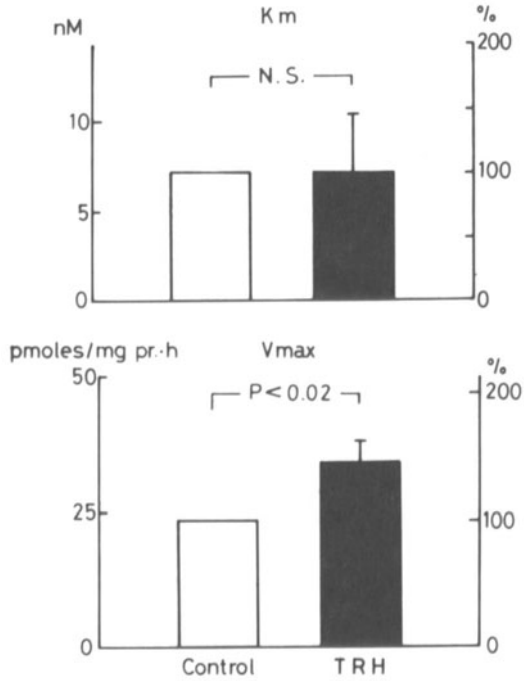

Fig. 5. Km and Vmax of pituitary 5'-deiodinase in thyroidectomized rats at 24 h after injection of a single dose of TRH.

DISCUSSION

The pituitary T_4 5'-deiodinase is known as low Km enzyme (3,8). In our hands, high Km 5'-deiodinase was also detected in the pituitaries when the same buffer was used as that for the enzyme in other tissues. The value of the Km in pituitary was lower than that of thyroid gland. After chronic treatment with TRH, Vmax of high Km enzyme was significantly decreased by TRH (Table 1), while that of low Km enzyme was not changed. As shown in Fig. 2, serum TSH was decreased by chronic treatment with TRH, as reported previously (9). The reduction of serum TSH shows the unresponsiveness (or desensitization) of pituitary to TRH, and, in that pituitary, the activity of T_4 5'-deiodinase (high Km) was found to be decreased. In normal pituitary, the low Km enzyme activity was very low. Therefore, experiments 2 and 3 were carried out, employing thyroidectomized rats in which the activity of low Km 5'-deiodinase increased (1-4).

The chronic treatment with TRH increased the Vmax of low Km T_4 5'-deiodinase in thyroidectomized rats and a single dose of TRH increased the Vmax significantly. This increase in the activity of the enzyme must be due to some biological effect of TRH, because the responsiveness to TRH was enhanced by thyroidectomy.

In summary, low Km and high Km T_4 5'-deiodinases were detected in normal rat pituitary. The decrease of Vmax of high Km enzyme was observed in the TRH-unresponsive pituitary and the increased Vmax of low Km enzyme was observed in the TRH-stimulated pituitary of thyroidectomized rats. The evidences of the stimulation of TRH to increase pituitary 5'-deiodinase have been reported recently by other laboratories (5,6). Those evidences show that TRH possibly regulates both high Km and low Km enzymes in rat pituitary.

REFERENCES

1. Larsen PR, Dick TE, Markovitz BP, et al. J Clin Invest 64: 117, 1979.
2. Cheron RG, Kaplan MM, and Larsen PR. J Clin Invest 64: 1402, 1979.
3. Kaplan MM. Endocrinology 106: 567, 1980.
4. Maeda M and Ingbar SH. J Clin Invest 69: 799, 1982.
5. Koenig RJ. Program and Abstracts of the American Endocrine Society 845, 1985.
6. St. Germain DL, Adler VA, and Galton VA. Endocrinology 117: 55, 1985.
7. Erickson VJ, Cavalieri RR, and Rosenberg LL. Endocrinology 111: 434, 1982.
8. Visser TJ, Kaplan MM, Leonard JL, et al. J Clin Invest 71: 992, 1983.
9. Nemeroff CB, Bissette G, Martin JB, et al. Neuroendocrinology 30: 193, 1980.

PERINATAL PITUITARY-THYROID FUNCTION AT HIGH ALTITUDE

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INTRODUCTION

Acute exposure of adult men to a high altitude environment results in transitory thyroid hyperactivity (1) and increased TSH secretion (2). The effect of high altitude on pituitary-thyroid function during perinatal life has little been studied. Normal low cord blood T_4 levels have been reported (3), and a hypoxemic condition of the fetus has been suggested by high cord blood hematocrits (4), hyperplasia of bone marrow erythroid cells (5), lower birth weight (6), and by a higher incidence of prematurity (6) and neonatal mortality (7).

The normal newborn infant rapidly becomes chemically hyperthyroid in the neonatal period due to an acute release of pituitary TSH (8), which evokes thyroid hormone secretion (9), and due to an abrupt increase in peripheral conversion of T_4 to T_3 (10).

The present study was conducted to explore further the pituitary and thyroid function of newborn infants acutely exposed to a high altitute environment characterized by hypoxia and lower temperature and barometric pressure.

MATERIALS AND METHODS

Two groups of healthy subjects have been studied. There were six native mothers and their newborn term infants at high altitude (Cerro de Pasco, 4,300 m) and five control mothers and their infants at sea level (Lima, 150 m). Maternal and cord blood samples were taken at delivery. The parents were informed and consent obtained. A polyethylene catheter was inserted under sterile conditions into the umbilical vein of the infant within a few minutes of birth, and 3 ml blood samples were obtained at 10, 30, 60, 90, and 120 minutes of life. The umbilical catheter was then removed. Additional samples were taken by venipuncture at 24 and 48 hours. Double antibody RIA measurements of TSH (11), Total T_4, and T_3 (12), and Free T_4 by solid-phase method (13) were conducted simultaneously on each maternal and newborn sample. Moreover, weight and height, Apgar index (14), gestational age (15), placental weight and volume (16), maternal

and cord blood microhematocrit (17), and maternal urinary iodine concentration (18), as previously described, were registered at birth.

All infants were products of full term, uncomplicated pregnancies and were delivered vaginally. The newborns were kept at an ambient nursery temperature (10°C at high altitude and 25°C at sea level), and mean rectal temperatures were monitored from birth to 120 minutes. The neonatal course was uneventful.

Statistical analysis was performed using standard methods for Student's t test (two tailed) and regression analysis. Variances from means are indicated as SDs. The rate of fall of serum TSH from the 30 min peak concentration was calculated by plotting on a logarithmic scale against age in minutes.

General data of mothers and their newborn children at delivery are shown in Table 1. Mean maternal hematocrit at high altitude (50.3 $\pm$ 7.0%) was significantly higher than at sea level (36.0 $\pm$ 2.3%). All high altitude mothers had normal mean urinary I^- excretion. Placental weight and volume were similar at both levels, although the ratio of placental weight: birth weight tends to be greater at high altitude (0.165 $\pm$ 0.010 vs 0.156 $\pm$ 0.018). With regard to perinatal data, infants born at high altitude differed from those at sea level by having a significantly lower mean weight (2.8 $\pm$ 0.2 vs 3.4 $\pm$ 0.2 kg; $p<.01$), mean height (49.2 $\pm$ 2.3 vs 53.4 $\pm$ 2.3 cm; $p<.05$), and mean rectal temperature (36.6 $\pm$ 0.7 vs 38.0 $\pm$ 0.4 °C), and greater cord blood hematocrits (60.3 $\pm$ 3.1 vs 48.4 $\pm$ 4.6%; $p<.01$).

RESULTS

The correlation between cord T_3 levels and the placental weight:birth weight ratio (placental coefficient), indicates that at the same placental placental coefficient, infants born at a high altitude had lower T_3 cord levels than infants born at sea level. There was no significant correlation between birth weight, placental coefficient, and cord hematocrit with respect to cord TSH concentration and cord thyroid hormones at either level, although newborns with greater weight tend to have higher cord T_4 and Free T_4 levels.

In contrast to newborns at sea level, fetal serum free thyroxine and TSH concentrations did not significantly exceed maternal values at term, with lower T_4 concentrations than their paired maternal levels.

The following data was obtained concerning neonatal changes of TSH and thyroid hormones during the first minutes and hours of life. The postnatal surge of TSH peaked at 30 minutes (108.5 $\pm$ 35.8 μU/ml) and was not significantly lower than at sea level (133.8 $\pm$ 30 μU/ml). The half time of disappearance of serum TSH from 30 minutes peak concentration was clearly slow at high altitude (108' vs 85'). While in both groups the maximum increase of T_4 and Free T_4 was peaked at 24 hours, the peaking up curves were delayed at high altitude, starting only after 60 minutes, whereas at sea level it began at 30 minutes of life. Free T_4 was significantly lower during the first 120 minutes, but total T_4 got significantly higher at 48 hours follow-up. The surge of T_3 was also delayed and significantly lower at high altitude, reaching its maximum peak (182 $\pm$ 44 ng/dl) at 24 hours, whereas at sea level it peaked (236 $\pm$ 30 ng/ dl) at two hours. The triiodothyronine:thyroxine ratio changes paralleled the T_3 changes, and was significantly lower and delayed at high altitude, reaching its peak at 120 minutes (0.011 $\pm$ 0.001), whereas at sea level it peaked (0.016 $\pm$ 0.002) at 90 minutes of life.

Table 1. General Data of Mothers and Newborn Children at Delivery

	Mother			Newborn					
	Age years	Ht %	Placental coefficient	Weight Kg	Height cm	Gestational age (wk)	APGAR	Rectal temperature	Cord Ht %
High altitude	26.6±	50.3±	0.165±	2.8±	49.2±	39.2±	10	36.6±	60.3±
	6.9*	7.0	0.010	0.2	2.3	0.7		0.7	3.1
Sea Level	23.2±	36.0±	0.156±	3.4±	53.4±	39.8±	10	38.0	48.4±
	4.6	2.3	0.018	0.2	2.3	0.3		0.4	4.6
P values	NS	<.01	NS	<.01	<.05	NS	NS	<.01	<.01

*$\bar{X}$ ± SD.

DISCUSSION

The postnatal surge of TSH was slightly lower in children born at a high altitude and, regardless of lower ambient and rectal temperature, they did not show more TSH surge. Since an early acute release of TSH in the newborn is mainly the result of extrauterine cooling and preformed pool of pituitary TSH (8), we can suggest that infants born at high altitudes have lower pituitary TSH reserve. In addition, the slower decline curve of serum TSH from the 30 minute peak concentration toward normal, as observed at high altitudes, suggests lower degradation rate of TSH.

Newborn children at a high altitude also showed delayed serum T_4 picking up levels and significant lower cord Free T_4 and during the first 120 minutes of life. Since neonatal elevation of T_4 and Free T_4 results mainly from TSH stimulus to the thyroid gland (19), we suggest that newborns at high altitudes have a decreased secretion of thyroid hormones. Moreover, it appears to be decreased T_4 clearance because its slower decline falls after the 24 hour peak concentration.

The surge of T_3 was also delayed and significantly lower in newborn children at high altitude, and it was parallel to changes in T_3/T_4 ratio. It has been suggested that the early increase of T_3 that occurs during the first hours of life is due to augmented thyroidal secretion (19) and rapid increase in peripheral conversion of T_4 to T_3 (20). Therefore, newborns of high altitude seem to have decreased thyroid response to TSH stimulus together with delayed maturation of tissue T_4 metabolism, namely of the T_4 to T_3 conversion system. In addition, placenta of high altitude seems to have impaired monodeiodinating activity as compared to those at sea level.

The pituitary and thyroid function of our infants born at high altitude are similar to those previously described in healthy, small for gestational age, and preterm infants delivered before full term maturation of the hypothalamic-pituitary-thyroid system and peripheral iodothyronine metabolism (21). Moreover, infants born at high altitudes seem to exhibit an apparent decreased thermogenesis response against cold exposure, which appears to increase thyroid activity and the rate of 5' monodeiodination (22).

Although fetal capillary pO_2 at high altitude has been described as not significantly lower than at sea level (23), the hypoxemic condition of our fetus at high altitude may be suggested by increased cord blood hematocrits and by lower height and weight at birth. Its possible effect on maturation of the hypothalamic-pituitary-thyroid system and peripheral iodothyronine metabolism deserves further comments.

The significance of this relatively lesser pituitary and thyroid activity shown by the newborn children at high altitude during the first hours of life is not yet clear. Since adequate thyroid function regulates the central nervous system (24) and lung surfactant maturation (25) and since a higher incidence of neonatal respiratory distress and mortality at high altitudes has been described (7), it appears that these children are handicapped when exposed to high altitude environmental conditions which should be discussed further.

In conclusion, our findings suggest that infants at high altitude seem to have: 1) lower pituitary TSH reserve, 2) lower and delayed secretion of thyroid hormones, and 3) delayed maturation of peripheral iodothyronine metabolism.

REFERENCES

1. Pretell EA, Llerena LA, et al. Abstracto II Congreso Bolivariano de Endocrinologia, Caracas, Venezuela, Oct. 1971, p 41.
2. Mordes J, Blume FD, et al. N Engl J Med 308: 1135, 1983.
3. Pretell EA, Abuid J, and Cateriano M. VII Jornadas Peruanas de Endocrinologia, Ica, Peru, Oct. 1977, p 42.
4. Loret de Mola L. Tesis de Bachiller, Facultad de Medicina, Universidad Nacional Mayor de San Marcos, Lima, Peru, 1955.
5. Reynafarje C. J Pediatr 54: 152, 1959.
6. Lichty JA, Ting RY, et al. Am J Dis Chil 93: 666, 1957.
7. Grahn D and Kratchman J. Am J Human Genet 15: 329, 1963.
8. Fisher DA and Odell WD. J Clin Invest 48: 1670, 1969.
9. Abuid J, Stinson DA, and Larsen R. J Clin Invest 52: 1195, 1973.
10. Fisher DA, Dussault J, et al. Prg Hormone Res 33: 59, 1977.
11. Spencer CA and Nicoloff JT. Clin Chim Acta 108: 415, 1980.
12. Challand GS, Ratcliffe WA, and Ratcliffe JG. Clin Chim Acta 60: 25, 1975.
13. Ekins RP. In RP Ekins (ed), Methods for the Measurement of Free Thyroid Hormones, Excerpta Medica, Ambsterdam, 1979.
14. Apgar V, Holaday, DA, et al. JAMA 168: 1985, 1958.
15. Dubowitz L, Dubowitz V, and Goldberg C. J Pediatr 77: 1, 1970.
16. Kadar K. Revista Peruana Ginecologia y Obstetrica 27: 3, 1971.
17. Mc Gover JJ, Jones AR, and Steimberg AG. J Med 252: 253, 1955.
18. Jolin T and Escobar del Rey F. J Clin Endocrinol Metab 25: 540, 1965.
19. Eremberg A, Phelps DL, et al. Pediatrics 32: 211, 1974.
20. Wu S-Y, Klein AH, et al. Endocrinology 103: 235, 1978.
21. Jacobsen BB, Anderson HJ, et al. Acta Paediatr Scand 66: 681, 1977.
22. Scammel J, Barney C, and Fregly M. J Appl Physiol 51 (5): 1157, 1981.
23. Sobrevilla LA, Casinelli MT, et al. Am J Obst Gynec 111: 1111, 1971.
24. Dunn JT. In JB Stanbury and RL Kroc (eds), Human Development and the Thyroid Gland, Plenum Press, New York, 1972, p 367.
25. Reddings RA, Douglas WH, and Stein M. Science 1975: 994, 1972.

HUMAN FETAL PROLACTIN BUT NOT TSH SECRETION IS AFFECTED BY DOPAMINERGIC STIMULI*

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In adult mammals, thyrotropin (TSH) is regulated by an integrated system in which circulating thyroid hormones and the intrapituitary conversion of T_4 to T_3 inhibit and thyrotropin-releasing hormone (TRH) stimulates TSH secretion. Furthermore, somatostatin (SRIH) and dopamine (DA) inhibit pituitary TSH release (1,2). We have reported that TRH (3) and SRIH (4) stimulate and inhibit, respectively, fetal TSH secretion. Metoclopramide, a DA receptor-blocking drug, given to parturient women does not affect cord blood (CB) TSH, suggesting that DA plays a minor role in the regulation of fetal TSH secretion (5). We have now investigated the effect of bromocriptine (BC), a DA agonist drug, given to women during labor on TSH and prolactin (PRL) secretion in the term fetus.

MATERIALS AND METHODS

Women with normal pregnancies were studied at term. During labor, 5 mg BC was given p.o. to 60 randomly selected women. Sixty-two women received placebo (control) during labor. Maternal serum (MS) was obtained before BC or placebo administration and at parturition. At birth, CB was collected from all newborns. Serum was frozen at -20°C until analysis. TSH, PRL, thyroxine (T_4), triiodothyronine (T_3), and reverse triiodothyronine (rT_3) concentrations were measured by RIA. All samples were assayed for each hormone in duplicate, in the same assay and in random order. MS and CB hormone values at parturition in women treated with BC or placebo were grouped according to the interval of time between BC or placebo administration and birth: 0-30, 30-60, 60-90, 90-120, 120-180, 180-240, 240-300, 300-425 min. Data were evaluated by comparing the results in MS or CB in the women treated with BC with those given placebo. Statistical analyses were carried out by the unpaired Student's t test, two-way analysis of variance (ANOVA), and regression analysis, as appropriate. All reported values are the mean $\pm$ SE.

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RESULTS

Maternal Serum Hormone Values

There were no significant differences in maternal serum T_4, T_3, rT_3, PRL, and TSH concentrations prior to BC vs placebo administration. At parturition, serum TSH concentration was significantly lower in the BC treated women as compared to values in the women receiving placebo (ANOVA, $p<0.006$). Serum PRL concentration was markedly decreased in the women previously treated with BC compared to the placebo treated women (ANOVA, $p<0.001$) and this decrease was significantly more pronounced with increasing time intervals between BC administration and delivery (regression analysis; $p<0.001$). No significant differences were observed in serum T_4, T_3, and rT_3 concentrations between the BC and placebo-treated women.

Cord Blood Serum Hormone Values

CB PRL concentrations in newborns whose mothers were treated with BC were significantly lower than those observed in women receiving placebo (ANOVA, $p<0.001$) (Fig. 1). There was a progressive decline in CB PRL values in newborns whose mothers had been treated with BC as the time between drug administration and delivery increased (regression analysis; $p<0.01$). There was no difference in CB TSH concentrations between BC and placebo-treated women (Fig. 2). CB T_4, T_3 and rT_3 concentrations were similar in the newborns whose mothers had received BC or placebo.

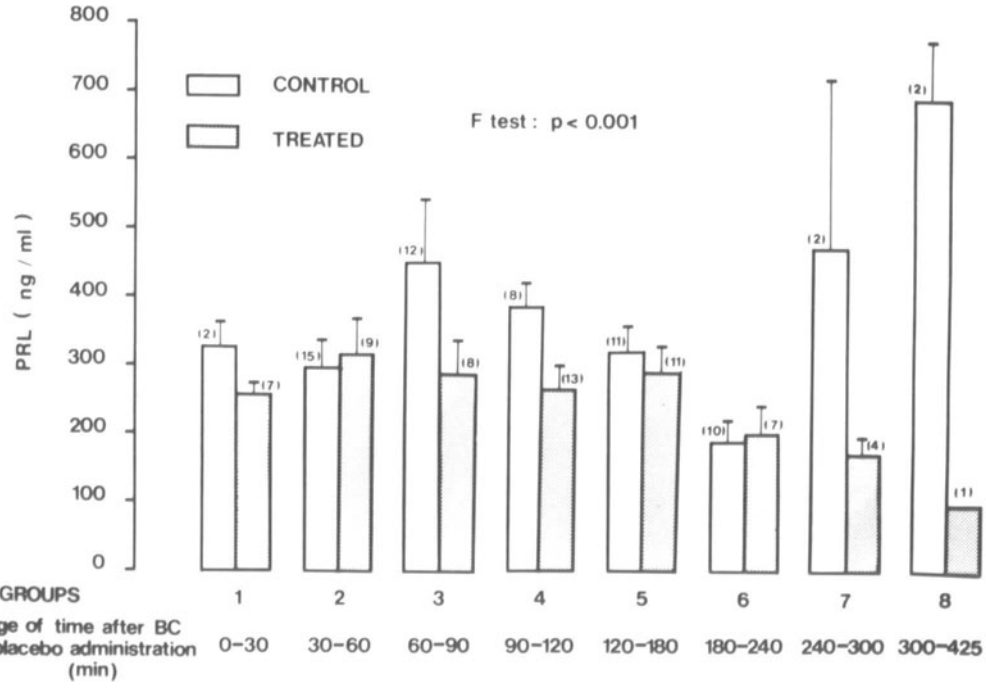

Fig. 1. Cord blood PRL concentrations at different intervals of time after the maternal administration of bromocriptine or placebo. The number of samples in each group is shown in parentheses. The bars represent the mean values and the brackets the SE.

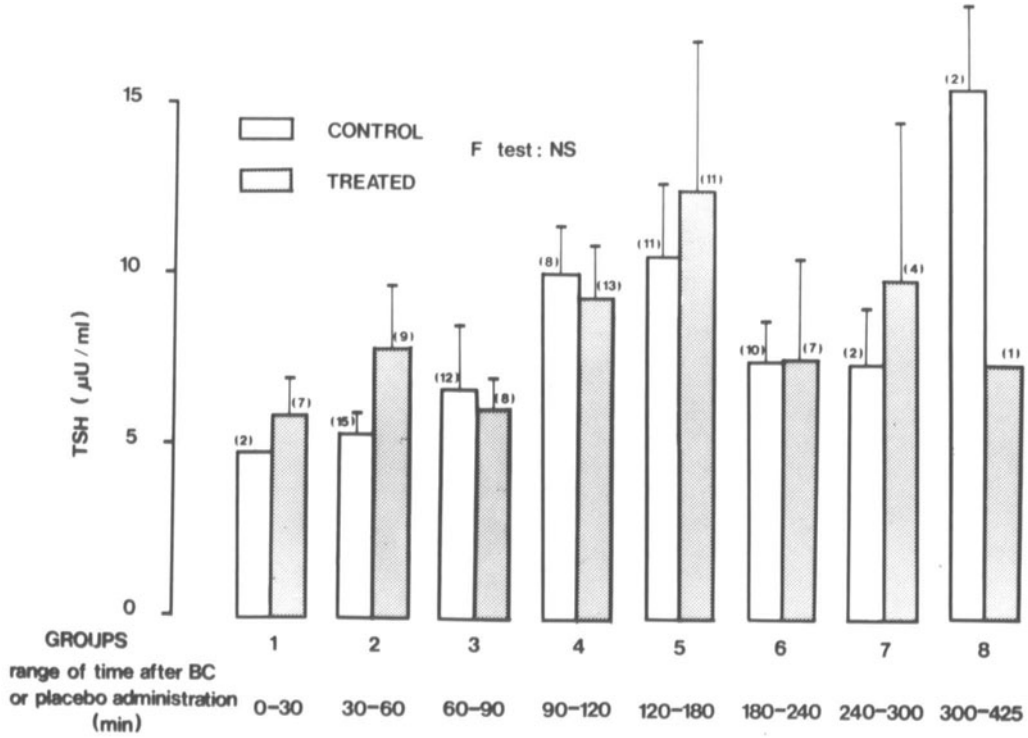

Fig. 2. Cord blood TSH concentrations at different intervals of time after the maternal administration of bromocriptine or placebo. The number of samples in each group is shown in parentheses. The bars represent the mean values and the brackets the SE.

DISCUSSION

Administration of DA and its agonists and DA antagonists to man and experimental animals inhibits and stimulates, respectively, PRL secretion (1). During pregnancy, markedly elevated serum PRL concentrations are usually present by the first trimester and continue elevated throughout pregnancy (6). Dopaminergic control of PRL secretion during pregnancy is suggested by the observations that the administration of antidopaminergic drugs to pregnant women significantly increases serum PRL concentrations (7) whereas the administration of dopaminergic drugs markedly decreases PRL secretion (8). In the present study, the administration of BC to women during labor markedly reduced serum PRL concentrations. The lowest PRL values were observed in maternal samples obtained 180-240 minutes after BC administration.

Radioimmunoassayable PRL is detected in fetal serum as early as 10-12 wks of pregnancy and after the 25th wk a progressive increase is observed until birth. At parturition, fetal PRL is higher than that observed in the mother (9). Evidence for an active role for DA in the regulation of PRL secretion during fetal and early neonatal life has been obtained in animals and man. Bromocriptine administration to an occasional pregnant woman has been reported to decrease fetal serum PRL concentration (10). We have demonstrated that BC administered to women during labor suppresses fetal PRL secretion in the fetus. This finding is somewhat at variance with the studies of others (11-13). TRH, a known stimulator of PRL release, readily crosses the human placenta, yet its administration to women in labor does not increase fetal PRL secretion (14). This latter observation does

not negate the role of TRH in the regulation of fetal PRL secretion, but rather suggests that PRL secretion is maximal in the term fetus and cannot be further stimulated.

In the adult, an inhibitory effect of DA on TSH secretion has been suggested by the observation that the administration of L-dopa, DA, and BC significantly reduces basal and TRH-stimulated serum TSH concentrations in euthyroid and hypothyroid subjects. Conversely, the administration of dopaminergic receptor blocking drugs significantly increases serum TSH concentrations (1,2). Studies to investigate the role of DA in the regulation of TSH secretion during pregnancy have yielded conflicting results. Ylikorkala et al. (15) reported that the oral administration of BC did not affect maternal basal serum TSH concentration but did lower the TSH response to TRH. Administration of metoclopramide to pregnant women did not induce a significant change in maternal serum TSH concentrations (5,7,13). We now report a small but significant decrease in maternal serum TSH concentrations following BC administration during labor, suggesting the presence of some dopaminergic control of TSH secretion in the term pregnant woman. Despite the effective transfer of BC across the placenta, as indicated by the decrease in fetal serum PRL, no effect of BC on fetal TSH concentration was observed. This finding is in agreement with previous results in one pregnant woman (10). These results suggest that dopaminergic control of TSH secretion is of minor importance in the term fetus which is in agreement with our preveous studies that the maternal administration of metoclopramide did not affect fetal serum TSH concentration (5). The failure of dopaminergic stimuli to inhibit TSH release might be partially responsible for the elevated serum TSH concentrations observed in the term human fetus since the other mechanisms inhibiting TSH release, such as circulating T_4 and T_3, the intrapituitary T_4 to T_3 deiodination and SRIH are normally active in the term fetus (4,16,17).

ACKNOWLEDGMENTS

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REFERENCES

1. Scanlon MF, Pourmand M, McGregor Am, et al. J Endocrinol Invest 2: 307, 1979.
2. Morley JE. Endocr Rev 2: 396, 1981.
3. Roti E, Gnudi A, Braverman LE, et al. J Clin Endocrinol Metab 53: 813, 1981.
4. Roti E, Robuschi G, Alboni A, et al. Acta Endocrinol (Copenh) 106: 393, 1984.
5. Roti E, Robuschi G, Emanuele R, et al. J Clin Endocrinol 56: 1071, 1983.
6. Albrecht BH. In D Tulchinsky and KJ Ryan (eds), Maternal-Fetal Endocrinology. WB Saunders, Philadelphia, London, and Toronto, 1980, p 97.
7. Kauppila A, Kivinen S, Ylikorkala O. J Clin Endocrinol Metab 52: 436, 1981.
8. Muller EE, Genazzani AR, Murru S, et al. Acta Endocrinol (Copenh) 86: 33, 1977.
9. Gluckman PD, Grumbach MM, and Kaplan SL. Endocr Rev 2: 363, 1981.
10. Bigazzi M, Ronga R, Lancranjan I, et al. J Clin Endocrinol Metab 48: 9, 1979.
11. Delitala G, Meloni T, Masala A, et al. J Clin Endocrinol Metab 46: 880, 1978.

12. Robuschi G, Emanuele R, d'Amato L, et al. J Endocrinol Invest 6: 107, 1983.
13. Arvela P, Jouppila R, Kauppila A, et al. Eur J Clin Pharmacol 24: 345, 1983.
14. Robuschi G, d'Amato L, Salvi M, et al. J Endocrinol Invest 7: 521, 1984.
15. Ylikorkala O, Kivinen S, Ronnberg L, et al. Clin Endocrinol (Oxf) 13: 253, 1980.
16. Fisher DA, Dussault JG, Sack J, et al. Recent Prog Horm Res 33: 59, 1977.
17. El-Zaheri MM, Braverman LE, and Vagenakis AG. Endocrinology 106: 1735, 1980.

CHOLINERGIC AGENTS AND INHIBITORS OF EXTRACELLULAR CALCIUM INFLUX SUPPRESS THE SECRETION OF TSH BY HUMAN THYROTROPIC ADENOMA CELLS MAINTAINED IN LONG-TERM CULTURE

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Recent reports from various centers have described effects of muscarinic agonists and blockers on anterior pituitary secretion in both animals and humans (1). Studies of intact organisms are limited, however, by the frequent significant stress such agents induce (2). Although important effects have been demonstrated for other pituitary hormones (3,4), reports of cholinergic effects on TSH secretion are scant (1). Thus, our laboratory has conducted ongoing studies of cholinergic effects on human TSH secretion in vitro in monolayer culture of cells obtained from TSH-secreting pituitary adenomas (5). In this paper, we report that incubation with acetylcholine inhibited basal and TRH-stimulated TSH secretion; only the latter effects were blocked by addition of atropine.

There has also been increasing evidence that free calcium, both extracellular and cytoplasmic, may be important in the physiology of TSH secretion especially that stimulated by TRH (6). To examine this issue, we studied the effects of inhibiting free calcium influx into these human thyrotropes. Verapamil produced a nonsignificant suppression of basal TSH. However, doses of EGTA, previously shown to complex virtually all free calcium (7), suppressed basal TSH but did not inhibit TRH-stimulated secretion.

METHODS

Fresh tissue was received at operation upon a 29 year old woman with TSH-induced hyperthyroidism. The presence of a pituitary adenoma was demonstrated by CT and MRI scans. Preoperative TSH was significantly elevated (20-30 μU/ml) and increased markedly after TRH (often to over 150 μU/ml), decreased into the normal range after dexamethasone (2 mg every 6 hr for 1 day) and minimally decreased after bromocriptine (20-30 mg for 6 months). No preoperative studies with cholinergic agents were performed. Adenoma tissue was immediately brought to the laboratory, washed in Ca^{++} and Mg^{++} free Hank's Balanced Salt Solution (HBSS), and minced to 1 mm pieces in a 0°C solution of 0.5 mg/ml trypsin (Worthington), 0.03 mg/ml collagenase (Worthington), 0.01 mg/ml DNAase (Sigma), 20 mM HEPES, and 2% chick serum

*The opinions expressed herein are the private views of the authors and are not to be construed to be official or reflecting the views of the Department of the Army or the Department of Defense.

(Gibco) in HBSS (8). This was then incubated 90 min at 37°C. The cells were then centrifuged and the solution removed by decantation and replaced with Medium 199 (Whittaker MA Bioproducts) with 20% fetal bovine serum (Gibco), 25 mM HEPES, 2 mM glutamine, 0.1 mg/ml ascorbic acid and antibiotic-antimycotic mixture (Gibco). Cells were further separated by vigorous pipetting and then distributed into 2.5 ml microwells (Limbro) at 10^6 cells per well (Total: 22 microwells; initial viability 93%) and cultured at 37°C under humidified 5% CO_2. Within 24 hours, cells were in monolayer, where they remained viable but without evident proliferation throughout the ensuing 98 days of this study. Media were changed after 72 hours of culture and then approximately twice weekly in addition to experiments conducted weekly. Media were replaced by the same mixture but without fetal bovine serum on the evening before each experiment and at 0 hours. At 3, 6, and 9 hours, media were changed with fresh serum-free media also containing experimental agent(s). After overnight incubation, these media were removed and medium without agent(s) but with fetal bovine serum were restored. All media removed were immediately frozen at -70°C for later TSH radioimmunoassay using agents supplied by the National Institute of Health (USA). Secretory rates, calculated as μU TSH per microwell per hr, were expressed as the percentage of the secretory rate in each well during incubation without agent(s) and normalized to the secretory rates of control wells. Data were analyzed by nonpaired Student's t test.

Table 1. Effects on TSH Secretion by Adenoma Cells in Monolayer Culture

Agent	% (± SEM) Stimulation
10^{-7}M TRH	112.3 ± 18.7*
10^{-5}M ACh, 10^{-5}M Physo	-27.6 ± 1.2*
10^{-5}M ACh, 10^{-5}M Physo, 10^{-6}M Atropine	-26.0 ± 8.0
10^{-5}M ACh, 10^{-5}M Physo, 10^{-9}M TRH	-28.1 ± 1.1*
10^{-5}M ACh, 10^{-5}M Physo, 10^{-7}M TRH	21.3 ± 13.6
10^{-5}M ACh, 10^{-5}M Physo, 10^{-7}M TRH, 10^{-6}M Atropine	83.1 ± 38.6*
10^{-8}M Somatostatin	66.1 ± 20.6
10^{-7}M Dopamine	19.1 ± 16.9
10^{-7}M Dopamine, 10^{-7}M TRH	31.1 ± 26.0
10^{-8}M Somatostatin, 10^{-7}M TRH	1.3 ± 19.2
10^{-4}M Verapamil	-75.2 ± 26.3
10^{-6}M LHRH	19.3 ± 9.3
$4x10^{-4}$M EGTA	-22.7 ± 14.4
$4x10^{-3}$M EGTA	-78.7 ± 24.9*
$4x10^{-4}$M EGTA, 10^{-7}M TRH	107.0 ± 29.4*
$4x10^{-3}$M EGTA, 10^{-7}M TRH	104.4 ± 38.2*

*$p<0.05$ versus control. Data are expressed as % stimulation vs baseline period and normalized to simultaneous control wells. Negative values: inhibition vs baseline; ACh: acetylcholine; Physo: physostigmine.

RESULTS

Initial TSH secretion was massive (10,600 ± 295 μU/well/day) and, although it declined, it persisted for 98 days. Sephadex G-100 gel chromatography of spent media collected after 72 hours of culture revealed a dominant peak that co-migrated with radiolabeled human standard TSH and small peak of apparent mol wt 62,000 (not shown). Although neither dopamine nor somatostatin suppressed basal TSH secretion, both agents blunted TRH-stimulated TSH secretion (Table 1). Unlike a previous report (9), LHRH had no effect on these thyrotopes. Both verapamil and the higher concentration of EGTA suppressed basal TSH, but EGTA did not blunt TRH-stimulated TSH. Acetylcholine (with physostigmine added to block cellular acetylcholinesterases) suppressed basal TSH secretion and blunted TRH-stimulated TSH secretion. When atropine was added to the incubation medium, there was a block of the suppressive effect of acetylcholine on TRH-stimulated TSH secretion, but no alteration of the effect on basal TSH secretion (Table 1).

DISCUSSION

This report, taken together with our laboratory's other recent studies (5), suggests that cholinergic mechanisms may play a role in modifying TSH secretion *in vitro*. In our previous report, acetylcholine suppressed basal TSH secretion in similar monolayer cultures of cells from a TSH-secreting pituitary adenoma that was not responsive to TRH. Atropine blocked these effects. However, we cannot fully explain why atropine blocked the acetylcholine effect on basal secretion in that TRH-unresponsive adenoma and, in the studies reported herein, blocked TRH-stimulated but not basal TSH secretion. Perhaps muscarinic mechanisms are chiefly modulators of the effects of other releasing substances and thus might have more evident effects in systems where TRH is active.

Although dopamine and somatostatin, in the doses we employed, failed to suppress basal TSH (similar to the *in vivo* results in this patient), we could demonstrate their well-known effects in suppressing TRH-stimulated TSH secretion. Our finding that incubation of thyrotropes with EGTA had no effect on TRH-stimulated TSH supports the notion that mobilization of intracellular calcium stores may be more important than calcium influx in mediating TSH release (6). Had we preincubated the cells with EGTA (to eventually deplete intracellular calcium), we might have observed a diminution of TSH secretion by TRH. Nevertheless, our findings of decreased basal secretion during incubation with agents that inhibit extracellular calcium influx supports other investigations (10) suggesting that this may also be an important physiological mechanism affecting TSH secretion.

REFERENCES

1. Delitala G. In GM Besser and L Martini (eds), Clinical Neuroendocrinology, Vol. 2, Academic Press, New York, 1982, p 67.
2. Lewis D, Sherman B, and Kathol R. J Clin Endocrinol Metab 58: 570, 1984.
3. Delitala G, Frulio T, Pacifico A, et al. J Clin Endocrinol Metab 55: 1231, 1982.
4. Rudnick M and Dannies P. Biochem Biophys Res Comm 101: 689, 1981.
5. Fein H, Richmond I, and Smallridge R. In Abstracts - 7th International Congress of Endocrinology, Excerpta Medica, Amsterdam, 1984, p 668.
6. Geras E and Gershengorn M. Am J Physiol 242: E109, 1982.
7. Gershengorn M, Hoffstein S, and Rebecchi M. J Clin Invest 67: 1769, 1981.
8. Baird C, Tharandt L, and Tamarkin L. Endocrinology 114: 1041, 1984.

9. Lamberts S, Oosterom R, Verleun T, et al. J Endocrinol Invest 7: 313, 1984.
10. Martin T and Kowalchyk J. Endocrinology 115: 1527, 1984.

COMPARISON OF A PITUITARY TSH-SECRETING MICRO- VERSUS MACROADENOMA

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While more than 40 cases of TSH-secreting pituitary adenomas have been reported in the medical literature (1,2), little attention has been devoted to structural variability--in particular that due to carbohydrate microheterogeneity--of thyrotropin molecules derived from different tumors and distinct physiological states. We recently evaluated two patients representing extremes within the spectrum of TSH-secreting pituitary adenomas and have contrasted several biochemical features of these tumors.

Patient A, a 23 year old female, presented with clinical hyperthyroidism and elevated serum TSH and α subunit levels. Imaging studies, including computer tomographic (CT) and nuclear magnetic resonance, showed no clear abnormality in the pituitary fossa. Venous sampling studies, however, showed step-up of TSH and α subunit in the left petrosal sinus. At surgery, a 0.8 cm x 0.8 cm left-sided pituitary microadenoma was resected. At one year follow-up, the patient is clinically cured with basal TSH in the normal range and undetectable α subunit.

Patient B, a 54 year old male, presented with clinical hyperthyroidism and elevated TSH and α subunit levels. CT scan of the pituitary fossa showed bony destruction by a large pituitary tumor invading the sphenoid sinus. At surgery, a 3 cm x 5 cm macroadenoma was partially resected. Postoperatively 5,200 rads of x-irradiation were delivered to the sella turcica; however, the patient required surgical debulking nine months later because of left eye blindness from tumor regrowth. At 18 month follow-up, he has radiologic evidence of persistent tumor; serum TSH is nonsuppressible at 20-30 μU/ml and α subunit continues to be elevated.

Pituitary tissue obtained from these two cases was freshly minced and incubated in Delbecco's Modified Eagles Medium with [^{35}S]methionine and either [^{3}H]glucosamine or [^{3}H]mannose at 37°C in an atmosphere of 95% O_2-5% CO_2 for 18 hours. Medium from this incubation was then sequentially immunoprecipitated with anti-hTSH-β to precipitate TSH dimers, followed by anti-hLH-β to remove LH subunits, and finally by anti-hTSH-α to precipitate

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remaining free α subunits, by modifiation of methods described previously (3). A portion of the immnoprecipitated TSH dimers and free α subunits was analyzed for subunit size on SDS-PAGE, while the remaining material was degraded by Pronase (Calbiochem) to generate TSH and free α glycopeptides. Free radioactivity was separated from glycopeptides by passage of the mixture over a Sephadex G-25 (Pharmacia) column, and the purified glycopeptides were then chromatographed on concanavalin A (con A)-agarose (Pharmacia) by the method of Cummings and Kornfeld (4). Asparagine-linked glycopeptides

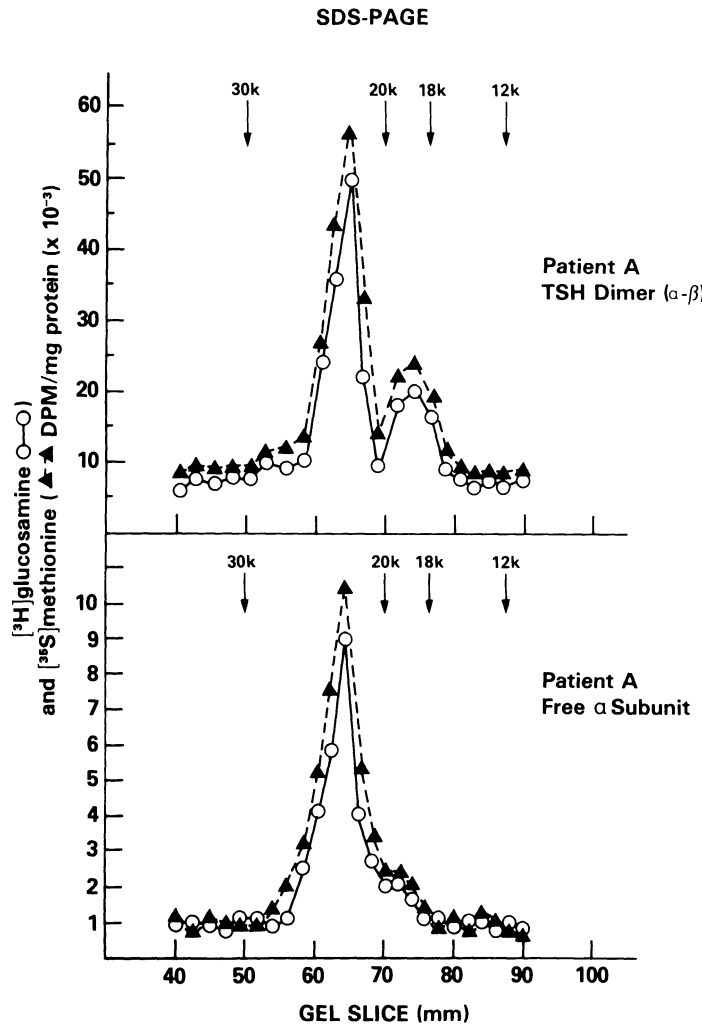

Fig. 1. Electrophoretic pattern of TSH and free in patient A. TSH and free α subunit were precipitated from medium incubated with tumor minces from patient A and analyzed by SDS-PAGE. The apparent molecular weights (MW) were 22,000 for TSH-α (top panel, left peak), 18,000 for TSH-β (top panel, right peak), and 22,500 for free α subunit (lower panel). All three subunits incorporated [^{3}H]glucosamine and [^{35}S]methionine. Arrows show position of MW markers.

that fail to bind to con A represent triantennary, tetraantennary, and bisecting complex forms; those that bind weakly and elute with 10 mM α-methylglucoside (α-MG) represent biantennary complex structures; those that bind strongly and require elution by 500 mM α-methylmannoside (α-MM) represent high mannose and hybrid structures. This study is the first in which newly synthesized, secreted human TSH has been proteolytically degraded into glycopeptides and analyzed by lectin chromatography methods, which permit more specific inferences of glycoprotein carbohydrate structure.

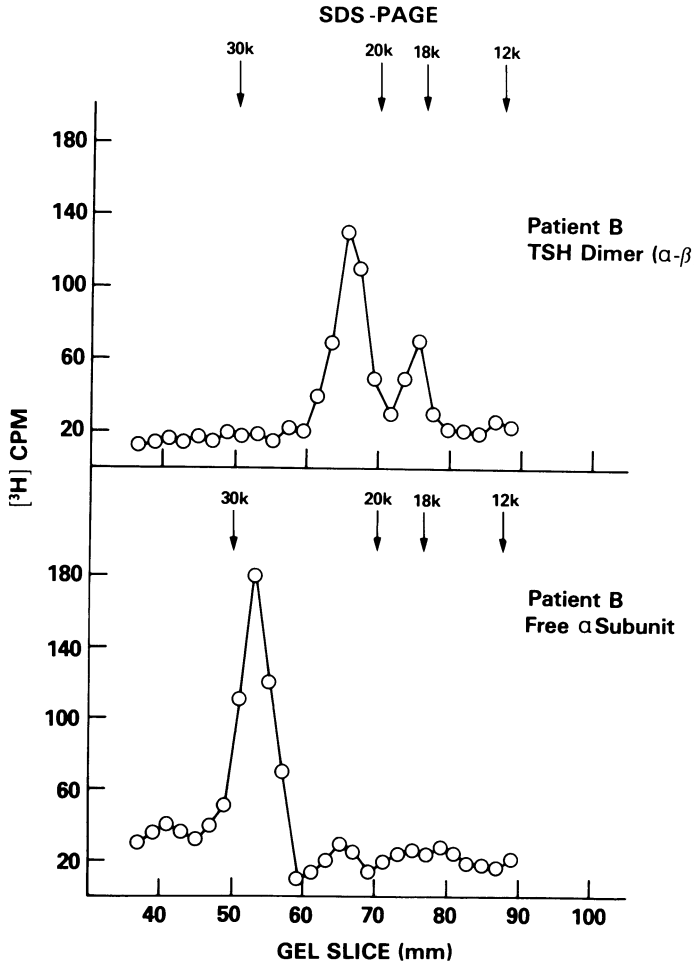

Fig. 2. Electrophoretic pattern of TSH and free α in patient B. TSH and free α subunit were precipitated from medium incubated with tumor slices from patient B and analyzed by SDS-PAGE. [^{3}H]glucosamine was the radionuclide label used in the incubation medium. TSH-α and TSH-β had apparent MW of 22,000 and 18,000, respectively (top panel). Free α subunit had a MW of 29,000 (lower panel), which is much larger than conventionally seen.

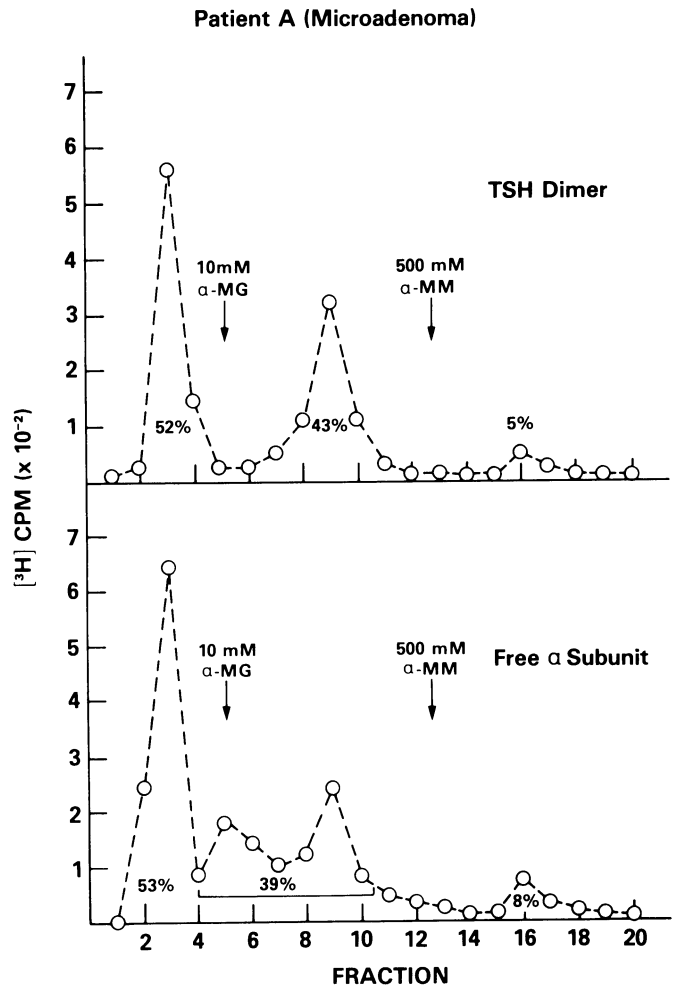

Fig. 3. Concanavalin A chromatography on patient A. [^{3}H]mannose-labeled glycopeptides were prepared as discussed in the text and chromatographed on con A-agarose. The arrows designate time points when eluting sugars α-methylglucoside (α-MG) and α-methylmannoside (α-MM) were applied to the columns. The first peak (from the left) represents glycopeptides that do not bind to con A; the second peak, glycopeptides that bind weakly to con A; and the third peak, glycopeptides that bind strongly to con A. In the free α subunit (lower panel), there is an additional class of glycopeptides that bind in a "retarded" fashion (second peak from left). Total [^{3}H] recovery from the con A columns was in excess of 85%.

As shown in Fig. 1 (top panel), in patient A, newly synthesized, secreted TSH-α had an apparent MW=22,000 and TSH-β an apparent MW=18,000, consistent with previous studies (5). Free α subunit (lower panel) had an apparent MW=22,500 and a broader base, suggesting greater molecular weight heterogeneity within the free α subunit. All three subunits showed comparable incorporation of [^{3}H]glucosamine relative the apoprotein label, [^{35}S]methionine.

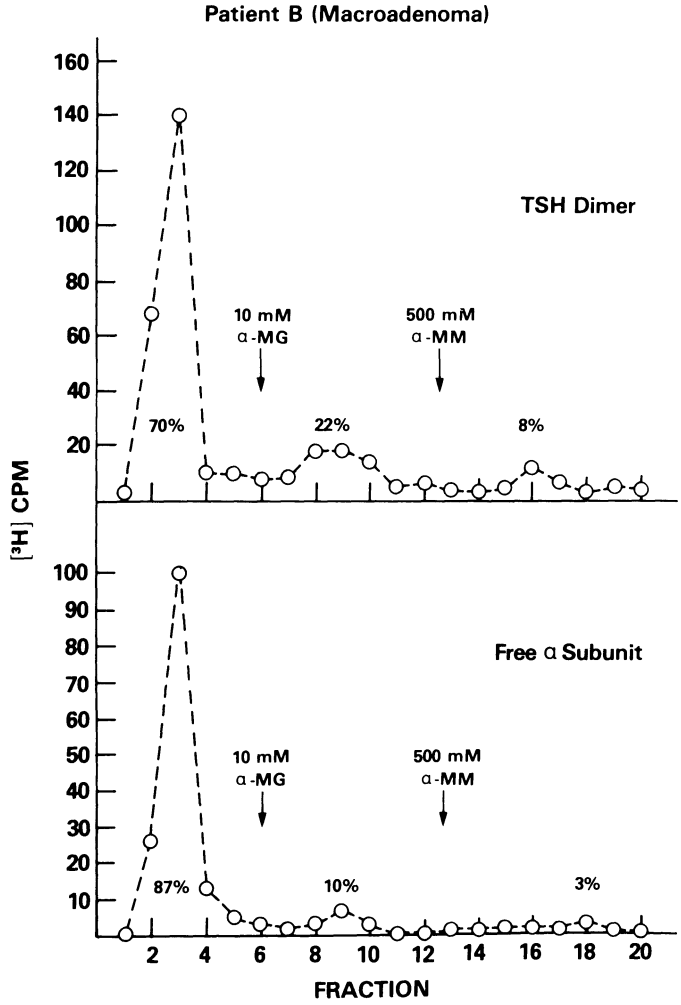

Fig. 4. Concanavalin A chromatography on patient B. [^{3}H]glucosamine-labeled glycopeptides were prepared as discussed in the text and chromatographed on con A-agarose. The arrows designate time points when eluting sugars α-methylglucoside (α-MG) and α-methylmannoside (α-MM) were applied to the columns. In the case of TSH dimer, only 30% of glycopeptides bind to con A (top panel), while for free α subunit, only 13% of glycopeptides bind to con A (bottom panel). Total [^{3}H] recovery from the con A columns was in excess of 85%.

Table 1. Biochemical Comparisons: TSH-secreting Micro- Versus Macroadenoma

	Patient A	Patient B
Diagnosis	Microadenoma	Macroadenoma
In vitro secretory response to TRH	Present	Absent
Apparent MW on SDS-PAGE of radiolabeled TSH	TSH-α 22,000 TSH-β 18,000 Free α 22,500	TSH-α 22,000 TSH-β 18,000 Free α 29,000
Percentage of [^{3}H] CHO-labeled glycopeptides binding to con A	48% TSH dimer 47% Free α subunit	30% TSH dimer 13% Free α subunit

In patient B, newly synthesized, secreted TSH-α and TSH-β had similar molecular weights as in patient A (Fig 2, top). However, free subunit had a much larger apparent molecular weight of 29,000 (Fig. 2, bottom). A second, smaller peak of MW=22,000 (Fig. 2, bottom) may represent the more conventional form of the free α subunit.

In order to investigate differences in carbohydrate structure in TSH and free α subunits secreted by these two tumors, newly synthesized glycopeptides were chromatographed on con A. In patient A, 52% of [^{3}H]mannose-labeled glycopeptides from TSH dimer failed to bind to con A, 43% bound weakly and were eluted by 10 mM α-MG, and 5% bound strongly and were eluted to 500 mM α-MM (Fig. 3, top). For free α subunit, 53% of [^{3}H]mannose-labeled glycopeptides failed to bind to con A, 30% bound weakly and were eluted by 10 mM α-MG, and 8% bound strongly and required elution with 500 mM α-MM (Fig. 3, bottom). Within the weakly bound glycopeptides there was seen a novel "retarded" peak (second peak from the left, Fig. 3, bottom); these glycopeptides, which are present on free α but not on combined subunits in this tumor, may represent a special class of N-linked biantennary complex structures that have decreased steric availability of core mannose residues (6).

In contrast to patient A, in patient B 70% of newly synthesized, secreted [^{3}H]glucosamine-labeled glycopeptides from TSH dimer failed to bind to con A, 22% bound weakly, and 8% bound strongly (Fig. 4, top). Free α subunit showed even less glycopeptide binding to con A; 87% of [^{3}H]glucosamine-labeled glycopeptides failed to bind, 10% bound weakly, and 3% bound strongly (Fig. 4, bottom). We have recently performed con A chromatography on TSH and free α glycopeptides labeled with either [^{3}H]mannose or [^{3}H]-glucosamine and have shown comparable binding patterns for both carbohydrate labels within a particular tumor. The dramatic changes in con A binding described here, therefore, are due to differences in carbohydrate structure of TSH and α subunit secreted by these two tumors.

In summary, these two tumors had dramatically different clinical behavior and also demonstrated distinctive biochemical features in vitro (Table 1). The macroadenoma secreted newly synthesized free α subunit that had an abnormally large molecular weight on SDS-PAGE and, when analyzed by glycopeptides, demonstrated little ability to bind to con A. Similarly, newly synthesized, secreted TSH dimer glycopeptides from the macroadenoma

had diminished con A binding (30%) when compared to those from the microadenoma (48%). Although not shown here, the microadenoma also had a brisk secretory response to TRH in vitro, which was blunted in the macroadenoma.

In conclusion, TSH and α subunit secreted by human pituitary adenomas demonstrate heterogeneity in carbohydrate structure. In this comparison, the more aggressive adenoma secreted TSH and α subunits with a larger proportion of bisecting and multiantennary complex oligosaccharide units. This observation supports the hypothesis that carbohydrate structure may predict the clinical behaviour of TSH adenomas and may modulate the bioactivity of the TSH molecule (7).

REFERENCES

1. Weintraub BD, Gershengorn MC, Kourides IA, et al. Ann Intern Med 95: 339, 1981.
2. Smallridge RC and Smith CE. Arch Intern Med 143 (3): 503, 1983.
3. Taylor T and Weintraub BD. Endocrinology 116: 1968, 1985.
4. Cummings RD and Kornfeld S. J Biol Chem 257: 11235, 1982.
5. Weintraub BD, Stannard BS, and Meyers L. Endocrinology 112: 1331, 1983.
6. Narasimhan S, Wilson JR, Martin E, et al. Can J Biochem 57: 83, 1979.
7. Joshi LR and Weintraub BD. Endocrinology 113: 2145, 1983.

DEFECTIVE TSH SUPPRESSION IN A CASE OF NON-NEOPLASTIC INAPPROPRIATE TSH SECRETION (ITSHS)

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The non-neoplastic inappropriate secretion of thyroid stimulating hormone (ITSHS) classification (1) includes also defective suppression of TSH secretion by somatostatin, dopamine, and other suppressors. These abnormalities have been postulated but not yet, at least contemporarily, documented in any reported case. The present study demonstrates the defective suppression of TSH secretion by dopamine, dopamine agonists, somatostatin, and corticosteroids, and also reports the measurement of plasma immunoreactive endogenous thyrotropin-releasing hormone (TRH) in a patient with ITSHS and thyroid hormone resistance (2).

MATERIAL AND METHODS

Case Report

A non-neoplastic ITSHS was diagnosed in a 44 year old man who had been previously hemithyroidectomized because of a cyst in the right lobe of the thyroid. The patient (162 cm, 64 kg) was judged clinically euthyroid on the basis of the absence of clinical and metabolic features of hypothyroidism, and on the normal serum total thyroxine (T_4) and marginally increased serum triiodothyronine (T_3) associated with increased serum TSH values (Table 1). Serum T_4-binding globulin and prealbumin were normal and anti-thyroid antibodies were absent. The x-ray enlarged sella turcica was revealed as an empty sella by means of computed tomography. Basal and dynamic (insulin tolerance test, TRH, LHRH) pituitary function (GH, PRL, FSH, LH) assessment gave normal results, as well as the evaluation of serum cortisol circadian rhythm. Serial estimation of liver enzymes, lipids, and T_4-binding globulin showed no significant variation after 2 or 3 week suprapharmacological thyroid hormone regimens (L-T_4 up to 800 µg/day; L-T_3 up to 120 µg/day). The administration of thyroid hormones induced poor increase in red blood cell glucose-6-phosphate dehydrogenase, in glucagon-stimulated cAMP, and in urinary hydroxyproline, giving evidence of a partial target resistance to thyroid hormone.

Assay Methods

Hormones were measured by commercial kits purchased from Biodata (Milan, Italy). The intra- and interassay coefficient of variation (CV)

Table 1. Selected Baseline Function Tests

Date	T4 (μg/dl)	T3 (ng/dl)	FT4 (pg/ml)	FT3 (pg/ml)	TSH (μU/ml)
12/3/81*	9.8	240	---	---	15.0
4/16/82*	10.8	270	---	---	25.0
6/10/82	6.2	263	---	---	33.0
9/15/82	6.5	214	7.6	4.5	30.0 (43)[a]
10/16/82	6.0	247	8.0	4.2	19.0
1/13/83	7.1	246	9.1	5.1	6.6
12/12/83	11.3	249	14.1	5.6	7.3 (24.4)[a]
1/9/84	9.5	195	---	---	7.1 (23.6)[a]
5/10/84	10.0	189	---	---	4.6 (14.7)[a]
12/14/84	12.9	212	15.3	6.0	15.3

*Assays performed in another laboratory. Right lobectomy was performed in March 1982. a: The TSH response after TRH is reported in brackets.

are 3.1 and 6.1 (T_4), 3.6 and 6.8 (T_3), 2.5 and 5.5% (TSH). Free thyroid hormones (FT_4 and FT_3) were measured by the Sclavo (Milan) kits based on column adsorption chromatography and RIA (CV for FT_4, 5.0 and 7.0; for FT_3, 4.9 and 6.5%). Serum alpha-TSH and TSH-beta were measured in Professor Giovanni Faglia's laboratory at Milan University according to his homemade method (3). Circulating endogenous TRH was measured according to the RIA method previously validated and published by one of us (4). Normal values for TRH are 31.9 $\pm$ 11 pg/ml (x $\pm$ SD); hypothyroid and hyperthyroid average values are 22.1 $\pm$ 16 and 49.1 $\pm$ 14 pg/ml, respectively.

RESULTS

Table 1 illustrates the spontaneous changes in serum total and free thyroid hormone and TSH levels during the entire observation period. Serum TSH ranged from normal to definitely high levels, and its subunits were repeatedly normal (alpha-TSH = 0.36 to 0.69 ng/ml, TSH-beta = 0.5 ng/ml). Identity of patient's TSH with the MRC 6838 TSH standard was proved by the parallel dilution curves of radioactive TSH displacement. Patient's TSH was biologically active as serum T_3 and FT_3 increased significantly following endogenous TSH surge after iv TRH. Serum TSH response to TRH ranged from normal to exaggerated (Table 1) when the patient was untreated. A further ther increase in TSH response to TRH was achieved after serum T_4 decrease induced by methimazole treatment. A similar normal stimulatory effect was exerted by dopamine agonists (domperidone and sulpiride).

The TSH response to suppressive agents was abnormal. In fact, full TSH suppression was never achieved, even in the presence of extremely increased FT_4 (73.9 pg/ml) and FT_3 (24 pg/ml) levels induced by the chronic administration of L-T_4 (800 μg) or L-T_3 (120 μg/day). Bromocriptine (0.5 to 5 mg/day, orally administered for three weeks) provoked a clear suppression of PRL release along with an unexpected enhancement in basal and TRH- (200 μg/iv) stimulated serum TSH. Another paradoxical increase in both TSH and PRL was observed after nomifensine (100 mg/day for 30 days). Also, dopamine iv

Table 2. Paradoxical TSH Response to Suppressive Agents Prior To and After 200 µg IV TRH (Δ TSH)

Agent	TSH (µU/ml)	Δ TSH (µU/ml)
Bromocriptine (0.5-5 µg/day/3 weeks)	9.9[a]	43.9
Nomifensine (100 mg/day/30 days)	8.0[a]	61.0
Dopamine (400 µg/kg/min/4 hrs)	14.3 - 22.0[c]	--
Somatostatin (800 µg/150 min)	7.9[b]	11.5
Dexamethasone (3 mg/day/3 weeks)	31.0[a]	43.9

TRH test was performed: a) on the last day of treatment, b) after 30 min infusion, or c) TSH was measured during infusion without TRH stimulation.

infusion (4 µg/kg/min) failed to suppress TSH secretion, as well as dexamethasone (3 mg/day for three weeks) and somatostatin (800 µg/250 min iv). The paradoxical responses are scheduled in Table 2.

Plasma TRH values fluctuated between 24 and 26 pg/ml prior to any treatment and between 22 and 26 during and after any of the drugs administered.

DISCUSSION

The clinical and biochemical characterization of this patient with empty sella and partial target organ resistance to thyroid hormones is discussed in detail in another extensive study (5). Strong arguments excluded the presence of a pituitary adenoma as the absence of thyroid hyperfunction, the normal alpha-TSH to TSH-beta ratio, and the normal responsiveness of TSH to appropriate stimuli.

In this patient, we also report normal plasma TRH values irrespective of the biochemical thyroid status or of the different substances administered. No immediate explanation for these results is available at the moment.

The inhibitory dopaminergic control on TSH secretion has been reported both in euthyroid (6) and hypothyroid patients (7). In our case, dopamine and its agonists failed to inhibit TSH secretion which was instead stimulated. Also, somatostatin, which is well known to inhibit TSH nocturnal peak and its response to TRH (8-10), failed to exert its suppressive effect. Other instances of unexpected effects provoked by different drugs have been described: serum TSH rose during T_3 (11) or T_4 (12). Also, partial or total ineffectiveness of bromocriptine in diminishing TSH levels has been reported (13) even very recently (14).

The present patient is the first in whom more of one paradoxical response was observed. We suggest, therefore, that this patient enters the classes II C 1-3 of the Weintraub et al. classification, in that he shows contemporarily defective TSH suppression by somatostatin, dopamine, and glucocorticoids.

REFERENCES

1. Weintraub B, Gershengorn MC, Kourides IA, et al. Ann Int Med 95: 339, 1981.
2. Refetoff S. Am J Physiol 243: E88, 1982.
3. Faglia G, Beck Peccoz P, Ballabio M, et al. J Clin Endocrinol Metab 56: 908, 1983.
4. Lombardi G, Panza N, Cei S, et al. Acta Endocrinol 87: 70, 1978.
5. Benvenga S, Sobbrio GA, Vermiglio F, et al. J Clin Endocrinol Metab, submitted for publication.
6. Leebaw WF, Lee LA, and Woolf PD. J Clin Endocrinol Metab 47: 480, 1978.
7. Feek CM, Sawers JSA, Brown NS, et al. J Clin Endocrinol Metab 51: 585, 1980.
8. Reichlin S. N Engl J Med 309: 1495, 1983.
9. Deemester-Mirkine N and Dumont JE. In M de Visscher (ed), The Thyroid Gland, Raven Press, New York, 1980, p 145.
10. Weeke J and Laurberg PJ. Clin Endocrinol Metab 43: 32, 1976.
11. Refetoff S, DeGroot LJ, and Barasano CP. J Clin Endocrinol Metab 51: 41, 1980.
12. Brooks MH, Barbato AT, Collins S, et al. Am J Med 71: 444, 1981.
13. Connel JMC, McCruden DC, Davies DL, et al. Ann Int Med 96: 251, 1982.
14. Sriwanatkul K, McCornick K, and Woolf PD. J Clin Endocrinol Metab 58: 225, 1984.

THYROTROPIN SECRETION IN THALASSEMIC PATIENTS DURING PUBERTY AND POSTPUBERTY

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INTRODUCTION

Hypogonadotropic hypogonadism attributed to hemosiderosis is frequent in patients with beta-thalassemia major (1,2). Data concerning the other pituitary and peripheral endocrine hormones are controversial. In a previous study (3), we found low thyroid hormone concentrations and/or increased serum TSH levels in a high percentage of clinically euthyroid patients. This situation was found in early childhood and did not change with increasing age. Compensated hypothyroidism with high or normal TSH levels which were hyperresponsive to TRH was described by several authors (4-6) in thalassemic patients, whereas others report normal indices of thyroid function (1,7).

These discrepancies may be due to differences in the selection of patients in regard to age, sex, puberty stages, and transfusion regimens. In order to evaluate whether pituitary hemosiderosis affects TSH secretion or whether primary thyroid function is involved, we studied TSH secretion and thyroid hormone concentrations in a group of patients in pubertal or postpubertal age treated with the same transfusion and chelating regimen. The data were evaluated separately during the different stages of puberty and in different age groups.

PATIENTS AND METHODS

Fifty patients, 31 females and 19 males, aged 13-23 with beta-thalassemia major participated in this study. Diagnoses were based on Hb electrophoresis and on hematological data. All had been transfused since early childhood on a monthly regimen to maintain the pretransfusional Hb levels at about 8 g % until 1982 and thereafter at a weekly regimen to maintain the pretransfusional Hb levels at about 10 g %. All were chronically treated with desferrioxamine, 40 mg/kg. Nineteen female patients were in puberty stage 1, six in stage 2, three in stage 3, and three in stage 4-5. Thirteen males were in stage 1, none in stage 2, and six in stage 3-4. Blood was drawn in the morning through an intravenous cannula kept patent by a slow infusion of saline. After 30 minutes, blood was taken for TSH, T_3, and T_4 determinations; TRH, 200 μg IV, was then given intravenously

and blood was collected for TSH after 30 and 60 minutes. Sera were stored at -20° until tested. TSH was measured by a double antibody RIA purchased from Biodata, Milan (inter- and intraassay variation coefficients: 7% and 3%, respectively). T_3 and T_4 were measured by a RIA from Biodata. All tests were performed at least two weeks after the last transfusion. The same measurements were carried out in a group of 22 age and sex matched healthy control subjects. Informed consent was obtained from the patients or their parents. Student's paired and unpaired t test and analysis of variance were performed to evaluate the data.

RESULTS

Mean baseline serum concentrations of TSH and its peak values after TRH administration are reported in Table 1. They were significantly higher in patients than in the group of sex and age matched control subjects. TSH peaked at 30 minutes in 44 patients and at 60 minutes in 6. The increase after TRH was blunted in only one patient. Basal and peak TSH levels were not different in male and female patients, whereas there was a significant difference between males and females in the normal subjects ($p<0.01$). TSH, both basal and TRH stimulated, did not differ when patients in different stages of puberty were compared (Table 2). Considering both basal and peak TSH values in different age groups separately, they were not different in males aged 14-16, 16-18, 18-23, and in females aged 14-16 and 16-18; a difference was found only between the youngest female group of 12-14 (basal TSH: 2.8 $\pm$ 1.7; peak TSH: 22.0 $\pm$ 11.2) and the oldest group of 18-21 years (6.3 $\pm$ 2.1 and 39.0 $\pm$ 10.8; $p<0.02$). No signifiant correlation could be found between TSH values, basal and stimulated, and the LH response to LHRH, total number of blood units transfused, or ferritin levels.

Serum levels of T_3 in the patients (1.5 $\pm$ 0.2 ng/ml, range 0.8-2.1) were not higher than in control subjects (1.4 $\pm$ 0.2, range 1.1-1.9); T_4 levels were within normal limits in all patients but the mean level was slightly lower (8.0 $\pm$ 1.5 µg/dl; range 4.3-10.0) than in the controls (9.5 $\pm$ 2.0, range 6.2-12.5, $p<0.05$).

DISCUSSION

These data indicate that in thalassemic patients TSH secretion is not affected by pituitary hemosiderosis. On the contrary, TSH secretion was increased in a high percentage of patients. Though serum levels of thyroid hormones were within the normal range in all patients, the mean value of serum T_4 was slightly, but significantly lower than in control subjects. Therefore, thalassemic patients frequently present a condition of "compensated" primary hypothyroidism. In a previous study carried out in prepubertal patients (3), we found that thyroid function indices were more severely impaired than in the present group. This difference may possibly be attributed to the severer condition of chronic anemic hypoxia in the former group of patients in whom the Hb levels were significantly lower, since they were transfused less frequently. It has been shown that hypoxia may potentiate the toxic action exerted by iron depositions in thyroid tissue on enzyme systems (8,9).

The lack of any difference between TSH levels in male and female patients which is present in normal subjects may be attributed to the impairment of gonadal function.

Longitudinal studies will be required to demonstrate whether TSH secretion tends to decrease with time and progressive iron overload.

Table 1. TSH Secretion in Male and Female Thalassemic Patients

	Patients			Controls		
	Whole group (50)	Males (19)	Females (31)	Whole group (22)	Males (9)	Females (13)
Basal TSH	3.9 ± 1.9[a]	3.9 ± 1.7[a]	3.9 ± 2.1[a]	2.4 ± 1.0	2.3 ± 0.2	2.5 ± 1.2
Peak TSH	29.5 ± 14.8[b]	28.2 ± 11.5[b]	30.0 ± 16.0[b]	14.2 ± 1.2	9.4 ± 2.8[c]	19.6 ± 7.1

[a]$p<0.05$ vs controls; [b]$p<0.01$ vs controls; [c]$p<0.01$ vs males. Basal serum TSH concentrations and peak values after TRH (μU/ml; Mean ± SD) in a group of 50 thalassemic patients and in 22 sex and age matched controls.

Table 2. TSH Secretion in Different Stages of Puberty

Puberty stage	Males		Females		
	1 (n=13)	3-4 (n=6)	1 (n=19)	2-3 (n=9)	4-5 (n=3)
Basal TSH	4.2 ± 1.5	3.7 ± 1.5	4.2 ± 2.3	3.2 ± 1.9	3.5 ± 1.4
Peak TSH	25.8 ± 8.9	32.5 ± 15.3	32.4 ± 17.8	25.4 ± 10.1	35.6 ± 22.1

Basal serum TSH concentrations (μU/ml, M ± SD) and peak values after TRH administration in 50 thalassemic patients in different stages of puberty. Differences were not significant.

REFERENCES

1. Landau H, Spitz IM, Cividalli G, et al. Clin Endocrinol 9: 163, 1978.
2. Keltzky OA, Costin G, Marrs RP, et al. J Clin Endocrinol Metab 48: 901, 1979.
3. Madeddu G, Dore A, Marongiu A, et al. Clin Endocrinol 8: 359, 1978.
4. Cavallo L, Licci D, Acquafredda A, et al. Acta Endocrinol 107: 49, 1984.
5. Flynn MD, Fairey A, Jackson A, et al. Arch Dis Child 51: 828, 1976.
6. Spitz IM, Hirsch HJ, Landau H, et al. J Endocrinol Invest 7: 495, 1980.
7. De Luca F, Melluso R, Sobbrio G, et al. Arch Dis Child 55: 389, 1980.
8. Kuo B, Zaino OE, and Roginsky M. J Clin Endocrinol 28: 805, 1968.
9. Necheles TF, Chung S, Sabbah R, et al. Ann NY Acad Sci 232: 179, 1974.

THYROID FUNCTION AND PROLACTIN SECRETION IN WOMEN WITH MENSTRUAL DISORDERS AND/OR INFERTILITY

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SUMMARY

Secretion patterns of TSH and prolactin (PRL) after stimulation tests with TRH and metoclopramide (MCP), respectively, were investigated in 313 women with infertility and/or menstrual disorders. Stimulated TSH and PRL serum levels, but not their basal levels, revealed a statistically significant correlation ($p<0.05$), also indicating an interdependence between hyperprolactinemia and hypothyroidism ($p<0.05$). A PRL stimulation test with MCP appears to reflect patho- and physiological conditions of these patients more accurately than does that with TRH.

INTRODUCTION

It is well established that the secretion of TSH and PRL from the anterior pituitary is controlled by hypothalamic factors. Since TRH has been shown to stimulate both TSH and PRL secretion, a PRL stimulation test with TRH has been employed for a number of studies (1,2).

However, the control mechanisms of TRH and PRL secretion are not identical, and a dissociation of secretion patterns of these hormones can be easily elicited by administration of particular drugs (3).

In this study, we performed both TSH and PRL stimulation tests with TRH and MCP, respectively, on patients with infertility and/or menstrual disorders, and their responses to each stimulation test were analyzed.

PATIENTS AND METHODS

In 313 women, aged 16 to 40 years (average 29.7), TSH stimulation tests using 400 μg TRH (Henning, W. Germany) and PRL stimulation tests using 10 mg MCP (Kali-Chemie, W. Germany) were performed during the early follicular phase (4,5). Blood samples were taken before and 25 minutes after the TRH and MCP bolus injection. Serum TSH, T_4, and PRL were measured by radioimmunoassay (5).

Patients were classified according to the TSH secretion patterns and the T_4 levels as "normal", "latent hypo"-, or "hypo"-thyroid. Classification was based on observations made in conception cycles (6).

"Normal": basal TSH $\leq$ 3 μU/ml and peak TSH $\leq$ 18 μU/ml (n=238, 76%).

"Latent hypo": basal TSH $\leq$ 3 μU/ml and peak TSH > 18 μU/ml (n=71, 22.6%). All patients of this group had normal T_4 levels (> 50 ng/ml).

"Hypo": basal TSH > 3 μU/ml and the difference between basal TSH and peak TSH > 15 μU/ml. These patients had subnormal T_4 levels (n=4, 1.2%).

Patients were also classified in a similar way according to the PRL concentrations (1,6); normal upper limits of PRL of 15 ng/ml (basal) and 200 ng/ml (after MCP) were used for this classification.

All results were expressed as mean $\pm$ SEM. Linear regression analysis, the Pearson product moment coefficient of correlation, and the Chi square test were used for statistical analysis.

RESULTS

The mean basal and stimulated TSH levels in the patients studied were 1.6 $\pm$ 0.4 and 14.2 $\pm$ 0.5 μU/ml, respectively; a significant correlation between the two was observed (r=0.2987, p<0.001). A similar correlation (r=0.3109, p<0.001) was observed between basal and MCP-stimulated PRL levels (14.6 $\pm$ 0.8 and 214.9 $\pm$ 6.2 ng/ml, respectively). Stimulated TSH and PRL levels, but not their basal levels, were positively correlated (p<0.05) (Fig. 1).

Abnormal TSH secretion was seen in a total of 75 patients (23.9%). Most of them suffered from latent hypothyroidism (71 patients) and only 4 women (1.2%) had hypothyroidism. None of the patients was hyperthyroid. Hyperprolactinemia was observed in 183 women (58.4%); 103 patients (32.9%)

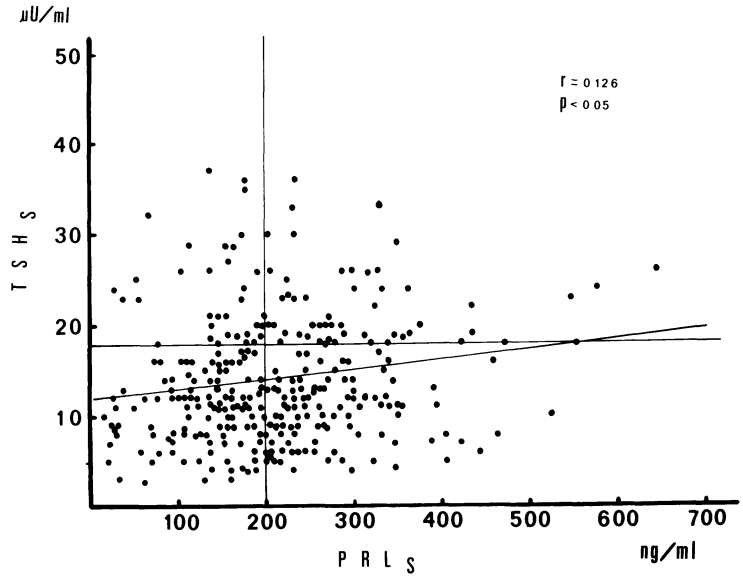

Fig. 1. Correlation between TSH concentrations after stimulation with TRH and PRL concentrations after stimulation with metoclopramide. The vertical and horizontal lines indicate the upper limit of normal levels of each hormone.

Table 1. Thyroid Function and PRL Secretion of the Subjects*

Thyroid function	Prolactin secretion			Total
	Normal	Latent hyper	Hyper	
Normal	110*	74	54	238
Latent hypo	19	28	24	71
Hypo	1	1	2	4
Total	130	103	80	313

$X^2 = 10.081$; $p < 0.05$. *Criteria of the classification of thyroid function and PRL status are mentioned in the text. **Number of patients.

had only an excessive PRL response after stimulation (latent hyperprolactinemia), and basal hyperprolactinemia was found in 80 women (25.5%). Thyroid function and PRL secretion showed a significant interdependence (Table 1).

In 41 galactorrheic patients, 14 (34.1%) showed an increased TSH secretion. The incidence of galactorrhea was somewhat higher in women with abnormal TSH levels than in euthyroid women; 11.3% (27/238) euthyroid patients, 18.3% (13/71) of the patients with latent hypothyroidism, and 1 of 4 patients with hypothyroidism had galactorrhea, although this was not statistically significant.

DISCUSSION

The present study demonstrates a close relationship between TSH and PRL in women with infertility and/or menstrual disturbances. The secretion of these hormones from the anterior pituitary is governed by a variety of neurotransmitters, some of which are common for regulation of both hormones. Indeed, replacement of thyroid hormones in primary hypothyroidism resulted in a decrease in PRL response to TRH (7). However, TRH is not a physiological regulator of PRL secretion. For instance, TRH-stimulated PRL levels showed no correlation with elevated PRL levels during suckling in puerperal women (8), as did MCP-stimulated PRL levels (9).

Besides hyperprolactinemia, primary hypothyroidism has been shown to be a major factor in galactorrhea for 2-6% of cases (10). This is underlined by this study, in which 34.1% of the galactorrheic patients had latent hypo- or hypothyroidism. The high incidence of galactorrhea in the women with latent hypo- and hypothyroidism indicates that the evaluation of thyroid function by means of the TRH test is mandatory in women with galactorrhea.

REFERENCES

1. Bohnet HG and McNeilly AS. Horm Metab Res 11: 533, 1979.
2. Pepperell RJ. Fertil Steril 35: 267, 1981.
3. Refetoff S, Fang VS, Rapoport B, et al. J Clin Endocrinol Metab 35: 450, 1974.
4. Bohnet HG, Naber NG, Hanker JP, et al. Horm Metab Res 12: 390, 1980.

5. Bohnet HG, Fiedler K, and Leidenberger FA. Lancet II: 1278, 1981.
6. Bohnet HG, Kato K, Trapp M, et al. Fertil Steril 1986, in press.
7. Yamaji T. Metabolism 23: 745, 1974.
8. Bohnet HG. In C Schirren and K Semm (eds), Prolaktin und seine Bedeutung fur die Frau, Grosse Verlag, Berlin, 1981, p 64.
9. Bohnet HG and Kato K. Obstet Gynecol 65: 789, 1985.
10. Sakiyama R and Quan M. Obstet Gynecol Surv 38: 689, 1983.

α-ADRENERGIC CONTROL OF TSH SECRETION IN MAN

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INTRODUCTION

It is well known that TSH secretion is under the control of different factors (1). On thyrotrophs, there is a direct negative control exerted by thyroid hormones and dopamine, while TRH released by hypothalamic neurons has a stimulatory effect on TSH secretion. These neurons are probably under the positive control of the noradrenergic system (1,2).

In man, data about α-adrenergic control are scanty (3-5). Therefore, we decided to study the effect of clonidine, an α-adrenergic agonist, on serum TSH and PRL levels in three different groups of subjects: hypothyroid patients, healthy volunteers, and euthyroid patients who received iopanoic acid for oral cholecystography. This last compound is known to inhibit T_4 to T_3 peripheral and pituitary conversion, and to increase TSH levels and its response to TRH (6). We studied the third group because we expected higher TSH responsiveness than in untreated subjects.

MATERIALS AND METHODS

Six normal volunteers (4 men and 2 women) aged 20-28 years, and 6 euthyroid patients (3 men and 3 women) aged 25-52 years, who were administered with iopanoic acid for oral cholecystography 3-5 days before tests, were studied. In addition, 4 patients affected by primary hypothyroidism took part in this study. Thyroid status was assessed both clinically and biochemically (7). The duration of hypothyroidism, as assessed by clinical history, ranged between 10-24 months. The primary hypothyroidism was subsequent to idiopathic thyroid failure in 3 patients and to subtotal thyroidectomy for thyroid carcinoma in the last one. Each subject gave informed consent to the study.

At 8:30 a.m., with subjects recumbent after an overnight fast, a venous catheter was inserted in a forearm vein and patency was maintained by a slow infusion of normal saline. After a rest period of 30 min, 2 baseline samples were collected (-30 and 0 min). In all subjects, samples were then taken every 30 min for 4 hours after drug administration. Each patient was tested twice in random order: once with placebo (normal saline, 2 ml im) and once with clonidine (0.3 mg im).

Serum T_3, T_4, TSH, and PRL were measured by RIA, using commercial kits furnished by Biodata (Italy). Assay sensitivities were 0.125 ng/ml, 6.2 ng/ml, 0.5 μU/ml, and 1.5 ng/ml, respectively. All intra- and inter-assay CV were less than 8%.

All specimens from any individual were measured in duplicate in the same assay. Sera of hypothyroid patients were conveniently diluted before TSH assay with TSH-free human serum, so as to obtain a final concentration between 5-10 μU/ml.

Statistical analysis was carried out using Student's t test for paired data.

RESULTS

In normal subjects (Fig. 1a), clonidine administration induced an increase in serum TSH levels, statistically significant at +60 ($p<0.05$), +90 ($p<0.02$), and +120 ($p<0.05$). A comparison between placebo and clonidine secretory areas also demonstrated a significant difference ($p<0.02$).

In patients who received iopanoic acid (Fig. 1b), clonidine determined a marked rise of serum TSH levels (more evident with respect to normal volunteers).

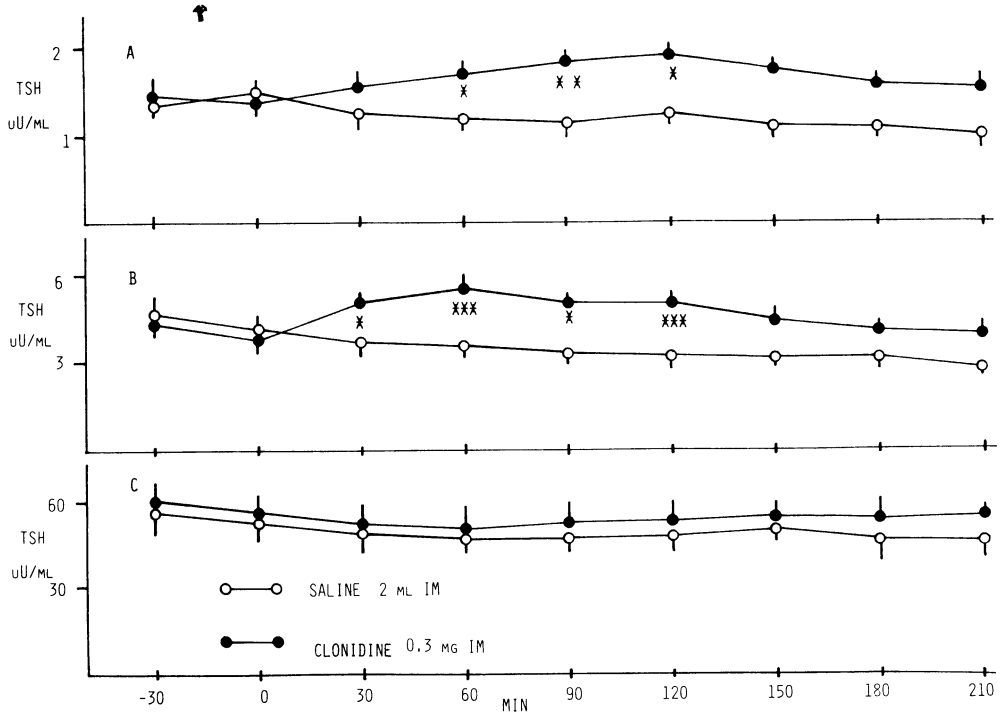

Fig. 1. Serum TSH (mean ± SE) following clonidine and placebo (saline) administration. A: six normal volunteers. B: six euthyroid patients administered with 3 g of iopanoic acid 3-5 days before tests. C: four hypothyroid patients. Statistics: *$p<0.05$; **$p<0.02$; ***$p<0.01$ vs the corresponding control (saline).

In hypothyroid patients, no significant variation in TSH levels was seen after drug administration with respect to placebo (Fig. 1c).

No changes in serum PRL, T_3, and T_4 were demonstrated (data not shown).

DISCUSSION

Our data show that clonidine administration induces an increase in serum TSH levels in normal volunteers. In the euthyroid subjects who took iopanoic acid (well-known inhibitor of the conversion of T_4 to T_3), we noted a more evident increase in TSH levels with respect to normal volunteers, the same as we would expect to occur in hypothyroid patients after clonidine administration. On the contrary, we observed no significant change in TSH levels. We must consider two distinct problems: the first is the different responsiveness of TSH to clonidine in normal volunteers and hypothyroid patients; the second is the apparent contrast in TSH response between hypothyroid and iopanoic acid-treated euthyroid patients.

For the first question, we must keep in mind that clonidine may act at a hypothalamic level, probably on TRH-producing neurons (8). In hypothyroid patients, the lack of effect of the drugs on TSH levels does not seem in agreement with this hypothesis. The absent response of TSH to clonidine in these subjects may be explained by a reduced responsiveness of TRH neurons to α-adrenergic stimuli, or by reduced secretion of TRH (this problem is today unresolved, 9). The drug may act, as α-adrenergic agonist, directly on thyrotrophs (10), but this effect would be mild, because of the overflow of TSH secretion.

The hypothesis of the reduced secretion and/or responsiveness of TRH in hypothyroid patients could also explain the difference between these subjects and iopanoic acid-treated patients. The responsiveness of TRH neurons of the latter group may be similar to the normal, because the reduction of thyroid hormones is of recent establishment and of lesser intensity. Moreover, reduced serum T_3 levels in iopanoic acid-treated subjects determines higher responsiveness of TSH with respect to normal subjects.

Another question is that clonidine, in spite of likely stimulation on TRH secretion, has no effects on PRL levels. This may be due to a direct inhibitory action on lactotrophs, as demonstrated by *in vitro* studies using α-adrenergic stimuli (11). The absent response of PRL to clonidine may also be explained by a higher threshold of pituitary lactotrophs to TRH with respect to thyrotrophs.

On the basis of our data, we cannot exclude other possible mechanisms of clonidine action, such as an interaction with opioid or serotoninergic system.

REFERENCES

1. Peters JR, Foord SM, Dieguez C, et al. Clin Endocrinol Metab 12: 669, 1983.
2. Krulich L. Neuroendocrinology 35: 139, 1982.
3. Nilsson KO, Thorrel JI, and Hokfelt B. Acta Endocrinol (Copenh) 76: 24, 1974.
4. Yoshimura M, Hachiya T, Ochi Y, et al. J Clin Endocrinol Metab 45: 95, 1977.
5. Zgliczynski S and Kaniewski M. Acta Endocrinol (Copenh) 95: 172, 1980.

6. Burgi H, Wimpfheimer C, Burger A, et al. J Clin Endocrinol Metab 43: 1203, 1976.
7. Evered DC, Ormston BJ, Smith PA, et al. Br Med J 1: 657, 1973.
8. Krulich L, Mayfield MA, Steele, et al. Endocrinology 110: 796, 1982.
9. Scanlon MF, Rees Smith B, and Hall R. Clin Sci 55: 1, 1978.
10. Klibanski A, Milbury PE, Chin WW, et al. Endocrinology 113: 1244, 1983.
11. Lancranjan I, del Pozo E, and Ohnhaus E. J Clin Endocrinol Metab 47: 671, 1978.

TSH AND THYROIDAL RESPONSES TO CONTINUOUS AND INTERMITTENT TRH ADMINISTRATION

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INTRODUCTION

Several hypothalamic-releasing hormones have been isolated, and the responses to them characterized. Single doses cause transient dose-dependent increases in the serum levels of the respective pituitary hormones. The responses to prolonged administration vary, depending on the method and frequency of administration, and are determined by factors such as inhibition by target gland hormones, depletion of hormone stores, and down-regulation of pituitary receptors. With regard to TRH, short-term infusions raise serum TSH for the duration of the infusion, and prolonged intrathecal TRH administration causes sustained increases in serum TSH, T_4, and T_3 (1-5). However, repeated individual intravenous or oral doses of TRH result in reduced TSH responses after several days (6-9). This study was undertaken to compare the TSH and thyroidal responses to the same total TRH dose given intermittently and continuously in the same subjects.

MATERIALS AND METHODS

Study Protocol

Six normal men, aged 20-28, were studied. The study was approved by the local Human Rights Committee and informed consent given by each subject. None had taken any medications within four weeks of the study. The men were studied in hospital and were fed an *ad lib* diet. Each initially had a baseline TRH test, in which an intravenous dose of 400 µg TRH was given and blood samples were collected for 120 min. Two or more weeks later, the men received, in random order at least 4 weeks apart, starting at 0800-0900 hr: 1) repetitive bolus doses of 200 µg TRH every 4 hr for 48 hours, during which blood samples were collected before and 30, 60, and 120 min after each dose of TRH; 2) continuous infusion of 0.83 µg TRH/min for 48 hr, during which samples were collected every 2 hr. At 48 hr in each study a final 400 µg bolus dose was given and multiple samples collected for 120 min.

Hormone Analyses

Blood was allowed to clot at room temperature, and serum stored at -5°C. All samples from an individual subject were analyzed concurrently

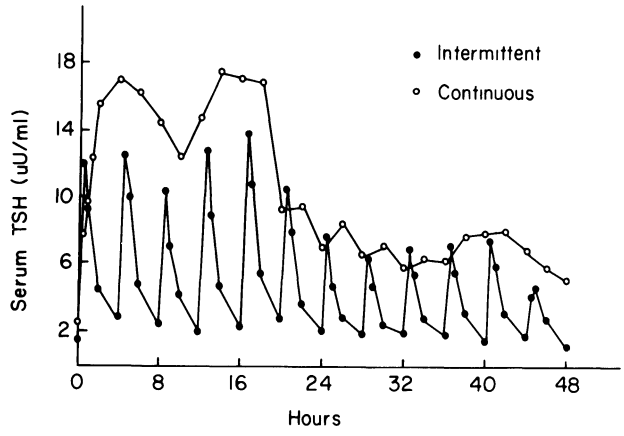

Fig. 1. Mean serum TSH concentrations in 6 normal men during intermittent (200 μg every 4 hr) and continuous (0.83 μg/min) TRH administration for 48 hr.

in duplicate in each assay. Serum T_4, T_3, and TSH were measured by RIA (10). The intra- and interassay coefficients of variation were, respectively, 11% and 13.3% for T_4, 6.9% and 14.6% for T_3, and 10.4% and 11.8% for TSH. TSH secretion was calculated by the trapezoidal rule.

RESULTS

Figure 1 shows the mean TSH concentrations during the two 48 hr periods of TRH administration. Serum TSH increased in response to each four hourly bolus dose of TRH. The mean peak post-TRH serum TSH concentration was highest, 14.0 ± 4.1 (SE) μU/ml, at 16 1/2 hr. The peak serum TSH increase after each dose of TRH then declined progressively to 6.3 μU/ml at 28 1/2 hr and it was 4.1 ± 1.5 μU/ml after the final 200 μg TRH dose given at 44 hr. The mean TSH before each dose did not change; for example, it was 1.5 μU/ml before the first TRH dose and 1.3 μU/ml before the final dose.

Mean serum TSH concentrations increased promptly after initiation of the continuous infusion, reaching 17.0 ± 4.3 μU/ml in 4 hr, and the peak was 17.6 ± 6.5 μU/ml at 14 hr. Serum TSH declined rather abruptly between 18 and 20 hr, from 16.9 to 9.3 μU/ml, and thereafter gradually declined to 5.1 ± 1.5 μU/ml at 48 hr.

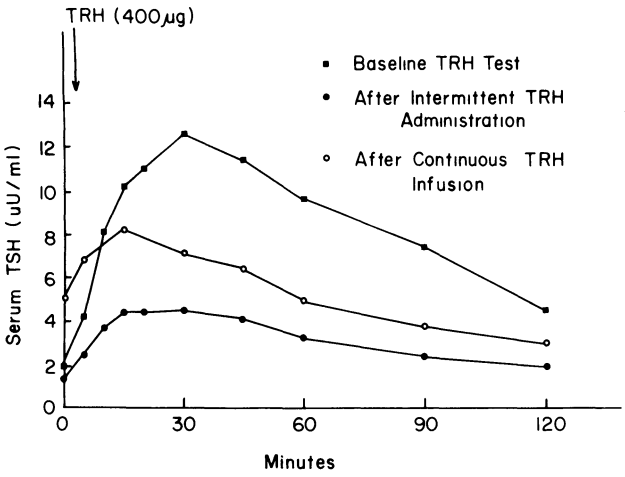

Fig. 2. Mean serum TSH responses to a 400 μg bolus TRH dose before and at the end of the two prolonged TRH administration studies.

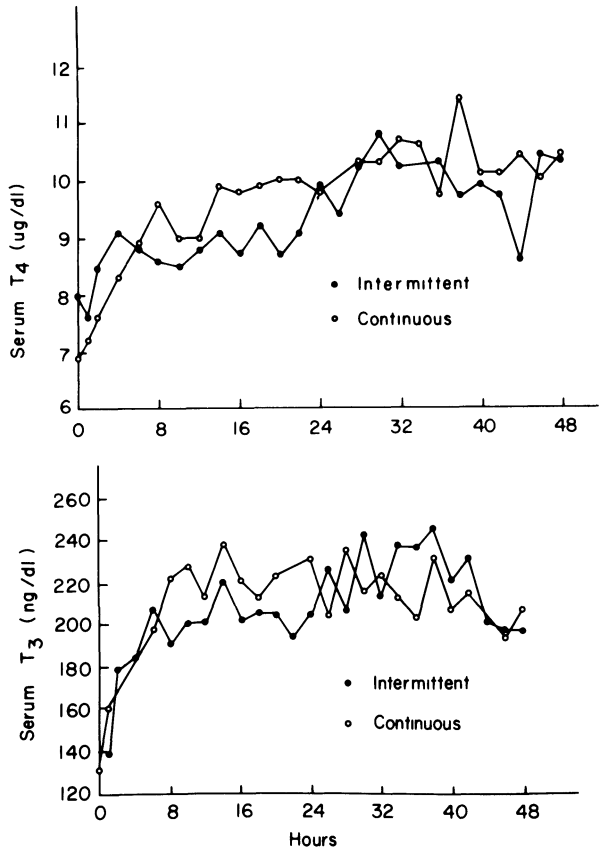

Fig. 3. Mean serum T_4 (top) and T_3 (bottom) concentrations during intermittent (200 μg every 4 hr) and continuous (0.83 μg/min) TRH administration for 48 hr.

Net TSH secretion, determined by calculation of the area under the TSH response curves from 0 to 48 hr, was 218 ± 64 μU/ml·hr during repetitive TRH administration and 498 ± 143 μU/ml·hr during continuous TRH administration ($p<0.001$).

Figure 2 shows the mean responses to the 400 μg TRH doses given before and at the end of the two 48 hr TRH study periods. The mean increments in serum TSH at these times were 10.7 ± 2.6 μU/ml (baseline), 3.2 ± 1.9 μU/ml after repetitive TRH administration, and 3.1 ± 1.8 μU/ml after continuous TRH administration. Net TSH secretion in the 120 min periods after these 400 μg TRH doses was 17.6 ± 4.2 μU/ml·hr (baseline, 6.3 ± 1.0 μU/ml·hr after intermittent TRH administration, and 10.6 ± 3.1 μU/ml·hr after continuous TRH administration (both $p<0.01$ vs baseline).

The serum T_4 and T_3 responses are shown in Fig. 3 and Table 1. The increments and patterns of increase in mean serum T_4 and T_3 in the 2 studies were similar. Mean serum T_4 increased significantly after 4 hr in both studies and further increased by 1 to 2 μg/dl thereafter. Mean serum T_3 increased earlier and somewhat more than did those of T_4, and the mean increases thereafter were small, approximately 10 ng/dl.

DISCUSSION

Three major findings emerged from this study. One, serum TSH concentrations were considerably higher during continuous than during repetitive administration of the same total TRH dose. Two, serum TSH declined rather

Table 1. Serum T_4 and T_3 Concentrations During Prolonged TRH Administration

	0	4 hr	8 hr	24 hr	48 hr
Intermittent TRH					
Serum T_4 (μg/dl)	7.9 ± 0.7[a]	9.1 ± 0.7[b]	8.6 ± 0.6[b]	9.9 ± 0.8[b]	10.3 ± 1.2[b]
Serum T_3 (ng/dl)	131 ± 13	184 ± 26[b]	191 ± 24[b]	205 ± 24[b]	197 ± 25[b]
Continuous TRH					
Serum T_4 (μg/dl)	6.9 ± 0.6	8.3 ± 0.8[b]	9.6 ± 0.7[b]	9.8 ± 0.7[b]	10.4 ± 1.2[b]
Serum T_3 (ng/dl)	131 ± 11	200 ± 23[b]	222 ± 25[b]	231 ± 23[b]	207 ± 24[b]

[a]Mean ± SE; [b]$p<0.05$.

abruptly after 20 to 24 hr of TRH administration. Three, the thyroidal responses were similar, despite the disparity in the serum TSH concentrations during the two studies.

The patterns of serum TSH responses to repetitive and continuous TRH administration were, not surprisingly, different. TRH given repetitively produced an increase in serum TSH after each dose, whereas TRH given continuously resulted in a sustained increase in serum TSH. In both instances, the peak response was reached within 20 hr, and thereafter both peak serum TSH responses after TRH given repetitively and serum TSH concentrations during continuous TRH administration were about 50% of those during the first 4 to 20 hr. Despite the fact that the total TRH dose was the same during the two 48 hr studies, the total serum TSH response was twofold higher when TRH was given intravenously.

The finding that continuous TRH administration resulted in secretion of more TSH is most simply explained by sustained stimulation of the thyrotrophs due to sustained increase in serum TRH concentrations. Therefore, sustained occupancy of thyrotroph TRH receptors did not lead to greater down-regulation of them than did intermittent TRH administration, if indeed any down-regulation occurred. The greater TSH response during continuous TRH administration also suggests that acute depletion of TSH stores did not occur. If it had, a greater response to intermittent TRH would have been expected.

There was a rather abrupt decline in serum TSH from 18 to 24 hr during continuous TRH administration and a similar decline in the peak serum TSH responses after intermittent TRH administration from 16 to 24 hr. Thereafter, the serum TSH responses changed little. These reduced serum TSH responses to TRH most likely were due to the increases in serum T_4 and T_3 which occurred within 4 hr after the start of the study, in accord with previous studies indicating that small increments in serum T_4 and T_3 inhibit TSH responses to TRH within 24 hours (6). Why the TSH responses did not decline progressively during the period from 24 to 48 hr is unknown, unless it was because further increases in serum T_4 and T_3 after the first 4 hr were small.

Two aspects of the thyroidal responses deserve comment. First, why did most of the increase in serum T_4 and T_3 occur in the first 4 hr, even though TSH secretion remained high for 16 to 18 hr? It is possible there is only a small readily releasable thyroidal T_4 and T_3 pool, and/or some down-regulation of thyroidal TSH receptors occurs. Second, the pattern and magnitude of the increase in serum T_4 and T_3 in the two studies were quite similar, althogh serum TSH concentrations and the calculated overall TSH responses were considerably higher during continuous TRH administration. Thus, episodic TSH secretion was clearly more effective in stimulating T_4 and T_3 secretion than was continuous TRH administration.

REFERENCES

1. Wartofsky L, Dimond R, Noel G, et al. J Clin Endocrinol Metab 42: 443, 1976.
2. Chan V, Wang C, and Yeung R. J Clin Endocrinol Metab 49: 127, 1979.
3. Spencer C, Greenstadt M, Wheeler W, et al. J Clin Endocrinol Metab 51: 771, 1980.
4. Mongioi A, Aliffi A, Vicari E, et al. J Clin Endocrinol Metab 56: 904, 1983.
5. Kaplan M, Munsat T, Taft J, et al. Prog Endocrine Soc, 1985, abst. 718.
6. Snyder P and Utiger R. J Clin Invest 52: 2305, 1973.

7. Rabello M, Snyder P, and Utiger R. J Clin Endocrinol Metab 39: 571, 1974.
8. Staub J, Girard J, Mueller-Brand J, et al. J Clin Endocrinol Metab 46: 260, 1978.
9. Pavasuthipaisit K, Norman R, Ellinwood W, et al. J Clin Endocrinol Metab 56: 541, 1983.
10. Shulkin T and Utiger R. J Clin Endocrinol Metab 60: 1076, 1985.

MEASUREMENTS OF THYROTROPIN-RELEASING HORMONE (TRH)-LIKE IMMUNOREACTIVITY IN HUMAN BLOOD IN THYROID DISEASES

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SUMMARY

Human blood TRH-like immunoreactivity levels were estimated by a new, highly specific and sensitive RIA in various abnormal thyroid states. Blood samples were extracted by cold methanol. All results were expressed in fmoles/ml (mean $\pm$ SD).

The TRH-like immunoreactivity levels were 44.5 $\pm$ 5.2 in 9 normal adult subjects and 50.4 $\pm$ 4.1 in 6 euthyroid subjects with simple goiter. Blood TRH-like immunoreactivity was found significantly increased in 19 patients with M. Basedow or with autonomy (132 $\pm$ 33; $p<0.01$), and significantly decreased in 12 hypothyroid patients (21 $\pm$ 3; $p<0.01$). These findings suggest that peripheral thyroid hormones may be involved in the T_4-TRH regulation mechanism and the hypothesis of a possible positive feedback of T_4 at hypothalamic level can, thus, be confirmed.

INTRODUCTION

Despite a decade of research, the physiology and the precise role of TRH in thyroid diseases remains controversial (1-3).

We studied the TRH-like immunoreactivity levels in various abnormal thyroid function states by a new, highly specific and sensitive RIA.

MATERIAL AND METHODS

Fasting blood samples were collected at about 8:00 a.m. and extracted for TRH in cold methanol. TRH-like immunoreactivity levels were measured in 15 euthyroid adult subjects (6 of these patients with simple goiter), 12 patients with M. Basedow or autonomy, and 12 patients with primary hypothyroidism. All patients were studied prior to the institution of any therapy.

The RIA for TRH was generated using antiserum produced against a conjugate of TRH coupled with diazotized benzidine to a carrier protein, a seed

globulin of the sunflower. The performance of the assay (specificity, sensitivity, recovery parallelism) was previously reported (4,5).

RESULTS

All results are expressed in fmol/ml. Blood TRH-like immunoreactivity levels were 44.5 ± 5.2 (mean ± SD) in the group of normal subjects and 5.4 ± 4.1 (mean ± SD) in the subjects with simple goiter (Fig. 1). No difference was observed between males and females.

Blood TRH-like immunoreactivity levels in hyperthyroid patients were found significantly elevated (132 ± 33, $p<0.01$). In contrast, hypothyroid patients had significantly lower levels of TRH-like immunoreactivity (21 ± 3, $p<0.01$, Fig. 2).

DISCUSSION

We have determined the blood levels of TRH-like immunoreactivity in normal subjects, as well as in patients with hyperthyroidism. Despite the fact that we have not yet arrived at a satisfactory answer to the question of the origin of TRH-like immunoreactivity in human blood, we were able to document (in comparison to normal subjects) the occurrence of significantly elevated values of TRH-like immunoreactivity in hyperthyroid patients and significantly reduced levels in the hypothyroid group. The peripheral blood TRH-like immunoreactivity ranges in various thyroid function states parallel to those occurring in thyroid hormones. These findings agree with the T_4-TRH regulation mechanism proposed by Reichlin (6) which predicted a positive feedback between these hormones.

Considering the biological significance of our results, we plan to utilize this very sensitive TRH-RIA for further studies of TRH secretory physiology in humans.

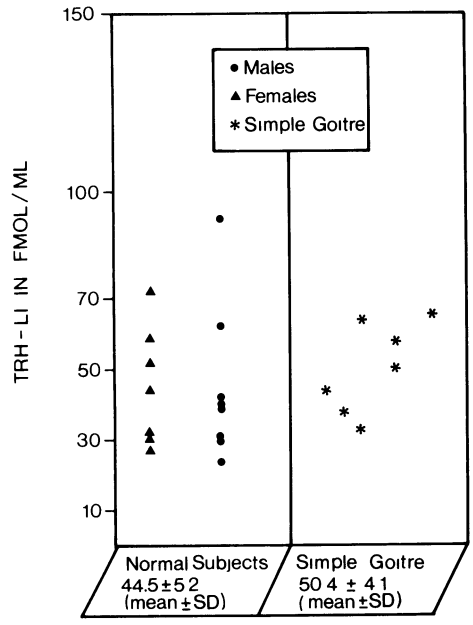

Fig. 1. TRH-like immunoreactivity levels in 15 euthyroid adults (6 of these patients with simple goiter).

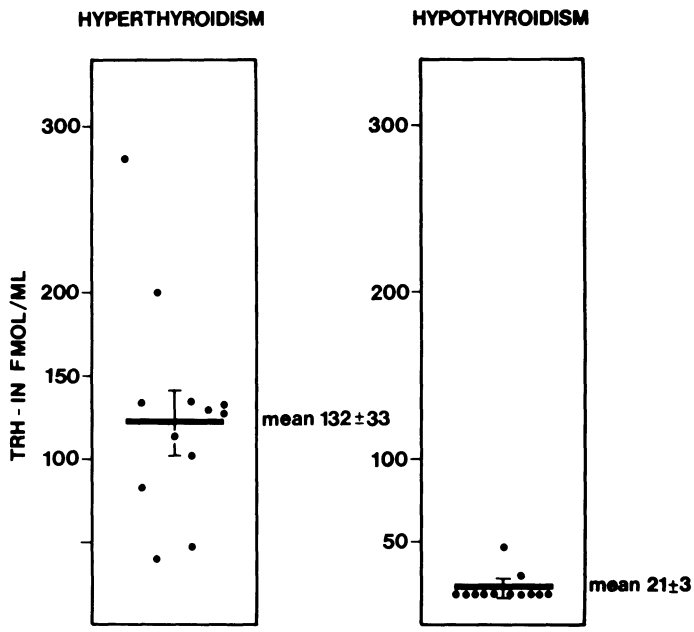

Fig. 2. TRH-like immunoreactivity levels in 12 patients with hyperthyroidism and in 12 patients with hypothyroidism.

REFERENCES

1. Mitsuma T, Hirooka Y, and Nihei N. Acta Endocrinol 83: 225, 1976.
2. Malik TK, Wilber SF, and Peguez S. J Clin Endocrinol Metab 54: 1194, 1982.
3. Lombardi G, Panzya N, Cei S, et al. Acta Endocrinol 87: 70, 1978.
4. Duntas L, Loos U, Grouselle D, et al. XV International Congress of the International Society for Psychoneuroendocrinology (ISPNE), Vienna, 1984.
5. Grouselle D, Faivre-Baumann A, and Tixier-Vidal A. Neuroscience Letters 7: 7, 1978.
6. Reichlin S, Martin JB, Mitnick M, et al. Rec Progr Horm Res 28: 229, 1972.

IMMUNOREACTIVE TRH AND TRH-OH IN RAT HYPOPHYSIAL PORTAL BLOOD

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INTRODUCTION

The release of the hypothalamic thyrotropin-releasing hormone (TRH) can be studied in the rat directly by radioimmunoassay in blood collected from the hypophysial portal vessels. Since TRH is rapidly inactivated in blood, it is necessary to collect hypophysial portal blood (HPB) in a way that minimizes TRH inactivation. In the present study, we have tested the validity of two different methods of collection of HPB for studying TRH secretion. Thereafter, we have measured TRH and its putative metabolite TRH-OH (Pyroglu-His-Pro-OH or Deamido-TRH) in HPB under various physiological and pharmacological conditions.

MATERIALS AND METHODS

Adult male rats of the Sprague-Dawley strain (250-300 g/b.w.) were anesthetized with sodium pentobarbital (4 mg/100 g/b.w.). The ventral diencephalon and pituitary stalk was sectioned. After removal of the entire pituitary gland, blood was collected from the stalk according to two different methods:

Method I (1): after cannulation of the stalk with a polyethylene cannula, blood was withdrawn with a Harvard Syringe pump at the rate of 7 µl/min. Portal blood in the cannula remained at room temperature during the first four minutes of the collection. The collecting tubing was then immersed in an ice bath throughout the experiment. At the end of the collection, blood was centrifuged. Plasma was separated and mixed with three volumes acidified methanol (methanol 90 : acetic acid 1 : distilled water 9). After centrifugation, the supernatant was evaporated to dryness with a Speed Vac (Savant Instruments).

Method II (modified from 2 and 3): blood from the pituitary stalk was taken off by fine pipette every 15 seconds and dropped immediately into a tube containing 0.7 ml acidified methanol. This method allows the collection of 151 $\pm$ 5.4 µl blood per 20 minutes (mean $\pm$ SEM; n=255). The tube was mixed in a vortex, centrifuged, and the supernatant was evaporated as described above.

TRH levels in HPB of intact rats, as well as TRH recovery during the first hour after injection of 10 µg synthetic TRH into the lateral ventricle, were measured using both methods of collection. It has been shown previously that TRH is transported rapidly from the cerebrospinal fluid into the hypophysial portal vessels (4).

Thereafter, Method N°2 was used for collecting HPB in the following experimental groups of rats: control, 3- and 30-day thyroidectomized, L-thyroxine treated (5 µg/100 g i/p. for 3 days), and after acute hypothermia (body temperature lowered to 18-20°C with a refrigerated blanket). HPB samples were collected every 20 minutes for one hour. At the end of the collection, a blood sample was obtained from the femoral artery.

TRH and TRH-OH were measured by two different and specific radioimmunoassay methods in dried blood extracts as previously described (5,6).

RESULTS AND DISCUSSION

High TRH levels are detected in HPB of intact rats when blood has been collected rapidly and dropped immediately into an acidified methanol solution that blocks the enzymatic inactivation of this peptide. As shown in Table 1, method II allows high recovery of TRH and HPB and is more appropriate than method I for studying secretion of this peptide. TRH levels in HPB are lower in our study (402 - 1177 pg/ml or 111 - 210 pg/hour) than in other reports: 5300 pg/ml (7), 4500 pg/hour (8), and 1041-2034 pg/ml (9). Differences in the specificity of the assay may account for these variations. Unlike Sheward et al. (8), we have observed that immunoreactive TRH in dried extracts of portal blood is recovered after high performance liquid

Table 1. Comparison Between Method I and II of HPB Collection for Studying TRH Secretion

Assay of endogenous TRH (intact rats)	
Method I (n=7):	<2.5 pg/20 min or <35 pg/ml
Method II:	0-20 min : 73 ± 24 pg/20 min
	or 1177 ± 377 pg/ml
	20-40 min : 47 ± 5 pg/20 min
	or 504 ± 54 pg/ml
	40-60 min : 37 ± 10 pg/20 min
	or 402 ± 157 pg/ml
Recovery of TRH during 1 hr after injection of 10 µg synthetic TRH into the lateral ventricle	
Method I (n=8):	112.38 ± 24.35 ng
Method II (n=9):	267.84 ± 34.34 ng

Results are given as pg TRH/20 min collection or ml plasma.

Table 2. TRH Levels in Hypophysial Portal Blood (HPB) and TRH-OH Levels in HPB and Systemic Blood

Group		TRH in HPB	TRH-OH in HPB	TRH-OH in systemic blood
First experiment:				
Control	0 - 20 min	857 ± 377	833 ± 155	321 ± 58.4
	20 - 40 min	814 ± 154	513 ± 148	315 ± 16.3
	40 - 60 min	500 ± 157	352 ± 57	282 ± 16.6
3 days thyroidectomized				
	0 - 20 min	752 ± 309		
	20 - 40 min	557 ± 149		
	40 - 60 min	185 ± 135		
30 days thyroidectomized				
	0 - 20 min	684.8 ± 172	1102 ± 220	131.5 ± 54
	20 - 40 min	661 ± 319	1045 ± 136	305.7 ± 28
	40 - 60 min	426 ± 136	840 ± 97	240 ± 36
Acute hypothermia				
	0 - 20 min	598.8 ± 307	1114 ± 99	340 ± 43
	20 - 40 min	386 ± 124	855 ± 137	475 ± 37
	40 - 60 min	523 ± 75	1429 ± 500	435 ± 36
Second experiment:				
Control	0 - 20 min	191.4 ± 44		
	20 - 40 min	167.14 ± 37.6		
	40 - 60 min	255.7 ± 41		
$L-T_4$ treatment				
	0 - 20 min	172 ± 12.4		
	20 - 40 min	84.3 ± 10.3		
	40 - 60 min	110.7 ± 11.5		

Results are given as pg/ml plasma; mean ± SEM; 6-8 rats per group. TRH levels in systemic blood range between 5 and 10 pg/ml without any significant variations between all the groups.

chromatography in one single peak which coelutes with synthetic TRH (unpublished results). Nevertheless, the percentage of hypothalamic TRH released/hour in HPB (5%) is similar to that of 41-CRF (4%) (results from our laboratory) and higher than that of LH-RH (0.1 - 0.3%) and Somatostatin (0.2%) (10). TRH in systemic blood is barely detectable (5-10 pg/ml). TRH levels in HPB do not vary after thyroidectomy and the administration of thyroxine (Table 2), a finding which supports the hypothesis that the feedback of thyroid hormones occurs mainly at the level of the pituitary gland. The lack

of increase of TRH in HPB after acute hypothermia may be due to the experimental procedure, since stress can block TSH increase during cold exposure (11). Unlike TRH, TRH-OH is detectable both in portal and systemic blood, its level in HPB being only two to threefold higher than in systemic blood (Table 2). The source(s) and meaning of TRH-OH in portal and systemic blood remain to be established.

SUMMARY

Immunoreactive TRH can be measured in HPB collected in the rat under conditions that minimize its degradation. TRH levels in HPB do not vary after thyroidectomy or treatment with L-thyroxine, which suggests that thyroid hormone feedback occurs mainly at the level of thyrotrophs.

ACKNOWLEDGMENTS

The authors thank Mrs. Sallen and Mrs. Vaillant for their excellent secretarial assistance.

REFERENCES

1. Porter JC and Smith KR. Endocrinology 81: 1182, 1967.
2. Worthington WC. Nature 210: 710, 1966.
3. Fink G and Jamieson MG. J Endocrinol 68: 71, 1976.
4. Oliver C, Ben-Jonathan N, Mical RS, et al. Endocrinology 97: 1138, 1975.
5. Eskay RL, Oliver C, Warbeg J, et al. Endocrinology 98: 269, 1976.
6. Dutor A, Ouakif LH, Castanas E, et al. Life Sciences, 1985 (in press).
7. De Greef WJ and Visser TJ. J Endocrinol 91: 213, 1981.
8. Sheward WJ, Harmar AJ, Fraser HM, et al. Endocrinology 113: 1865, 1983.
9. Ching MCH and Utiger RD. J Endocrinol Invest 6: 347, 1983.
10. Fink G, Koch Y, and Ben Aroya N. Brain Res 243: 186, 1982.
11. Mannisto PT, Saarinen A, and Ranta T. Endocrinology 99: 875, 1977.

ONTOGENY OF HUMAN PANCREATIC THYROTROPIN-RELEASING HORMONE

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INTRODUCTION

It is known that thyrotropin-releasing hormone (TRH) is present in hypothalami, as well as in other cerebral areas and in other tissues including the gastrointestinal tract and, particularly, the pancreas (1-7). In rats, the islets of Langerhans are the main source of pancreatic TRH (6) and it has been shown immunohistochemically that TRH is localized especially in marginal islet cells (8,9). The role of pancreatic TRH is not well established (10), but it has been shown to enhance arginine-induced glucagon release in the adult dog (11). Ontogenetic studies in rats have demonstrated that pancreatic TRH content is higher in the neonatal period and progressively declines after the first weeks of life (5,12-14). No data are available on the ontogeny of pancreatic TRH in humans and this represents the aim of the present study.

MATERIALS AND METHODS

Pancreases were taken within 2-8 hours after death from two infants who died at one year from respiratory infection, and from 11 fetuses of 15-36 weeks of gestation after pregnancy interruption for medical or accidental events. No history of diabetes mellitus was present in the mothers. The whole pancreas was immediately frozen and TRH extracted using a 90% methanol solution, as previously described (6). The dried extracts were diluted in phosphate buffered saline 0.01 M, pH 7.6; TRH was measured by a specific and sensitive (2 pg/tube) radioimmunoassay (15) and insulin by a commercial radioimmunoassay kit (Corning Immunophase, Milan, Italy). All samples were analyzed in duplicate and at three different dilutions in a single run in order to avoid interassay variation. The results were expressed as pg/organ and mU/organ for TRH and insulin, respectively. The degradation of synthetic and pancreatic TRH to proteolytic enzymes was analyzed by measuring the immunoassayable TRH before and after incubation with fresh human serum at 37°C for two hours. Pancreatic TRH was further submitted to Sephadex G-10 or BioGel P2 column chromatography and TRH was measured by radioimmunoassay in eluted fractions. The elution pattern was compared with that of synthetic TRH.

Table 1. TRH and Insulin Content in the Pancreas of Human Fetuses at Various Weeks of Gestation and in One Year Old Infants

Sample N.	Age	TRH pg/organ	Insulin mU/organ
Fetuses	(weeks)		
1	15	109	24.1
2	17	171	23.2
3	18	151	39.1
4	21	136	76.1
5	22	191	108.2
6	23	294	136.7
7	26	1,755	459.1
8	34	11,636	550.1
9	36	5,464	609.5
10	36	4,756	452.5
11	36	4,091	700.0
Infants	(year)		
12	1	3,208	2,090
13	1	3,300	2,180

RESULTS

The data are reported in Table 1. TRH was present in all pancreases examined. TRH extracted from the pancreas of fetuses at various weeks of gestation and of infants showed immunological, chromatographic, and serum inactivation properties similar to those of synthetic TRH. TRH was detected in the pancreas as early as 15 weeks in the fetus with a value of 109 pg/organ, increased progressively, reaching a maximal value of 11,636 pg/organ in the 34 week fetus, and then decreased to the mean value of 3,250 pg/organ in the two 1 year old infants. Insulin was present in pancreatic tissue of the 15 week fetus with a value of 24 mU/organ, and a continuous and progressive increase was observed during the entire period of gestation and in one year old infants.

DISCUSSION

The results of the present study demonstrate that human fetal pancreatic TRH has immunological, chromatographic, and serum inactivation properties similar to those of synthetic TRH. These findings confirm previous observations (3,4,6-8,12,13,16) that pancreatic TRH in rats is identical to synthetic TRH, although no similarity between extrahypothalamic and synthetic TRH has been reported by Youngblood et al. (17). Furthermore, our study demonstrates that TRH is present in the fetal pancreas at 15 weeks

of gestation, reaching the maximal value during the late period of pregnancy, and decreasing during the perinatal period and in the first year of life. A similar ontogenetic pattern of pancreatic TRH after birth was observed in rats by us (13) and others (5,12,14), who reported that pancreatic TRH content is higher during the early neonatal period and progressively declines, reaching adult value at 30-45 days of age. At present, there is no explanation for the ontogenetic development of pancreatic TRH in humans, although a possible maturation of TRH inactivating enzymes should be taken into account.

In the present study, the insulin content of fetal pancreas increases during gestation and, in contrast to TRH, remains elevated in the pancreas of fetuses during the last weeks of pregnancy and in one year old infants. These findings indicate that these two hormones have different ontogenetic patterns in humans.

REFERENCES

1. Morley JE. Endocrine Rev 2: 396, 1981.
2. Jackson IMD. N Eng J Med 306: 145, 1982.
3. Morley JE. Life Sci 25: 1539, 1979.
4. Leppaluoto J, Koivusalo F, and Kraama R. Acta Physiol Scand 104: 175, 1978.
5. Engler D, Scanlon MF, and Jackson IMD. J Clin Invest 67: 800, 1981.
6. Martino E, Lernmark A, Seo H, et al. Proc Natl Acad Sci USA 75: 4265, 1978.
7. Koivusalo F. J Clin Endocrinol Metab 53: 734, 1981.
8. Koivusalo F, Leppaluoto J, Kniff M, et al. Acta Endocrinol 97: 398, 1981.
9. Kawano H, Daikoku S, and Saito S. Endocrinology 112: 951, 1983.
10. Nielsen JH. Acta Endocrinol (Suppl 266) 108: 27, 1985.
11. Morley JE, Levin SR, Penhlevanian M, et al. Endocrinology 114: 137, 1979.
12. Aratan-Spire S, Wolf B, Portha B, et al. Endocrinology 114: 2369, 1984.
13. Martino E, Seo H, Lernmark A, et al. S Proc Natl Acad Sci USA 77: 4345, 1980.
14. Koivusalo F and Leppaluoto J. Life Sci 24: 1655, 1979.
15. Martino E, Seo H, and Refetoff S. Endocrinology 103: 1655, 1978.
16. Spindel E and Wurtman RJ. Brain Res 201: 279, 1980.
17. Youngblood WW, Humm J, and Kizer JS. Brain Res 163: 101, 1979.

DOPAMINE AND L-DOPA, PRECURSORS OF NOREPINEPHRINE: EFFECTS ON THYROXINE RELEASE

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Norepinephrine (NE), epinephrine, and their precorsors have been shown to enhance iodide organification in calf thyroid tissue through alpha-adrenergic receptors (1). In an _in vitro_ mouse thyroid preparation, NE concomitantly stimulated iodide organification and inhibited thyrotropin (TSH)-stimulated thyroxine (T_4) release (2,3). Recently such inhibition of TSH-stimulated T_4 release was confirmed _in vivo_ (4). In the present study, dopamine (DA) and L-DOPA also inhibited TSH-stimulated T_4 release through alpha-adrenergic and dopaminergic receptors; cAMP appeared to be an unlikely mediator.

TSH stimulated T_4 release by mouse thyroid _in vitro_ (2,3). DA when added to TSH inhibited T_4 release. The alpha-adrenergic antagonists, phentolamine (PHENT), Prazosin (PZ), and yohimbine (YOH), all reversed DA inhibition. The beta-antagonist, L-Propranolol (PROP), was ineffective. DA alone did not change T_4 release above control levels. Therefore, DA inhibition of T_4 release involved alpha-adrenergic but not beta-adrenergic receptors (Table 1, expt. 1). PHENT, PZ, YOH, and PROP, alone or with DA, did not stimulate T_4 release.

Apomorphine (APO), a dopaminergic agonist, but unlike DA not an NE precursor, also blocked TSH-stimulated T_4 release. Like DA inhibition, APO inhibition was reversed by PHENT, PZ, and SULP, again indicating involvement of alpha and dopaminergic receptors (Table 1, expts. 3 and 4).

The dopamine beta-hydroxylase inhibitors, diethyldithiocarbamate and DU-18288 (5), did not prevent DA inhibition of TSH-stimulated T_4 release. Conversion of DA to NE would, therefore, not be a prerequisite for inhibitory activity.

L-DOPA inhibited TSH-stimulated T_4 release. PHENT reversed this inhibition, indicating, like for DA, involvement of alpha-receptors. The aromatic L-amino acid decarboxylase inhibitor, carbidopa (CARB), reversed this inhibition, indicating prior conversion to DA was required for inhibition (Table 2).

Like TSH, $(Bu)_2$cAMP stimulates T_4 release by mouse thyroids _in vitro_ (3). DA and L-DOPA inhibited this stimulation. PHENT reversed DA inhibition of $(Bu)_2$cAMP action. Results suggest that DA and L-DOPA inhibition, and PHENT reversal, all occurred at a locus beyond cAMP generation (Fig. 1).

Table 1. Inhibition of TSH-stimulated T_4 Release by Dopamine and Apomorphine, and Reversal by Adrenergic and Dopaminergic Antagonists

Expt. 1		Expt. 2	
Additions	T_4 ng/thyroid		T_4 ng/thyroid
Control	(7) 5.8 ± 0.9	(6)	4.8 ± 1.4
TSH	(8) 17.4 ± 1.0*	(6)	18.8 ± 2.1*
TSH + DA	(7) 5.5 ± 0.5**	(6)	4.2 ± 0.4**
TSH + DA + PHENT	(7) 13.9 ± 1.1***		---
TSH + DA + PRAZ	(6) 19.7 ± 1.7***		---
TSH + DA + YOH	(6) 12.5 ± 1.4***		---
TSH + DA + PROP	(5) 7.4 ± 0.8		---
TSH + DA + SULP	---		10.7 ± 1.7***
Expt. 3		**Expt. 4**	
Control	(7) 3.4 ± 0.6	(6)	0.5 ± 0.2
TSH	(7) 21.7 ± 2.5*	(8)	14.5 ± 0.7*
TSH + APO	(6) 2.5 ± 0.4**	(9)	1.8 ± 0.2**
TSH + APO + PHENT	(5) 10.6 ± 1.8***		---
TSH + APO + PRAZ	(4) 7.4 ± 0.5***		---
TSH + APO + SULP	---	(10)	3.6 ± 0.5***

3 hr incubation of excised mouse thyroids with larynx and trachea in medium described (2) but containing 10^{-6} M NaI. No. vessels in parenthesis. TSH 1 mU/ml DA, APO and SULP 5 x 10^{-4} M, PHENT 5 x 10^{-5} M, PRAZ 10^{-5} M, YOH 10^{-4} M. T_4 determined by Mallinckrodt RIA SPAC T_4 kit. Mean ± SE. *p<.001 vs control, **p<.001 vs TSH, ***p<.01 vs TSH with DA or APO.

Table 2. Inhibition of TSH-stimulated T_4 Release by L-DOPA, and Reversal by Phentolamine and Carbidopa

Additions	T_4 ng/thyroid
Control	(6) 2.4 ± 0.5
TSH	(5) 15.0 ± 0.8*
TSH with DOPA	(6) 7.4 ± 0.8**
TSH with DOPA and PHENT	(6) 14.5 ± 1.3***
TSH with DOPA and CARB	(6) 12.5 ± 1.3***

Conditions as in Table 1. TSH 1 mU/ml, L-DOPA and CARB, 5 x 10^{-4} M, PHENT 5 x 10^{-5} M. Mean ± SE. *p<.001 vs control, **p<.001 vs TSH, ***p<.01 vs TSH with DOPA.

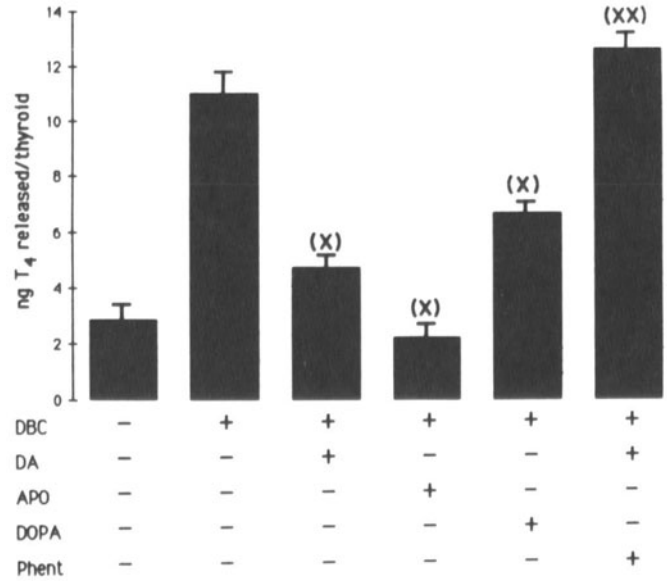

Fig. 1. Inhibition $(Bu)_2$cAMP (DBC) stimulated T_4 release by DA and L-DOPA, reversal by PHENT. Conditions as in Table 1. DBC 10^{-3} M. DA and L-DOPA 5 x 10^{-4} M. PHENT 5 x 10^{-5} M. Mean ± SE of 4-8 vessels (X) $p<.001$ vs DBC, (XX) $<.001$ vs DBC with DA.

TSH 1 mU/ml increased cAMP in excised mouse thyroid lobes after 30 min incubation (3). DA inhibited this stimulation. Unlike data on T_4 release, however, PHENT and SULP were unable to reverse this inhibition. DA alone did not increase cAMP above control values (Fig. 2).

The current study demonstrated that not only alpha receptors (as for NE) but also dopaminergic receptors were involved in DA effects. This action may have been direct and not through conversion to NE, since it persisted in the presence of blockers of dopamine beta-hydroxylase.

Dopaminergic action on adrenergic receptors was further confirmed when APO blocked TSH-stimulated T_4 release via alpha-receptors even though APO is not a precursor of NE.

L-DOPA, like DA, inhibited TSH-stimulated T_4 release via alpha-adrenergic receptors. CARB, an inhibitor of aromatic L-amino acid decarboxylase, reversed this inhibition. Absence of biological activity of L-DOPA would, therefore, indicate that prior conversion to DA was required.

Two sets of data would seem to exclude cAMP as an intermediary in DA inhibition of T_4 release: a) DA and APO inhibited the $(Bu)_2$cAMP-induced T_4 release in the presence of excess amounts of cAMP ester, and b) the NE and DA inhibition of the TSH-induced rise in cAMP was not reversed by alpha-blockers.

Dissociation between physiological effects of DA and effects on cAMP generation have been reported in other systems. Furthermore, thyroid cAMP may not necessarily be the mediator for all TSH actions on thyroidal iodine metabolism (3).

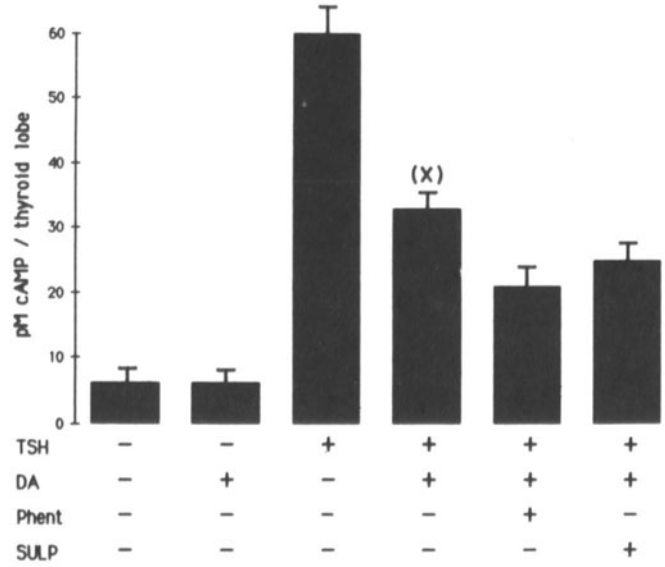

Fig. 2. Inhibition of TSH-stimulated thyroidal cAMP content by DA, and absence of reversal by PHENT or SULP. 30 min incubation of single thyroid lobes in medium containing 10^{-2} M theophylline (3). TSH 1 mU/ml, PHENT 5 x 10^{-5} M, SULP 5 x 10^{-4} M. Mean $\pm$ SE of 4-8 vessels. (X) $p<.001$ vs TSH.

CONCLUSIONS

Dopamine acts on the thyroid through alpha-adrenergic and dopaminergic receptors. L-DOPA has to be converted to dopamine to exert its thyroidal effect. cAMP is unlikely to be an intermediate in dopamine action.

REFERENCES

1. Maayan ML, Shapiro R, and Ingbar SH. Endocrinology 92: 912, 1973.
2. Maayan ML, Debons AF, Krimsky I, et al. Endocrinology 101: 284, 1981.
3. Maayan ML, Volpert EM, and From A. Endocrinology 109: 930, 1981.
4. Ahren B. Acta Endocrinol 108: 184, 1985.
5. Krulick L, Mayfield MA, Steele MK, et al. Endocrinology 110: 796, 1981.

SHORT-TERM IN VITRO STIMULATORY EFFECT OF GLUCAGON ON T_4 SECRETION IN RAT AND HUMAN THYROID

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Although hyperthyroidism is rarely associated with diabetes mellitus (1), it is often complicated by impairment of glucose tolerance (2). High concentrations of plasma glucagon are often found in hyperthyroidism (3). In poorly controlled diabetes mellitus, as in other acute episodes of severe diseases, the plasma T_3 level is reduced, whereas the plasma reverse T_3 level is elevated (1). Hyperglucagonemia, often found in such catabolic states (4,5), does not seem to be involved in this reduction of the plasma T_3 level (6). Furthermore, glucagon reduces the number of T_3 nuclear receptors in rat hepatocytes (7), and the thyroid hormones modify the plasma cyclic AMP response to glucagon (8).

Catecholamines have been shown to stimulate organification of iodine and synthesis of thyroid hormones *in vitro* (9), as well as their secretion *in vivo* (10). In stress situations, there is marked increase in glucagon secretion as in catecholamine secretion. However, the direct effect of glucagon on thyroid secretion has never been reported. In our study, we demonstrate *in vitro*, using perifused rat and human thyroid fragments, that glucagon directly stimulates thyroxine secretion.

MATERIALS AND METHODS

We used the technique of perifused thyroid fragments, described in detail elsewhere (11). Briefly, the system consisted of four 950 µl perifusion chambers at a constant temperature of 37°C. Krebs-Ringer bicarbonate glucose solution (KRBG) with human albumin (final pH - 7.45), gassed with 93% O_2 and 7% CO_2, was used and sent through silicone tubes to a peristaltic pump at a constant flow rate of 600 µl/min. Silicone tubes also connected the pump to the bottom of the chambers and received (through a connecting branch) the substances tested, i.e., in the present case, different concentrations of glucagon at a constant flow rate of 275 µl/min.

Rat and Human Thyroid Tissue

Thyroid lobes, taken from male Wistar rats (240-300 g) anesthetized i.p. with phenobarbital, were immediately immersed in the KRBG solution. For each experiment, three chopped thyroid lobes weighing in all 40 $\pm$ 2 mg were placed in each of the 4 perifusion chambers. Less than 10 minutes

elapsed between the end of the thyroidectomy and the beginning of the perifusion.

Experiments were carried out with human thyroid tissue removed surgically from patients undergoing laryngectomies for cancer. Normality of the piece removed was verified by light microscope examination. It was then immediately chopped into fragments. The same amount of tissue was used as that for the experiments with rats (40 mg). Less than 20 minutes elapsed between removal of the thyroid piece and perifusion.

Experimental Protocol and Timing

In 3 of the perifusion chambers, thyroid fragments were first perifused with KRBG alone for 30 min (washing period), then stimulated with glucagon (Novo, Paris) for 20 min, and again washed with KRBG alone. The fourth chamber received only KRBG and served as a control. Samples were collected every 2-6 min and were frozen at -20°C until assay.

T_4 was assayed by RIA (RIA gnost T_4, Behring Institute, Marburg, West Germany).

Expression of Results

The response was analyzed by comparing T_4 release during the stimulation period and basal release of T_4. As T_4 release with KRBG alone decreased slowly, the basal rate of release (B) (expressed in ng/min) was considered to be the mean value for the last sample of the washing period (Bi) and the first sample taken 10 min after the stimulation period. The mean rate of release of T_4 (R) was defined as the ratio of the mean amount of T_4 released per minute during the 20 min stimulation period over the B value for the same perifusion chamber. The kinetic pattern of the response was analyzed by index I, defined as the ratio of the T_4 released per minute in a given sample over the Bi value for the same chamber. Imax and $I_{0\longrightarrow 6}$ represented, respectively, the peak and the mean response during the first six minutes.

Results were expressed as means $\pm$ S.E.M. Comparisons were made using the unpaired Student's t test.

RESULTS

Rat Thyroid Fragments

With KRBG alone, the mean rate of release (R) was always less than 1. The mean R value for 15 experiments was 0.82 ± 0.05. The I value decreased exponentially during the stimulation period. Glucagon was tested at four different concentrations. A dose-response correlation was found between R and the glucagon concentration (Fig. 1). A glucagon concentration as low as 0.07 ng/ml induced a significant response : $R = 1.28 \pm 0.07$, $n = 12$ ($p<0.001$ vs KRBG). The highest response was obtained with 7 ng/ml glucagon : $R = 1.61 \pm 0.13$, $n = 16$.

The peak Imax was reached early, generally within the first six minutes (Fig. 2). Like $I_{0\longrightarrow 6}$, it increased as the glucagon concentration was raised and was maximal for 7 ng/ml (Fig. 1). After the sixth minute of the stimulation period, the response was almost the same for all the glucagon concentrations (gradual decline) (Fig. 2).

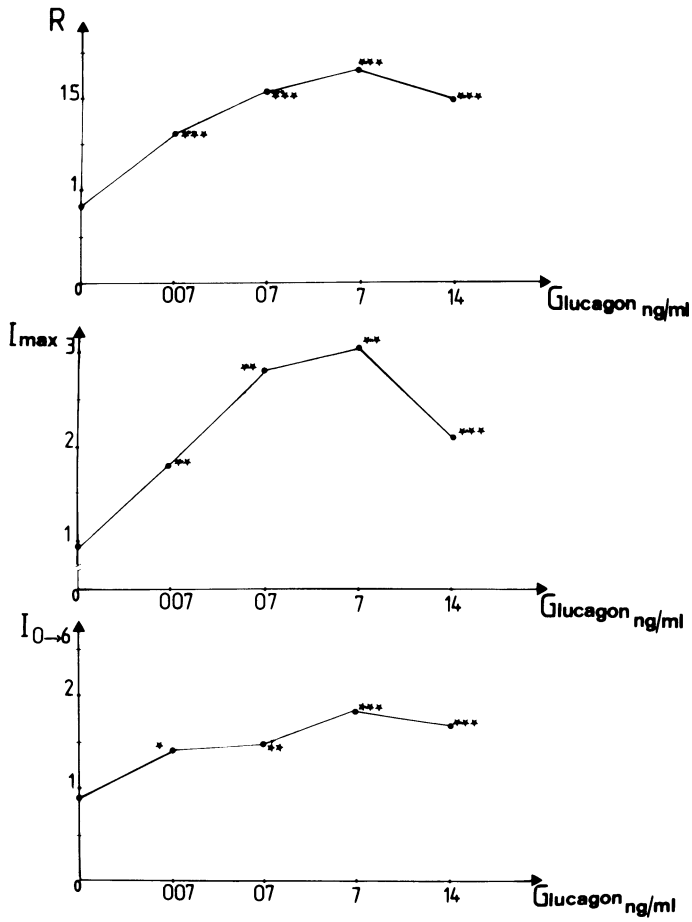

Fig. 1. Perifusion of rat thyroid fragments. Mean rate (R) and peak (Imax) of T_4 release, and mean response of T_4 during the first 6 minutes ($I_{0 \longrightarrow 6}$), with increasing concentrations of glucagon. Comparison with KRBG : P value *<0.025; **<0.005; ***<0.001.

10^{-3} mol/l theophylline (n = 7) induced a significant response vs KRBG : R = 1.04 $\pm$ 0.10 ($p<0.05$), Imax = 1.44 $\pm$ 0.17 ($p<0.001$). The peak was reached early and the response was brief (Fig. 2).

10^{-3} mol/l theophylline perifused simultaneously with glucagon enhanced the response induced by 0.07 ng/ml and 0.7 ng/ml glucagon but not the response with 7 ng/ml (Fig. 2). The difference was significant for 0.7 ng/ml glucagon.

Human Thyroid Fragments

As with rat thyroid fragments, the T_4 response to KRBG alone gradually decreased. The mean R value for eight experiments was 0.77 $\pm$ 0.08.

Two different glucagon concentrations were tested. A stimulatory effect was found with 3 ng/ml glucagon (R = 1.11 $\pm$ 0.39, n = 3) and with 7 ng/ml (R = 2.02 $\pm$ 0.30, n = 6, $p<0.001$ vs KRBG). As with the rat fragments, the response decreased as soon as the stimulation ceased.

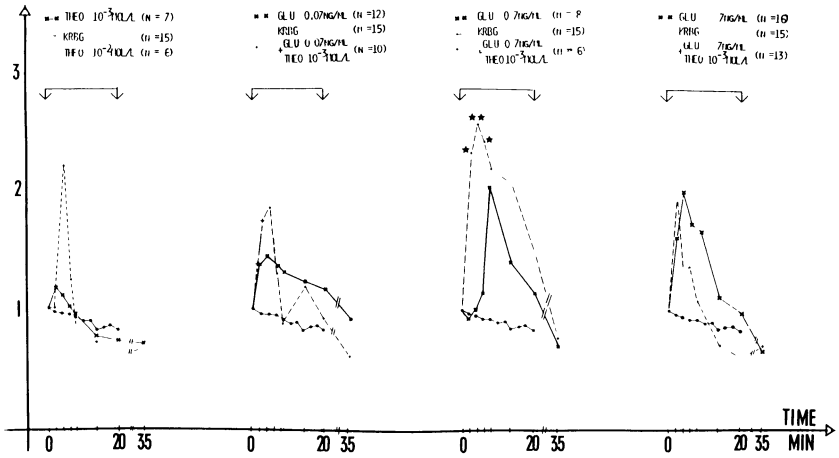

Fig. 2. Perifusion of rat thyroid fragments. Kinetic pattern of T_4 response to different theophylline (THEO) and glucagon (Glu) concentrations and to a simultaneous perfusion of Glu + THEO. Each stimulation period lasted 20 min. Comparison with 0.7 ng/ml Glu: *$p<0.05$; **$p<0.025$.

DISCUSSION

The present study shows evidence of the direct stimulatory effect of glucagon on T_4 secretion, for both rat and normal human thyroid fragments. The T_4 response rapidly rises, and ceases at the end of the stimulation period. the kinetic pattern is similar to that for theophylline and bovine TSH which we have reported in a previous study (11). Moreover, the response is enhanced by simultaneous stimulation of theophylline and glucagon, but only for the lower concentrations of glucagon. The results of the kinetic pattern for glucagon alone and the potentiation of the glucagon effect by theophylline argue in favor of activation of the adenylcyclase system by glucagon in thyroid cells as well as in hepatocytes (12). It has been shown that glucagon does not interfere with TSH binding on thyroid cell membrane receptors (13). However, the exact mechanism involved in the action of glucagon on thyroid secretion remains to be clarified.

The fact that glucagon stimulates T_4 release in vitro at concentrations close to physiological plasma concentrations argues in favor of the possibility of a similar effect in vivo. Such an effect could be compared with the effect induced by catecholamines and other stress hormones.

In stress situations there is a marked increase in glucagon secretion as in catecholamine secretion. Hyperglucagonemia might thus cause transient hyperthyroxinemia. These findings have potentially important implications with regard to the role played by glucagon, as well as catecholamines, in triggering thyrotoxicosis and certain kinds of transient euthyroid hyperthyroxinemia (14).

REFERENCES

1. Mouradian M and Abourizk N. Diabetes Care 6: 512, 1983.
2. Doar JWH, Stamp TCB, Wynn V, et al. Diabetes 18: 633, 1969.
3. Kabadi UM and Eisenstein AB. Clin Res 26: 630A, 1978.

4. Gerich JE, Langlois M, Noacco G, et al. J Clin Invest 58: 320, 1976.
5. Fisher M, Sherwin RS, Hendler R, et al. Proc Natl Acad Sci USA 73: 1735, 1976.
6. Gavin LA, Mc Mahon FA, and Moeller M. Diabetes 30: 694, 1981.
7. Dillman WH, Bonner RA, and Oppenheimer JH. Endocrinology 102: 1633, 1978.
8. Madsen N. Acta Endocrinol 85: 760, 1977.
9. Maayan ML and Ingbar SH. Endocrinology 87: 588, 1970.
10. Melander AE, Rankley E, Sundler F, et al. Endocrinology 97: 332, 1975.
11. Attali JR, Darnis D, Valensi P, et al. J Endocr 102: 43, 1984.
12. Pohl SL. J Biol Chem 246: 4447, 1971.
13. Goldfine ID, Amir SM, Ingbar SH, et al. Biochim Biophys Acta 448: 45, 1976.
14. Borst GC, Eil C, and Burman KD. Ann Intern Med 98: 366, 1983.

EFFECTS OF PHORBOL ESTERS ON PROTEIN PHOSPHORYLATION AND FREE T_3 RELEASE BY MOUSE THYROID LOBES

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Phospholipid-dependent calcium-activated protein kinase, previously identified and termed protein kinase C (1), is known to be widely distributed among various tissues. Although many proteins can be substrates of protein kinase C, little is known about its physiological role. Diacylglycerol, a major degradative product of phosphatidylinositol (PI) turnover, was reported to stimulate protein kinase C in vitro (2). Previously, Igarashi and Kondo (3) have clearly shown that a transient increase of diacylglcerol is elicited by TSH in hog thyroid follicles. We, therefore, wished to know whether thyroid protein kinase C has a role in thyroid physiology. In the present paper, we report some aspects of protein kinase C in mouse thyroid lobes.

MATERIALS AND METHODS

Chemicals

12-Q-Tetradecanoyl-phorbol-13-acetate (TPA), 4β-phorbol-13-acetate, phorbol-12-13-didecanoate, and phorbol were purchased from Sigma Chemical Co. ^{32}P-(PO_4) was obtained from New England Nuclear.

^{32}P-(PO_4) Labeling of Thyroid Glands

Mouse thyroid lobes were resected. Labeling of lobes was performed by incubating them for 1 h at 37°C with 1.0 mCi/ml of carrier-free ^{32}P-(PO_4) in fresh phosphorylation buffer (0.14 M NaCl, 15 mM Tris-HCl (pH 7.5), and 5.5 mM glucose) as described by Lyon et al. (4). Next, the lobes were immersed in the phosphorylation buffer without ^{32}P-(PO_4), and then the incubation was carried out at 37°C for 30 min with or without the stimulators. The incubation was terminated by transferring the lobes into 200 µl of stop solution, which contained 5% sodium dodecyl sulfate and 10% 2-mercaptoethanol, after which the lobes were immediately homogenized with glass-glass homogenizers. The samples were heated to 100°C for 3 min, and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli et al. (5). The gels were stained with Coomassie brilliant blue. For autoradiography, destained gels were dried and exposed to Kodak AR films.

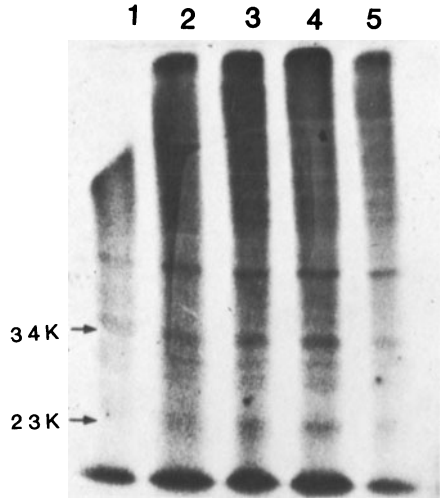

Fig. 1. Effects of phorbols on phosphorylation of proteins from mouse thyroid lobes. After labeling the lobes, TPA and other phorbol derivatives at the concentration of 100 ng/ml were added to the incubation medium. Thyroid homogenates were subjected to SDS-PAGE and then to autoradiography. The separating gel was 15% acrylamide containing 0.1% SDS. Lane 1, phorbol; Lane 2, phorbol-13-acetate; Lane 3, phorbol-12-13-didecanoate; Lane 4, TPA; Lane 5, control.

Free T_3 Release

Thyroid lobes (one thyrotracheal unit/tube) were placed in 5 ml tubes containing 0.5 ml Krebs-Ringer bicarbonate buffer (pH 7.4) and 50 mg/100 ml glucose. The tubes were gassed with 95% O_2-5% CO_2, capped, and incubated at 37°C for 4 h. Free T_3 released into the medium was measured by commercially available radioimmunoassay kits from Amersham. Statistical analysis of the significance of difference between groups was done by means of Student's t test.

RESULTS

Fig. 1 shows that 34 K and 23 K proteins were phosphorylated by adding TPA at a concentration of 100 ng/ml. The phosphorylation of these proteins was stimulated by TPA, followed by phorbol-12-13-didecanoate, 4β-phorbol-13-acetate, phorbol, and control. Fig. 2 shows the stimulating effect of TPA on free T_3 release by mouse thyroid lobes. The lowest concentration which significantly stimulated free T_3 release was 50.0 ng/ml. TPA at concentrations up to 200 ng/ml also enhanced the free T_3 release. In contrast, a higher concentration of TPA at 500 ng/ml had little effect.

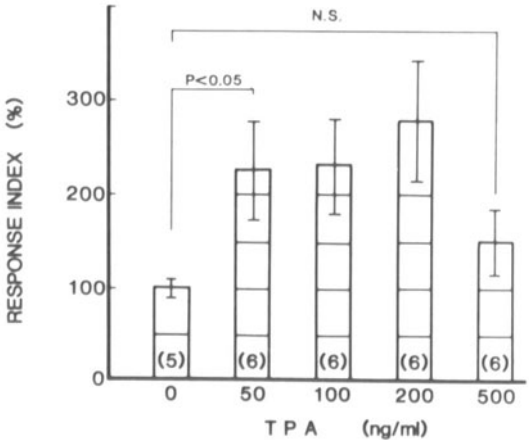

Fig. 2. Effects of varying concentrations of TPA on free T_3 release by mouse thyroid lobes. Bars and vertical brackets indicate mean ± SEM. The numbers of determinations made for each group are indicated in parentheses.

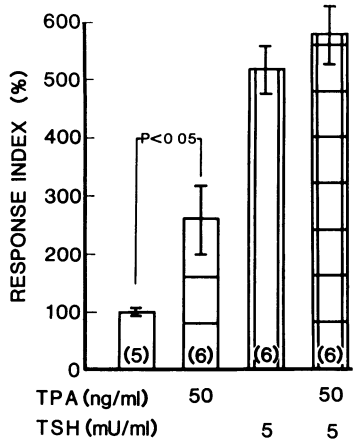

Fig. 3. Failure of TPA to affect TSH-stimulated free T_3 release by mouse thyroid lobes. The concentrations of TSH and TPA are indicated in the figure.

Fig. 3 shows the effect of TPA on TSH-induced free T_3 release by mouse thyroid lobes. TSH at a concentration of 5 mU/ml caused 5-fold increase in free T_3 release. On the other hand, TPA was not so potent as TSH in stimulating free T_3 release. TPA did not potentiate TSH-stimulated free T_3 release.

Fig. 4 shows the effect of TPA on DBC-stimulated free T_3 release. TPA at a concentration of 50 ng/ml was as potent as DBC at a concentration of 200 μg/ml in stimulating the free T_3 release. TPA had no effect on DBC-stimulated free T_3 release, either.

Fig. 5 shows the autoradiographic comparison of TPA and A23187-stimulated phosphorylation. A23187 (5 μg/ml with 20 mM calcium) did not significantly stimulate free T_3 release (control, 100 $\pm$ 21% vs A23187, 123 $\pm$ 10%) (unpublished data). On the other hand, the same concentration of A23187

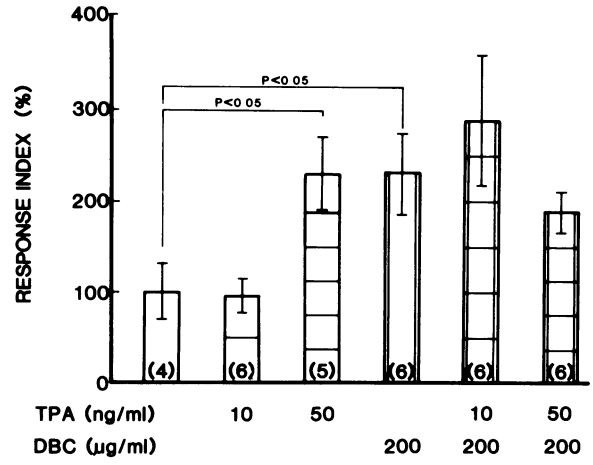

Fig. 4. Failure of TPA to affect DBC-stimulated free T_3 release by mouse thyroid lobes. The concentrations of DBC and TPA are indicated in the figure.

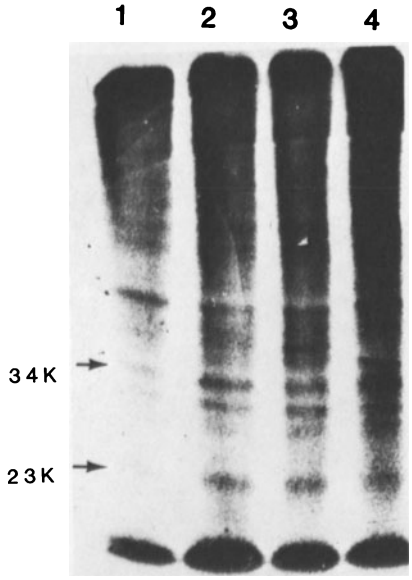

Fig. 5. Effect of A23187 on the phosphorylation of proteins from mouse thyroid lobes. A23187 (5 μg/ml) and calcium (20 mM) were added to the incubation medium. Lane 1, control; Lane 2, A23187 with 20 mM calcium; Lane 3, TPA; Lane 4, A23187 with TPA + 20 mM calcium. The separating gel was 15% acrylamide gel.

and calcium significantly stimulated protein phosphorylation in mouse thyroid lobes. It was also observed that the autoradiographic pattern in the presence of A23187 was similar to that of TPA.

DISCUSSION

It has been reported that tissue distribution of phorbol diester receptors is the same as that of protein kinase C activities (6). Therefore, TPA was thought to bind protein kinase C directly and to activate it. Biological responses reportedly induced by TPA include the stimulation of aggregation and serotonin release by platelets (7) and increased secretion of prolactin. In the present study, 34 K and 23 K proteins were specifically phosphorylated by active phorbols. The stimulating activity of phorbol on 34 K phosphorylation was proportional to the stimulatory activity and to the affinity of solubilized protein kinase C (8). Thus, TPA appears to stimulate free T_3 release by activating protein kinase C and phosphorylating its endogenous substrates. TPA did not potentiate DBC- or TSH-stimulated free T_3 release. Hence, TPA appears to occupy the same pathway as TSH and DBC in stimulating the hormone secretion.

Although the autoradiograph of A23187-induced protein phosphorylation was similar to that of TPA, A23187 itself was not effective in stimulating free T_3 release. The data suggest that this phosphorylation process alone is not enough to cause hormone secretion.

SUMMARY

The effects of phorbols on the degrees of phosphorylation of 34 K and 23 K proteins in our experiments were proportional to those previously reported for the protein kinase C activators. Among the phorbols tested, TPA was the strongest stimulator of protein phosphorylation. In addition, TPA stimulated free T_3 release from mouse thyroid lobes. These results suggest that protein kinase C may play a stimulatory role in thyroid hormone secretion by phosphorylating endogenous substrates.

REFERENCES

1. Takai Y, Kishimoto A, Iwasa Y, et al. J Biol Chem 254: 3692, 1979.
2. Kishimoto A, Takai Y, More T, et al. J Biol Chem 255: 2273, 1973.
3. Igarashi Y and Kondo Y. Biochem Biophys Res Commun 97: 759, 1980.
4. Lyon RM, Stanford N, and Majerus PW. J Clin Invest 56: 924, 1975.
5. Laemmli UK. Nature (Lond.) 227: 680, 1970.
6. Naka M, Nishizuka M, Adelestein RS, et al. Nature 306: 490, 1983.
7. Castagna M, Takai Y, Kaibuchi, et al. J Biol Chem 257: 7847, 1982.
8. Ganonico PL, Judo LM, Koike K, et al. Endocrinology 116: 218, 1985.

ACUTE IN VITRO STIMULATORY EFFECT OF THYROTROPIN RELEASING HORMONE (TRH) ON T_4 SECRETION IN RAT AND HUMAN THYROID

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TRH distribution is ubiquitous. In addition to the hypothalamus, TRH has been found in different parts of the brain, the islets of Langerhans, the testis, the placenta, and amniotic fluid (1). Evidence of TRH immunoreactivity has also been found in the porcine and human thyroid gland (2). Rat thyroid concentrates the injected labeled TRH (3). These findings suggest that TRH may have a direct effect on the thyroid gland. Prolonged intermittent or continuous infusion of TRH in rhesus monkeys at first induced a rise in TSH and T_4, but while TSH went down to its basal value by the third day, T_4 increased up to the ninth day (4). Similar results were reported in fasting humans (5). The late TSH decrease could result from the negative feedback of T_4, but the fact that T_4 continued to rise while TSH decreased suggests a stimulatory effect of TRH on T_4 secretion.

The aim of this work was to test the hypothesis that there is a direct stimulatory effect of TRH on rat and human thyroid. The system we used was thyroid fragment perifusion. One of the advantages of this system is that it permits detailed kinetic studies during short-term stimulations.

MATERIALS AND METHODS

The technique of perifusion of thyroid fragments has been described in detail elsewhere (6). Briefly, the system consisted of four 950 µl perfusion chambers at a constant temperature of 37°C. Krebs-Ringer bicarbonate glucose solution (KRBG) with albumin (final pH = 7.45), gassed with 93% O_2 and 7% CO_2, was used. It was sent through silicone tubes to a peristaltic pump at a constant flow rate of 600 µl/min. Silicone tubes connected the pump to the chambers and also received TRH at different concentrations, at a constant flow rate of 275 µl/min.

Rat and Human Thyroid Tissue

Thyroid lobes were taken from anesthetized male Wistar rats. For each experiment, 3 chopped thyroid lobes weighing in all 40 $\pm$ 2 mg were placed in each of the perifusion chambers. Less than 10 minutes elapsed between the end of the thyroidectomy and the beginning of the perifusion.

Human thyroid tissue was removed surgically from patients undergoing laryngectomies for cancer. Normality of the piece removed was verified by light microscope examination. It was then chopped into fragments. The same amount of tissue was used as that for the experiments with rats. Less than 20 minutes elapsed between removal of the thyroid piece and perifusion.

Experimental Protocol and Timing

In three of the perifusion chambers, thyroid fragments were first perifused with KRBG alone for 30 min (washing period), then stimulated with TRH (Protireline, Roche, France) for 20 min and again washed with KRBG alone. In some instances, after a 60 min washing period, a second similar stimulation was carried out. The fourth chamber received only KRBG and served as a control. Samples were collected every 2-6 min and frozen at -20°C until assay. T_4 was assayed by RIA (RIA gnost T_4, Behring Institute, Marburg, West Germany).

Expression of Results

The response was analyzed by comparing T_4 release during the stimulation period and basal release of T_4. The mean rate of release of T_4 (R) was defined as the ratio of the mean T_4 released per minute during the 20 min stimulation period (Pm) over the T_4 released per minute in the last sample of the preceding washing period (B) : $R = Pm/B$. the kinetic pattern of the response was analyzed by index I, defined as the ratio of the T_4 released per minute in a given sample over the B value for the same chamber. Imax represented the peak value.

Results were expressed as mean $\pm$ S.E.M. Comparisons were made using unpaired and paired Student's t tests.

RESULTS

Perifusion of Rat Thyroid Fragments

With KRBG, T_4 secretion decreased progressively. The mean R value for 15 experiments was 0.82 ± 0.05.

For a TRH concentration as low as 1.7×10^{-11} M, T_4 response was significant: $R = 1.41 \pm 0.34$ ($n = 5$, $p<0.005$ vs KRBG). The response was related to the TRH concentration. The highest R value was obtained with an 8.5×10^{-9} M concentration. The R value for 1.7×10^{-8} M was significantly lower than R for 8.5×10^{-9} M ($p<0.02$), and only slightly above the value obtained with KRBG. Thus, the dose-response curve was bell-shaped.

The kinetic study of the response showed that T_4 secretion rose from the beginning of the TRH stimulation. An early peak was reached in the first six minutes and was followed by one or more peaks. For nearly all the TRH concentrations tested, T_4 release was maintained throughout the stimulation and even 15 minutes after the end of the stimulation. Like R, the highest mean Imax value (Fig. 1) and the highest late T_4 release were obtained with an 8.5×10^{-9} M concentration.

Perifusion of Human Thyroid Fragments

With KRBG, T_4 release also decreased progressively. The mean R value for eight experiments was 0.77 ± 0.08. Two different TRH concentrations were tested. With 1.7×10^{-9} M TRH, the T_4 response was significantly higher than with KRBG : $R = 1.19 \pm 0.17$ ($n = 11$, $p<0.05$). A second stimulation with the same TRH concentration after a 60 minute washing period

with KRBG induced a response which was not significantly different from that with the first one: in 4 experiments, first stimulation : R = 1.01 ± 0.12, second stimulation : 1.12 ± 0.14.

With 1.7 x 10^{-7} M TRH, the T_4 response was higher than with 1.7 x 10^{-9} M: R = 1.32 ± 0.20 (n = 8, $p<0.02$ vs KRBG). A second stimulation with the TRH concentration induced a lower response: R = 0.94 ± 0.08 ($p<0.05$).

The kinetic pattern of the response showed an early peak as with rat thyroid. With 1.7 x 10^{-7} M TRH, T_4 release was maintained throughout the stimulation and even 15 min after the end of it (Fig. 2).

DISCUSSION

The present study demonstrates that TRH directly stimulates T_4 secretion in vitro in both rat and human thyroid. In our perifusion system, human thyroid seems less sensitive than rat thyroid. However, we have not yet tested concentrations as low as 1.7 x 10^{-11} M on human thyroid.

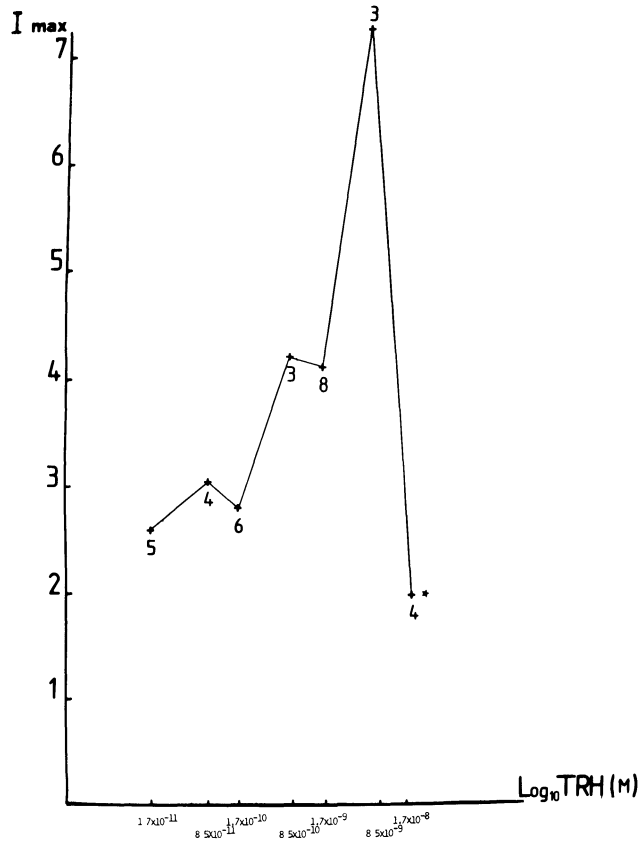

Fig. 1. Perifusion of rat thyroid fragments. Peak value of T_4 release (Imax) with increasing concentrations of TRH. The number of experiments is shown for each concentration; comparison with 8.5 x 10^{-9}: *$p<0.01$.

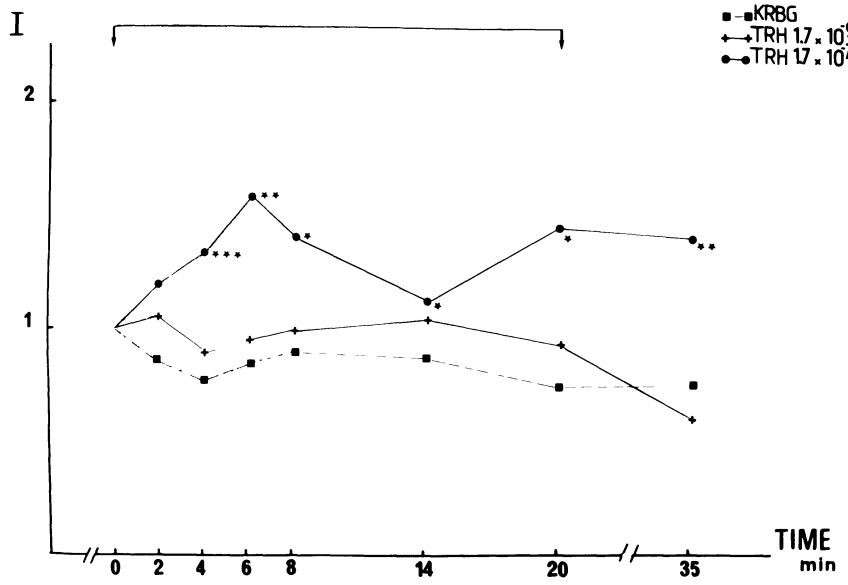

Fig. 2. Perifusion of human thyroid fragments. Kinetic pattern of T_4 response to a first stimulation with 1.7×10^{-9} M TRH (+——+: mean of 11 experiments) and with 1.7×10^{-7} M TRH (●——●: mean of 8 experiments). The ■——■ line represents the kinetic pattern of T_4 secretion with KRBG alone, in 8 experiments. Comparison with KRBG: *$p<0.05$; **$p<0.025$; ***$p<0.001$.

The lowest TRH concentration we tested on rat thyroid fragments (1.7×10^{-11}M, i.e., 3.3 pg.ml) is close to rat or normal human plasma concentrations. This suggests that TRH has a physiologic action on thyroid secretion.

In the rat thyroid experiments, the descending part of the dose-response curve could result from the presence of TRH in excess of the number of thyroid receptors available.

The kinetic response to TRH differs from the responses to theophylline and TSH which we had observed previously using the same perifusion technique (6). With the latter agents, the peak came earlier and the response ceased immediately after the stimulation or even before the end of it. Like TSH of theophylline, TRH might act on the thyroid gland via cyclic AMP. However, the differences in the kinetics of the responses suggests that other mechanisms may be involved in direct TRH stimulation of the thyroid, similar to those already described in TRH stimulation of pituitary TSH secretion. pituitary TSH secretion. In dog thyroid in vitro, TRH inhibited cyclic AMP accumulation induced by TSH or cholera toxin, and this effect was suppressed in a medium deprived of calcium (7). Activation of the adenylate cyclase system by TRH can thus be ruled out. An enhancement of calcium influx could be involved in TRH stimulation of T_4 release.

The direct TRH action on the thyroid gland can be compared with the direct action of another hypothalamic hormone, LHRH, on testis secretion (8,9). Such hypothalamic-releasing hormones which are involved in the regulation of their corresponding peripheral glandular secretions could be considered to be pituitary and peripheral releasing hormones (P.P.R.H.). These findings concerning the double effect of TRH can contribute to a better understanding of certain pituitary and thyroid disorders.

REFERENCES

1. Morley JE. Life Sci 13: 1539, 1979.
2. Iversen R, Laurberg P, and Weeke J. Ann Endocrinol 44: 90A, 1983.
3. Steiner H, Knuzi H, and Studer RO. Experientia 80: 1096, 1974.
4. Pavasuthipaisit K, Norman RL, Ellinwood WE, et al. J Clin Endocrinol Metab 56: 541, 1983.
5. Spencer CA, Lum SMC, Wilber JF, et al. J Clin Endocrinol Metab 56: 883, 1983.
6. Attali JR, Darnis D, Valensi P, et al. Endocr 102: 43, 1984.
7. Delbeke D, Van Sande J, Cochaux P, et al. Biochim Biophys Acta 761: 262, 1983.
8. Hunter MG, Sullivan MHF, Dix CJ, et al. Mol Cell Endocrinol 27: 31, 1982.
9. Bambino TH, Schreiber JR, and Hsueh AJW. Endocrinology 107: 908, 1980.

EFFECT OF CALMODULIN INHIBITORS ON THYROID HORMONE SECRETION

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It is well known that the intracellular free Ca^{2+} plays a role in TSH-induced thyroid hormone secretion (1). Onaya et al. have reported that chlorpromazine inhibits TSH-stimulated endocytosis and glucose oxidation in canine thyroid slices (2). Chlorpromazine inhibits the action of calmodulin which is contained in the thyroid and may act on tubulin assembly-disassembly (3). These findings led us to postulate that calmodulin plays a role in TSH-induced thyroid hormone secretion.

In this study, we investigated the effect of calmodulin inhibitors on TSH-induced thyroid hormone secretion from the rat thyroid *in vivo* and *in vitro* and from dibutyl cyclic AMP (DBC)-induced thyroid hormone secretion *in vitro*.

MATERIAL AND METHODS

The jugular vein of 200 g male Wistar rats was cannulated 24 hours before treatment with calmodulin inhibitors, trifluoperazine (TFP) and N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W-7), or N-(6-aminohexyl)-1-naphthalene sulfonamide (W-5) as the control substance for W-7. Thirty min after intraperitoneal injection of calmodulin inhibitors or the control substance, 2 IU TSH was administered through the jugular vein. Serum triiodothyronine (T_3) and thyroxine (T_4) were measured by radioimmunoassay kits.

To rule out any effect of calmodulin inhibitors on the conversion and degradation of T_4 in peripheral tissue, we studied its *in vitro*-conversion to T_3 by rat liver homogenate in the presence of calmodulin inhibitors, and *in vivo*-degradation of ^{125}I-T_4 after injection of calmodulin inhibitors. Conversion was studied using a modification of the procedure of Visser et al. (4). The degradation of ^{125}I-T_4 was determined, using 200 g male rats cannulated through the jugular vein. Thirty min after intraperitoneal injections of 5 mg W-7, 0.02 mCi ^{125}I-T_4 was administered. The serum taken at various intervals was precipitated by 10% TCA. The radioactivity in the precipitate was then counted.

To check the effect of calmodulin inhibitors on DBC-induced thyroid hormone secretion, one thyroid lobe removed from male Wistar rat was preincubated with 1.5 ml Krebs-Ringer bicarbonate buffer, pH 7.4, in a humidified 5% CO_2 : 95% O_2 atmosphere at 37°C for 30 min and then reincubated for 5 min with the same buffer containing calmodulin inhibitors. After the addition of DBC or TSH in saline into the incubation medium, the medium was taken at 4 hours to measure the concentration of free T_4 by radioimmunoassay.

RESULTS

Effect of Calmodulin Inhibitors on Thyroid Hormone Secretion

In rats pretreated with intraperitoneal injection of 5 mg W-7, a significant inhibition of T_4 secretion was observed at 2, 3, and 4 hours and so did TFP at 3 and 4 hours after injection of 2 IU TSH, whereas pretreatment with saline or 5 mg W-5 did not have this effect (Fig. 1). Pretreatment with 5 mg W-7 suppressed T_3 secretion at 2, 3, and 4 hours and so did TFP at 3 and 4 hours after TSH injection, whereas neither saline nor W-5 affected T_3 secretion (Fig. 2).

Effect of W-7 on Conversion of T_4 to T_3 and Degradation of ^{125}I-T_4

In vitro addition of 80 μM W-7 did not affect conversion of T_4 to T_3 as tested in rat liver homogenate (Table 1). This suggests that W-7 has no effect on deiodinase activity in peripheral tissues. ^{125}I-T_4 (0.02 mCi). was administered intravenously to rats pretreated with 5 mg W-7. W-7 treatment had no influence on degradation of ^{125}I-T_4 compared to the saline-treated control rats.

Effect of Calmodulin Inhibitors on DBC-induced Thyroid Hormone Release

Addition of 1 mg DBC to the incubation medium markedly enhanced release of free T_4 from rat thyroid as compared to TSH. Preincubation of rat thyroid with 100 M W-7 completely inhibited release of free T_4, enhanced by TSH or DBC (Table 2), but preincubation with saline did not.

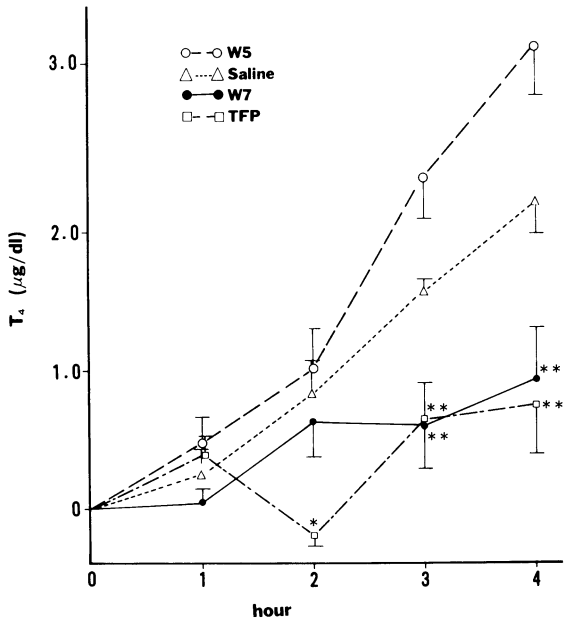

Fig. 1. In vivo effect of calmodulin inhibitors on T_4 release from rat thyroid. Calmodulin inhibitor [W-7 (5 mg, ●——●); TFP (5 mg, □——□), or W-5 (5 mg, o——o)] or saline △——△) was injected intraperitoneally to the rat. Thirty min later, 2 IU TSH were administered through the jugular vein. T_4 values subtracted basal T_4 value (the value at 0 time) are expressed as Δ T_4. Each value represents the mean ± S.E. (n=4). (*$p<0.05$ and **$p<0.01$): statistically significant (Student's t test).

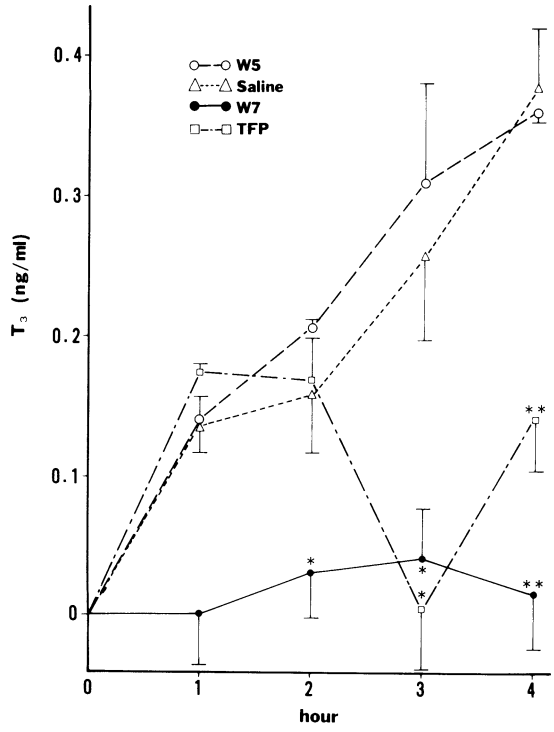

Fig. 2. In vivo effect of calmodulin inhibitors on T_3 release from rat thyroid. Methods and symbols are the same as Fig. 1. T_3 values subtracted basal T_3 value (the value at 0 time) are expressed as ΔT_3. Each value represents the mean ± S.E. (n=4). (*$p<0.05$ and **$p<0.01$): statistically significant (Student's t test).

DISCUSSION

We have shown that calmodulin inhibitors suppress TSH-induced secretion of thyroid hormones from rat thyroid in vivo and in vitro. However, W-5 which has a similar chemical structure to W-7, but no inhibitory effect on calmodulin, did not inhibit TSH-induced thyroid hormone secretion. These findings suggest that calmodulin plays a role in thyroid hormone secretion from the thyroid as Ollis et al. (5) and Kubota et al. (6) have suggested.

Table 1. Effect of W-7 on the Conversion of T_4 to T_3 by Rat Liver Homogenate

	T_3 concentration (ng/ml) incubated at 37°C	T_3 concentration (ng/ml) incubated at 0°C	T_3 production (ng/ml)
W-7 +	1.152 ± 0.023	0.576 ± 0.034	0.576 ± 0.057
W-7 -	1.113 ± 0.006	0.557 ± 0.022	0.556 ± 0.028

The reaction mixture (2.0 ml) contained 0.2 ml supernatant of rat liver homogenate and 0.1 μg T_4 in 50 mM Tris-HCl buffer. The reaction mixture was incubated with or without W-7 (80 μM) dissolved in saline. Each value represents the mean ± S.E. (n=4).

Table 2. Effect of W-7 on TSH- or DBC-induced Thyroid Hormone Secretion from the Incubated Rat Thyroid Lobe

	TSH (-)	TSH W-7	DBC (-)	DBC W-7	(-) (-)
Free T_4 (ng/ml/ 10 mg wet wt)	5.79 ± 0.34	2.74 ± 0.32*	5.80 ± 0.53	1.95 ± 0.21*	2.74 ± 0.24

The thyroid lobe was incubated with TSH (10 m IU/ml) or DBC (1 mg/ml) and W-7 (100 μM). Each value represents the mean ± S.E. (n=4). (*$p<0.01$) statistically significant (Student's t test).

Calmodulin activates not only adenylate cyclase (7,8) but also myosin light chain kinase (9) and related enzymes. Onaya et al. (10) and the authors confirmed that W-7 inhibited DBC-induced thyroid hormone secretion, suggesting that inhibitory site of W-7 for thyroid hormone secretion is subsequent to cyclic AMP formation. It is possible that calmodulin has multiple action sites in the process of TSH-induced thyroid hormone secretion. Further investigations will, however, be necessary to explain this mechanism.

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REFERENCES

1. Zor U, Lowe IP, Bloon G, et al. Biochem Biophys Res Commun 33: 64, 1968.
2. Onaya T, Solomon DH, and Davidson WD. Endocrinology 85: 150, 1969.
3. Marcum JM, Dedman JR, Brinkley BR, et al. Proc Natl Acad Sci 75: 3771, 1978.
4. Visser TJ, Van Der Does-Tobe I, Docter R, et al. Biochem J 150: 489, 1975.
5. Ollis CA, Davier R, Munro DS, et al. Bioscience Reports 4: 695, 1984.
6. Kubota N, Mitsuhashi T, Kuzuya N, et al. Folia Endocrinologica Japonica 59: 641, 1983 (abstract).
7. Valverde I, Vandermeers A, Anjaneyulu R, et al. Science 206: 225, 1979.
8. Le Donne NC and Coffe CJ. Ann NY Acad Sci 356: 402, 1980.
9. Hidaka H, Naka M, and Yamaki T. Biochem Biophys Res Commun 90: 694, 1979.
10. Onaya T, Takazawa K, and Shibata K. Acta Endocrinologica Japonica (in press).

THYROTROPIC ACTIVITY OF ACID ISOELECTRIC VARIANTS OF HUMAN CHORIONIC GONADOTROPIN FROM TROPHOBLASTIC TUMORS

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The occasional occurrence of hyperthyroidism in patients with trophoblastic diseases is widely unexplained and there is yet no proof that hCG is indeed the relevant thyrotropic factor (1,2). Therefore, we studied whether special tumor-derived forms of hCG with enhanced thyrotropic activity may exist.

MATERIAL AND METHODS

Thirty serum samples from 13 patients with nonseminomatous testicular germ cell tumors, 3 from women with choriocarcinoma, and 5 from patients with hydatidiform moles were examined. HCG (+hCG-β) was determined by ^{125}I-hCG anti-hCG (+hCG-β) RIA (3) and ^{125}I-hCG, anti-hCG RIA (cross reaction with hCG-β 2%), hTSH was determined by an ultrasensitive monoclonal hTSH IRMA (4), and free thyroid hormone concentrations of patients' sera by kits (Fa. Henning Berlin).

The biological activity of serum samples, isoelectric focused fractions, and hormone preparations were evaluated by a modified in vitro bioassay according to Atkinson and Kendall-Taylor (5), which determined the T_3 release from thyroid slices. Human and porcine slices were used. Pooled Graves' sera with high T_3-releasing activity, hTSH (3 IU/mg) bTSH (30 IU/mg) in a range from 0.1 to 10 IU/l and pooled negative sera served as controls. A positive response was defined if the T_3 release exceeded the 2 SD range of the pooled negative controls. The adenylate cyclase activity in human slices was determined according to Onaya (6).

The purity of hormone preparations was assessed by pore gradient SDS-electrophoresis (T = 4-22%; + β-mercaptoethanol) on gels (26 x 12.5 cm x 0.48 mm) according to Gorg (7) and stained with Coomassie R-250 or silver. IEF was done on polyacrylamide gels (26 x 12.5 cm; T = 7.7%; C = 3%; pH gradient 3-7; 10°C; 24 h; 2000 V). Thirty slices (7.5 mm) or 47 slices (5 mm) were eluted in 1 ml dest. water. For quantitative determination for acidic and neural pI variants in serum samples of patients, pI markers were visualized by staining. Gelslides pI 3.3-4.0 and 4.0-5.2 were combined, eluted each in 1 ml buffer, and determined by hCG-RIA.

HCG from patients with testicular cancer were purified from ultrafiltrated urine by DEAE-Trisacryl, SP-Trisacryl-M chromatography, gel filtration on Ultrogel AcA 44, and preparative isoelectric focusing. Purity was determined by SDS-electrophoresis (7).

RESULTS

In all but one serum from the tumor patients, but in none of 11 sera of pregnant women, T_3-releasing activity was found in vitro. Two patients with testicular cancer and one patient with molar pregnancy experienced episodes of frank hyperthyroidism. Isoelectric focusing on polyacrylamide gels (PAGIF) of tumor sera (n=15) revealed substantial amounts of acidic isoelectric variants, pI 3.3 to 3.9, which were only barely detectable in pregnancy sera. The percentage of acidic hCG variants pI 3.3-4.0 to total hCG pI 3.3-5.2, as determined by hCG (+hCG-β) RIA of the eluted fractions of PAGIF, varied from 12-45% in sera of tumor patients and from 0-4% in pregnant sera.

We purified the acidic variants of hCG with pI 3.6-3.8 (hCGav) from the urine of cancer patients. The β-subunit of purified hCGav had a slightly higher molecular weight (35,750) than that of hCG CR 119 (34,190) on polyacrylamide electrophoresis. The hCGav showed a dose-dependent stimulation of T_3 release and of cAMP generation from human thyroid slices, whereas the other hCG fractions of IEF had no thyrotropic effect in similar dose levels. The TSH-like activity of hCGav could be roughly estimated as 10 mIU TSH/IU hCGav. Anti-hCG (+hCG-β) antiserum, but not anti-hTSH antiserum, neutralized the biological activity of hCGav (Fig. 1).

DISCUSSION

Our own results underline studies of Carayon (8) that only very high doses of hCG have a weak thyrotropic effect. Therefore, our interest was

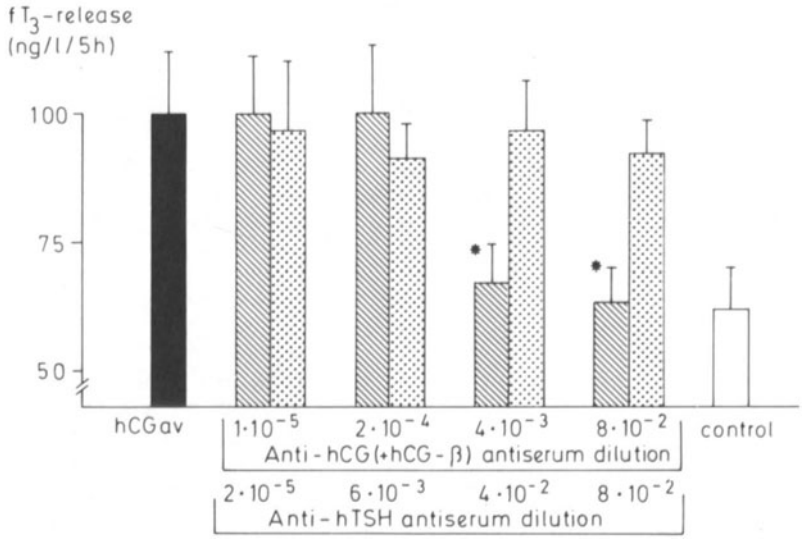

Fig. 1. T_3 release from human thyroid slices by acidic hCG variants pI 3.6-3.8 and neutralization of the thyrotropic activity by anti-hCG (+hCG-β) antiserum. Anti-hTSH antiserum had no effect. ($\bar{x} \pm$ SD, n=5); *p<0.01.

focused on qualitative differences of the hCG molecule in patients with trophoblastic diseases and pregnancy. Serum samples of these patients always contained significant amounts of acidic hCG variants and it was shown that these particulate forms are responsible for the thyrotropic effect in vitro. The biological activity of the purified acidic hCG was roughly estimated by dose response curves. The TSH-like activity of 1 IU hCGav was comparable to 0.01 IU TSH, whereas 1 IU hCG CR 119 (NIAMDD) had a potency of only 0.3 IU TSH. As the hCGav-mediated T_3 release and cAMP formation was inhibited by anti-hCG (+hCG-β) antibodies, but not by anti-TSH antibodies, we assume hCGav as relevant stimulator. However, the clinical significance of these hCG variants remains unclear. In contrast to the high incidence of thyroid-stimulating activity in vitro, we observed rarely frank hyperthyroidism in patients with trophoblastic diseases. The percentage of acidic to total hCG varies extremely under chemotherapy. Thus, in vivo hCGav may not stimulate the thyroid gland for a period long enough to initiate thyrotoxicosis.

REFERENCES

1. Galton VA, Ingbar HS, Jimenez J, et al. J Clin Invest 50: 1345, 1971.
2. Steigbigel NH, Oppenheim JJ, Fishman LM, et al. N Engl J Med 271: 345, 1964.
3. Mann K, Lamerz R, Hellmann T, et al. Oncodevelop Biol Med 1: 301, 1980.
4. Schnorr G, Seidel L, and Strecker H. In International Symposium on the Impact of Biotechnology on Diagnostics, Rome, Italy, 1985, Abstr. No. 84.
5. Atkinson S and Kendall-Taylor P. J Clin Endocrinol Metab 53: 1263, 1981.
6. Onaya T, Kotani M, Yamada T, et al. J Clin Endocrinol Metab 36: 859, 1972.
7. Gorg A, Postel W, Westermeier R, et al. J Biochem Biophys Methods 3: 273, 1980.
8. Carayon P, Amir S, Nisula B, et al. Endocrinology 108: 1891, 1981.

EFFECTS OF NEUROPEPTIDES AND TSH ON THYROID BLOOD FLOW AND HORMONE SECRETION

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The thyroid gland of the rat is now known to receive nerve fibers containing a variety of neuropeptides (NP's) including vasoactive intestinal peptide (VIP) (1), peptide HI (PHI) (2), neuropeptide Y (NPY) (3), and substance P (SP) (4). There is also evidence to indicate that some of these NP's can affect thyroid hormone secretion, particularly TSH-induced secretion. For example, VIP enhances thyroid colloid droplet formation and cyclic AMP production *in vitro* (1,5,6), and it increases the release of radioactive iodine from the thyroid *in vivo* (1). Using the latter index, NPY has been shown to modulate thyroid responsiveness to TSH (3). In contrast, SP is reported to increase thyroid hormone release *in vitro* (7), but apparently has no effect *in vivo* (4). Since the nerve fibers containing these NP's are distributed to both follicles and blood vessels (2), we have initiated some studies of the effects of NP's on thyroid blood flow and on plasma thyroid hormone concentrations in the presence and the absence of exogenous TSH. In our preliminary communications (8,9), we have reported increases in total thyroidal blood flow in response to some of these NP's. However, since flow is a function of both pressure and resistance (either of which may vary), we were not able to determine with certainty whether the NP's had a direct effect on the vascular smooth muscle of the thyroid vessels. We address this issue in the present communication, and we also consider the route by which the NP-containing fibers might reach the thyroid by measuring blood flow and hormone secretion in superior cervical ganglionectomized (SCGX) rats.

MATERIALS AND METHODS

Female Sprague-Dawley rats, weighing approximately 200-300 g, were housed under standardized conditions with access to chow and tap water *ad libitum*. All rats (including SCGX) were purchased from Hilltop, Scottsdale, PA.

Blood flows to the thyroid and other selected organs were determined from the distribution of ^{141}Ce-labeled microspheres after their injection directly into the left ventricle of ketamine-pentobarbital anesthetized rats, as previously described (8). Blood flows were calculated on the basis of a reference sample withdrawn at a known flow rate from the distal aorta at the time of microsphere injection. NP, TSH, or vehicle solutions were infused into a tail vein over two minutes; the volume infused was equal to

the volume withdrawn for the reference sample. Heparinized blood samples were collected before and after infusion for the measurement of plasma T_3 by radioimmunoassay.

Data were analyzed statistically by analysis of variance followed by Dunnett's test, with a $p<0.05$ being accepted as significant.

RESULTS AND DISCUSSION

We have recently reported that thyroid blood flow in the rat is increased by VIP (8) and by PHI (9), but is not significantly altered by equal amounts of NPY or SP (9). However, the systemic administration of these NP's may result in changes in arterial pressure which would alter thyroid blood flow independent of changes in thyroid vessel diameters. For example, administration of a NP might result in a fall in arterial pressure, but no change in thyroid blood flow. In this case, the NP must have dilated thyroid vessels, but flow did not increase due to the fall in arterial pressure. Since pressure is equal to the product of flow and resistance, it is possible to "normalize" flow measurements for changes in pressure by expressing such results as vascular resistance. Alternatively, the vascular conductance (the reciprocal of resistance) is often used for this purpose since it, and its units, are conceptually easier to appreciate. Another advantage of conductance is that it is directly related to flow, whereas resistance is inversely related to flow. In Fig. 1A, we present thyroid vascular conductance measurements during the infusion of several NP's at a total dose of 0.625 µg. These are referred to as "relative" conductances since we have used the mean left ventricular pressure, rather than the mean arterial pressure, in deriving them. The pattern of the results with the first four NP's

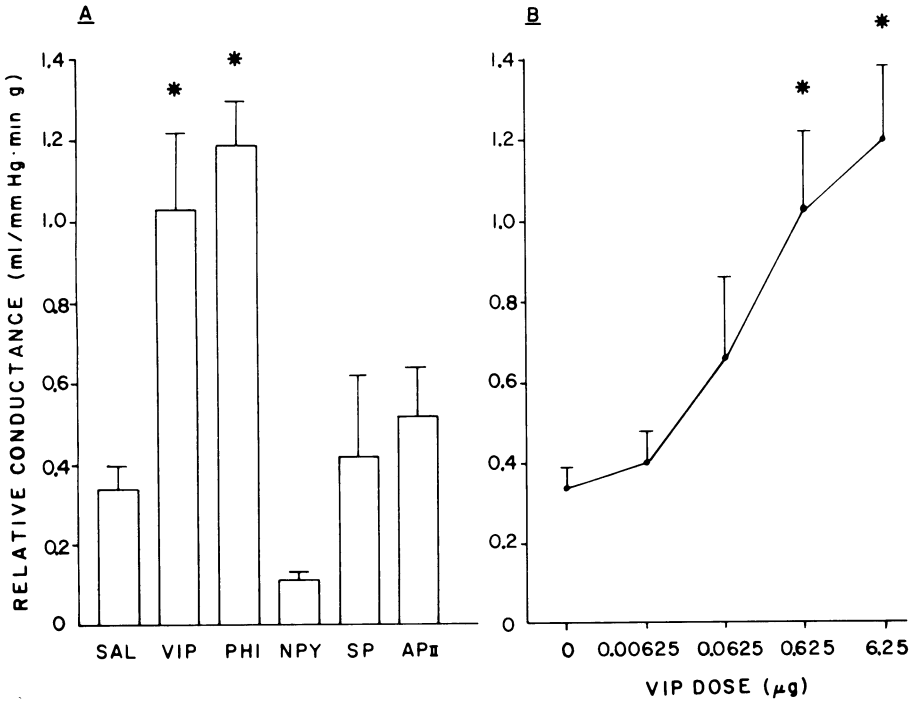

Fig. 1. Effects of NP's on thyroid vascular conductance (*=$p<.05$, n = 5-9).

is similar to our previously reported flow data in that the structural homologs, VIP and PHI, increase conductance and SP has no effect (8,9). This similarity indicates that under these conditions there were no complicating effects on pressure due to the systemic infusions. In the case of NPY, the conductance appears to be reduced, but this was not statistically significant. The additional peptide in Fig. 1A, atriopeptin II (APII), had no effect on thyroid vascular conductance, nor did it affect thyroid blood flow.

A dose-response curve for one of these NP's, NIP, is presented in Fig. 1B. In this case, there is a very clear divergence between these conductance data and the corresponding flow measurements (8). With the highest dose of VIP (6.25 μg), the flow is only approximately 70% of the flow measured at the next lower dose (0.625 μg). In contrast, vascular conductance continues to rise in approximate proportion to the log of the dose over this range. This indicates that thyroid vascular smooth muscle is still responding by vasodilation to VIP at the highest dose tested, but this cannot be detected experimentally when measuring only flow because pressure falls at this highest dose. Thus, the use of conductance (or resistance) instead of flow allows one to more closely relate these effects of systemic infusion of NP's to normal physiology. These are important considerations in the present experiments because the NP's of interest are located in perivascular (and interfollicular) nerve fibers and are, thus, more likely to affect flow (or secretion) by local mechanisms than by systemic distribution via the blood.

We have measured plasma T_3 and T_4 levels in the foregoing experiments and have observed no changes in response to any of the NP's tested. This was somewhat surprising since there is some evidence in the literature (using other indices of thyroid secretion) that several of these substances enhance thyroid hormone secretion (see Introduction). We are currently determining whether a more prolonged treatment with NP's is necessary to increase thyroid hormone concentrations. There is no problem with thyroid responsiveness per se, since TSH will greatly increase plasma T_3 levels under these conditions (see below).

Much of the available information concerning a role for thyroid nerves in modulating secretion suggests that these nerves might affect responsiveness to TSH rather than exerting much of a direct effect on their own. Given this information, and the fact that the experiments above revealed no effect of the NP's on plasma thyroid hormone levels, we have begun a series of experiments to determine the effects of these NP's in combination with TSH on both thyroid blood flow and hormone secretion. Chronic treatment with PTU (2 mg i.p./day for one week) induced a sixfold increase in endogenous TSH levels (secondary to the nondetectable T_3 and T_4 levels), and increased thyroid blood flow fivefold (8). Thus, our method of measurement can certainly detect TSH-induced changes in thyroid blood flow - at least with this prolonged exposure to high TSH. However, a two minute infusion of TSH that causes a significant elevation in T_3 levels fails to alter thyroid blood flow during infusion or two hours afterward. We are now testing longer infusions of TSH to find a treatment regimen that will increase both secretion and flow to be combined with NP's in subsequent experiments.

It is already known that certain thyroid nerve fibers, such as the NPY-containing fibers (3), originate in the superior cervical ganglion (SCG). In contrast, SP-containing fibers apparently do not derive from this ganglion, since sympathectomy fails to diminish the density of these fibers in the thyroid (4). To determine the potential importance of the SCG in regulating thyroid function, we have assessed thyroid blood flow and have measured basal and TSH-stimulated T_3 levels 10-15 days after surgical SCGX. As shown in Fig. 2A, there is no effect of this procedure on thyroid blood flow in the presence or absence of exogenous TSH. T_3 concentrations (basal

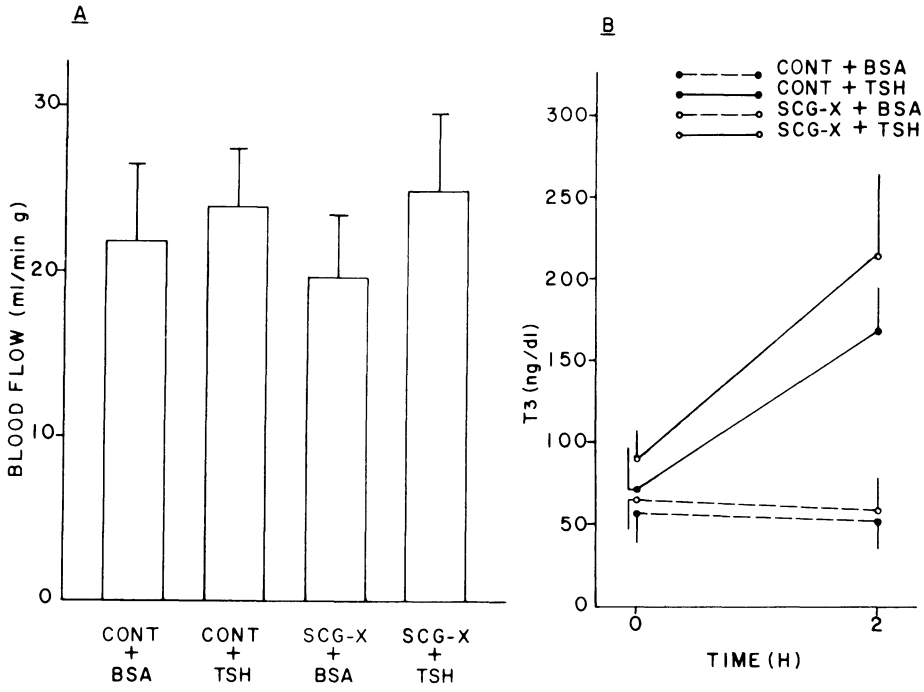

Fig. 2. Effects of SCGX on thyroid blood flow and plasma T_3 levels before and after 125 mU TSH/100 g (n = 9-13).

and stimulated) tend to be higher in SCGX rats (Fig. 2B), but this is not statistically significant. These flow data suggest that the thyroid nerve fibers containing NP's that did affect thyroid blood flow (VIP and PHI) are not likely to derive from the SCG.

REFERENCES

1. Ahren B, Alumets J, Ericsson M, et al. Nature 287: 343, 1980.
2. Hedge GA, Huffman LJ, Grunditz T, et al. Endocrinology 115: 2071, 1984.
3. Grunditz T, Hakanson R, Rerup C, et al. Endocrinology 115: 1537, 1984.
4. Ahren B, Grunditz T, Ekman R, et al. Endocrinology 113: 379, 1983.
5. Molinero P, Calvo JR, Goberna R, et al. Bioch Bioph Res Comm 128: 1336, 1985.
6. Toccafondi RS, Brandi ML, and Melander A. J Clin Endocrinol Metab 58: 157, 1984.
7. Yamashita K, Koide Y, and Aiyoshi Y. Life Sci 32: 2163, 1983.
8. Huffman L and Hedge GA. Endocrinology (in press).
9. Huffman L and Hedge GA. 67th Meet Endo Soc: 238, 1985 (abstract).

EFFECT OF PROTEIN KINASE C ACTIVATION ON THYROID CELL GROWTH AND FUNCTION*

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Activation of protein kinases and alterations in calcium fluxes are early events in the transduction of signals leading to cell proliferation and/or differentiation by hormones and other agents with cell-surface receptors (1). TSH is known to stimulate cAMP production and the cAMP-dependent protein kinase, protein kinase A. TSH stimulation results in many differentiated functions, e.g., iodide uptake and organification and thyroglobulin synthesis (2,3). In addition, there are reports of stimulation of Ca^{2+} efflux (3) and activation of another kinase known as protein kinase C (4) in response to TSH. We have previously shown that both the tumor-promoting phorbol ester, tetradecanoylphorbol acetate (TPA), a known stimulator of protein kinse C and epidermal growth factor (EGF) antagonize TSH-mediated differentiated functions, i.e., cAMP accumulation, iodide uptake and organification and thyroglobulin synthesis (5,6). This inhibition is not due to toxic effects on the cells, since both factors stimulate thyroid cell growth while inhibiting differentiation. The interaction of pathways stimulated by protein kinase A, protein kinase C, and Ca^{2+} remain unexplored in the thyroid gland. In several other systems, calcium mobilization and protein kinase C activation have been shown to act synergistically to produce a biological effect (1,7). In this paper we examine the effects of putative stimulators of protein kinase C on thyroid cell growth and differentiation.

METHODS

Sheep thyroid cells were cultured as previously described (5). FRTL-5 cells were a generous gift from Dr. L. Kohn (N.I.H.) and were maintained as described (8). The soluble analog of diacylglycerol, sn-1, 2-dioctanoylglycerol (diC8) was the generous gift of Drs. G. Ganong and R. Bell, Duke University. Oleylacetylglycerol (OAG) was obtained from Molecular Probes (Junction City, Oregon). Binding studies and iodide uptake and organification studies were performed as described previously (6,7) using 10^{-7} M NaI and 1 μCi/ml ^{125}I for the latter.

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Table 1. Effect of Protein Kinase C Stimulators on ^{125}I-EGF Binding

	cpm ^{125}I-EGF x 10^{-3} specifically bound	% control
Control	15.0 ± 0.5	100
OAG 19^{-4} M	10.0 ± 0.3	68
DiC8 2.5 x 10^{-4} M	10.3 ± 0.1	69
TPA 10^{-7} M	3.0 ± 0.2	20

Cells were preincubated with the compounds as noted for 60 minutes. Binding of ^{125}I-EGF was performed as previously described (5).

RESULTS

The EGF receptor is thought to be phosphorylated by protein kinase C which results in its down-regulation (9). Consequently, inhibition of binding of ^{125}I-EGF to thyroid cells is a measure of protein kinase C activation. The abilities of TPA and the synthetic diacylglycerols to down-regulate the EGF receptor are shown in Table 1. TPA, used at a concentration which stimulates growth in thyroid cells, caused a marked inhibition of ^{125}I-EGF binding. The synthetic diacylglycerols, diC8 and OAG, also produced significant down-regulation of the EGF receptor. However, they were not as effective as TPA in this assay.

We have previously shown that TPA and EGF dramatically inhibit iodine metabolism in sheep thyroid cells (5,6). In Fig. 1 we compare the potency

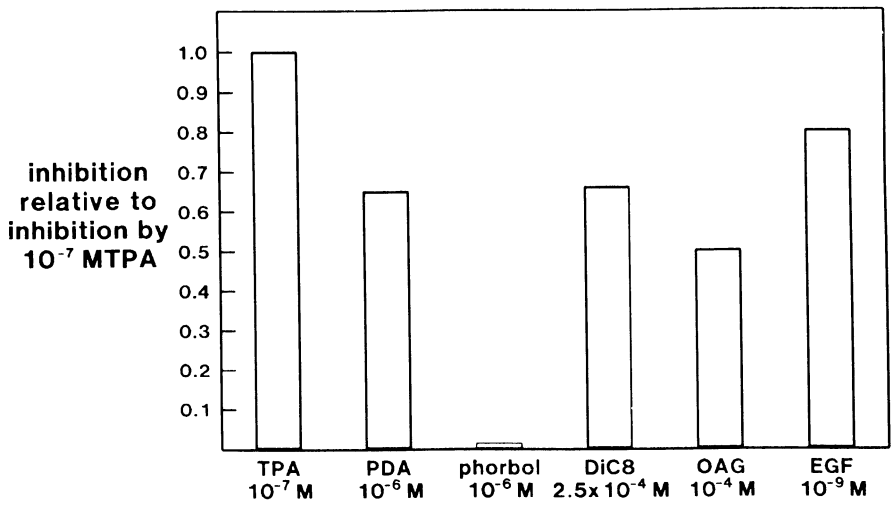

Fig. 1. The relative potency of putative protein kinase C stimulators on inhibition of iodine metabolism in sheep cells. The results are expressed as inhibition relative to inhibition by 10^{-7} M TPA.

of TPA and other phorbol derivatives and synthetic diacylglycerols on their ability to inhibit iodine metabolism. The results are expressed as relative inhibition compared to TPA. Although the synthetic diacylglycerols were effective inhibitors of iodine metabolism, their potencies were significantly lower than that of TPA. One explanation for this could be that at the relatively high concentration of TPA (10^{-7} - 10^{-8} M) used to effect cell growth and inhibition of iodine metabolism, TPA could also be altering membrane events and even Ca^{2+} mobilization. We, therefore, investigated the interaction of low concentrations of OAG and a calcium ionophore to determine if these compounds act synergistically to inhibit iodide uptake. In other systems, a synergistic reaction between calcium mobilization and protein kinase C activation has been shown to occur, e.g., in proliferation of BALB/C 3T3 cells (10) and macrophage-depleted human peripheral lymphocytes (11), in catecholamine release from bovine adrenal medulla, and in insulin release from rat pancreatic islet cells (1).

Although the calcium ionophores inhibited iodide uptake significantly at concentrations used in other systems (7), we did not see any synergistic effect between OAG and the ionophore, ionomycin, as shown in Fig. 2. Short-term experiments performed in salt solutions $\pm$ Ca^{2+} showed that absence of Ca^{2+} did not prevent either EGF or TPA inhibition of iodide uptake by sheep cells. These data argue against a role for calcium mobilization in inhibition of iodide uptake.

In the FRTL-5 cell line we found that calcium ionophores at 10^{-7}M had a small stimulatory effect on growth. However, we did not see a synergistic interaction between OAG and the ionophores. Both TPA and, to a lesser extent, OAG were mitogenic for these cells, although TSH was considerably more effective than either. We found no additivity between the effects of TPA and TSH on cell growth in these cells, suggesting (a) that TSH and TPA may share a common pathway for growth stimulation and (b) that stimulation of cAMP production plays an important role in FRTL-5 cell growth. We examined the substrates phosphorylated by TSH and TPA in sheep cells using SDS-polyacrylamide gel elecctrophoresis. In the cytosol fraction, prepared by

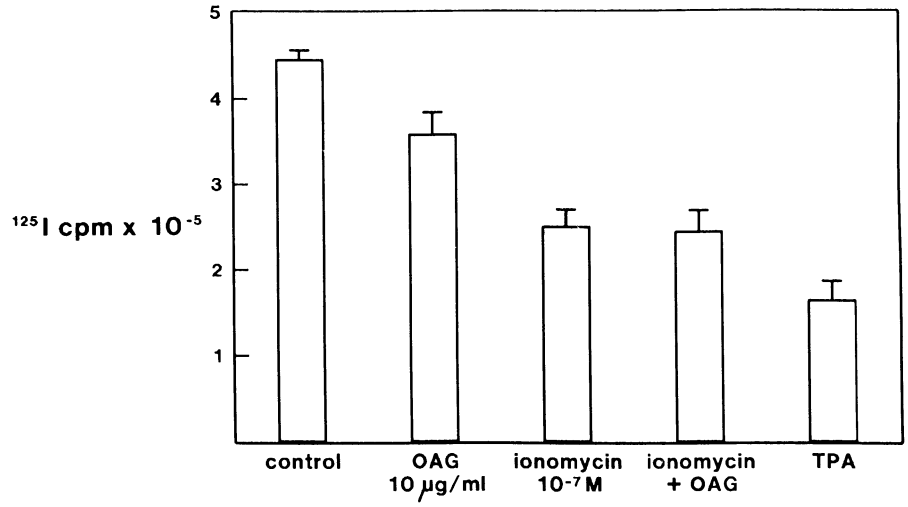

Fig. 2. Lack of synergism between oleylacetylglycerol (OAG) and ionomycin on inhibition of thyroid iodide uptake. Cells were preincubated with the compounds as noted for 4 h and labeled with 1 µCi ^{125}I in 10^{-7} M NaI for 2 h.

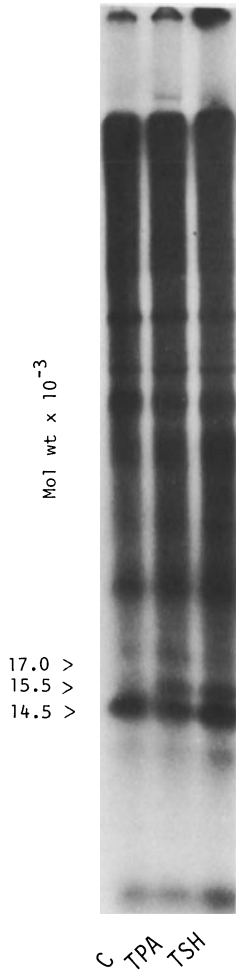

Fig. 3. Effect of TPA and TSH on ^{32}P-protein isolated from the nuclei of sheep cells. Cultures were incubated for 2 h in phosphate-free medium containing 20 µCi/ml ^{32}P-orthophosphate. After treatment with 10^{-7} M TPA for 20 minutes, nuclei were isolated by solubilizing the cell membrane in 0.1% Triton X-100 and pelleting the nuclei. Nuclei were precipitated with TCA and samples dissolved in SDS-PAGE sample buffer and analyzed on 10% polyacrylamide gels.

solubilizing ^{32}P-labeled cells with 0.1% Triton X-100, we found several differences in protein phosphorylation patterns within 2 minutes of TPA addition. In the nuclear fraction we saw several significant changes in the phosphorylation of low molecular weight polypeptides as shown in Fig. 3 and marked by arrows. These low molecular weight proteins were found to be present in large quantities when the gel was stained with Coomassic blue and probably represent histone fractions. These changes were evident 20 minutes after TPA or TSH addition. These protein phosphorylation patterns using synthetic diacylglycerols to stimulate protein kinase C, forskolin to stimulate protein kinase A, and calcium ionophores should reveal some of the interactions between these three pathways to stimulate growth and/or differentiation.

DISCUSSION

Treatment of thyroid cells with TPA, OAG, diC8, or EGF results in thyroid cell growth and inhibition of iodine metabolism. Although less potent than TPA itself, the synthetic diacylglycerols clearly show that activation of protein kinase C is responsible in part for these effects. The role of calcium mobilization in these processes is more difficult to ascertain. Calcium-calmodulin activation mediates the activity of many enzymes, e.g.,

phosphodiesterase and another protein kinase with substrates different from those of protein kinase A or protein kinase C. Both calmodulin and protein kinase C are thought to regulate Ca^{2+} efflux from cells (7). Although our studies with Ca^{2+} free media and using OAG with ionophores suggest no role for calcium in eithr stimulation of cell growth of inhibition of iodide uptake, the intracellular levels of calcium are not known. Further studies using the fluorescent reagent, quin 2, to measure intracellular Ca^{2+} levels are currently underway to examine this mechanism more precisely.

REFERENCES

1. Nishizuka Y. Nature 308: 693, 1984.
2. Errick JE, Eggo MC, and Burrow GN. In MC Eggo and GN Burrow (eds), Thyroglobulin - The Prothyroid Hormone, Raven Press, 1985, p 271.
3. Dumont JE, Roger P, Servis P, et al. In MC Eggo and GN Burrow (eds), Thyroglobulin - The Prothyroid Hormone, Raven Press, 1985, p 283.
4. Friedman Y, Poleck T, and Burke G. 67th Annual Meeting Endocine Soc, abstract 700, 1985.
5. Bachrach LK, Eggo MC, and Burrow GN. Endocrinology 116: 1603, 1985.
6. Eggo MC, Bachrach LK, Fayet G, et al. Mol Cell Endocrinol 38: 141, 1984.
7. Rasmussen H, Kojima I, Kojima K, et al. Adv in Cyclic Nucleotide and Protein Phosphorylation 18: 159, 1985.
8. Valente WA, Vitti P, Kohn LD, et al. Endocrinology 112: 71, 1983.
9. Fearn JC and King AC. Cell 40: 991, 1985.
10. Donnelly TE, Littler R, and Scholar EM. Biochem Biophys Res Commun 126: 741, 1985.
11. Kaibuchi K, Takai Y, and Nishizuka Y. J Biol Chem 200: 1366, 1985.

CONTROL OF PROLIFERATION AND DIFFERENTIATION IN PRIMARY CULTURES OF CALF THYROID CELLS*

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The proliferation of follicular cells and selected gene expression in the thyroid gland in vivo, is mainly controlled by hormones, neurotransmitters, and growth factors either directly or indirectly by means of second messengers (cyclic nucleotides...). Several in vitro thyroid models have been described (1-9). Studies of the effects of different substances on proliferation and differentiation using thyroid cell lines may have little relevance to in vivo situations, whereas data obtained from primary culture of thyroid cells of differing origins often give dissimilar or contradictory results. In addition, the probes available to study thyroglobulin gene expression originate from species different from those used for existing primary cultures. We have, therefore, developed a model consisting of calf follicular cells in primary culture which allows the study of important mechanisms such as proliferation and differentiation, and of the role played by cAMP or growth factors in both processes in vitro. This model gives us the opportunity to use bovine cDNA probes established in our laboratory (10) under conditions of perfect homology with the genetic material used. This will allow a functional and structural study at the molecular level of the thyroglobulin gene expression and of its control promoter in different conditions of stimulation.

Characteristics of the Culture System

Follicles were obtained by enzymatic digestion (collagenase 1 mg/ml, Dispase 5 mg/ml, DNAse 400 µg/ml) from minced calf thyroid tissue obtained from a local slaughterhouse and purified by several low speed centrifugations. They were seeded in Petri dishes and maintained in a medium consisting of DMEM + Ham's F_{12} + MCDB104 (2:1:1) (5) supplemented (1) with antibiotics, fungizone, 2 mM glutamine, 5 µg/ml insulin, 1.25 µg transferrin/ml, 10 ng glycyl-histidyl-lysyl-acetate/ml, 10 ng somatostatin/ml, 40 µg ascorbic acid/ml, and 0.1% fetal calf serum. The medium was changed 2 days after seeding and subsequently every 2 or 3 days. Calf thyroid cells were initially well differentiated; just after seeding they transported iodide (measured by radioiodide uptake) and expressed thyroglobulin gene (reflected by the amount of Tg mRNA). They progressively lost these characteristics in the absence of TSH. Many cultivated cells required serum

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†Research fellow of the Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture (I.R.S.I.A.).

to survive and proliferate. Seeded calf thyroid follicles adhered to the plastic substrate both in the absence and presence of serum. However, in the absence of serum or with 0.1% serum, cells remained quiescent in follicle-derived aggregates with few spread out cells. It is not known if these cell aggregates still contain functional follicle lumina. 0.1% serum allowed a better attachment and maintenance of the cells during the 14-18 days of the culture. In such poorly spread out aggregates, cells were morphologically very sensitive to TSH or agents which increase cAMP (Forskolin, cholera toxin). These compounds induced the typical cytoplasmic retraction and arborization observed in other thyroid cell systems (3, 5,11,12). When treated with agents which increase proliferation, the follicles collapsed and the cells spread out and divided. They reached confluency in several days and formed a monolayer with only sparse follicle-derived structures. Under these conditions, the morphological response to TSH was poor although they showed a striking response to Forskolin or cholera toxin. As shown in porcine thyroid cell monolayers (13), these observations could be explained by the inaccessibility of TSH receptors, as these basal pole receptors would be facing the bottom of the culture dish in confluent monolayers of polarized thyroid cells. Polarized monolayers remained, however, responsive to membrane-soluble hydrophobic compounds such as Forskolin, which activates adenylate cyclase without requiring TSH receptors.

Effects of TSH and Agents Which Increase cAMP Levels

TSH is the principal stimulator of specialized and differentiated metabolic pathways in thyroid cells. Most of its physiological actions are mediated by an increase in intracellular cAMP levels, and are mimicked by agents such as Forskolin and cholera toxin which activate their adenylate cyclase.

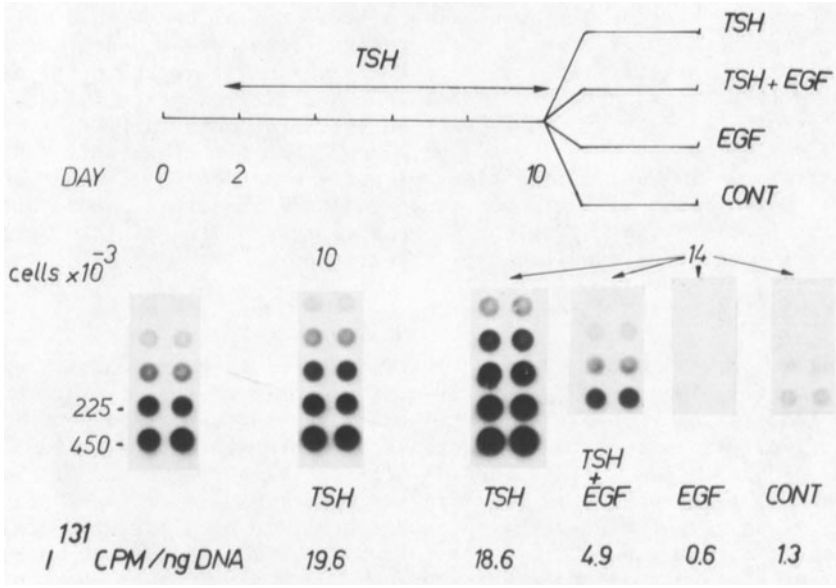

Fig. 1. Tg mRNA levels in cultured calf thyroid cells. Inhibition of Tg gene expressionand iodide trapping by EGF (25 ng/ml). Since day 2, cells were cultured in medium containing 0.1% serum and TSH (1 mU/ml). At day 10, cells were rinsed and cultured as indicated. The Tg mRNA levels were evaluated by cytoplasmic DOT BLOT using cloned bovine Tg cDNA probe ^{32}P labeled by Nick Translation (14).

TSH or Forskokin added the second day and maintained throughout the culture period allowed the calf thyroid cells to remain differentiated; they had a very high radioiodide concentration and high level of Tg mRNA (Fig. 1). The TSH concentration we most frequently used was 100 µU/ml. However, an almost maximal effect on iodide transport was already observed with 10 µU/ml, demonstrating the high sensitivity of bovine thyroid cells to bovine TSH. The cells remained differentiated so long as TSH or Forskolin were added to the medium; if they were removed, the cells eventually dedifferentiated. In the conditions tested, neither TSH nor Forskolin enhanced the proliferation of calf thyroid cells. However, cholera toxin, another high specific permanent activator of adenylate cyclase, potently stimulated DNA synthesis and proliferation. This effect was observed in the presence but not in the absence of 1% serum. Our preliminary data concerning the control of proliferation of calf thyroid cells by TSH and its second messenger cAMP remain, therefore, unconclusive. The discrepancy could be due to differences in cAMP levels induced by the different activators, kinetic differences in cAMP accumulation, biphasic failure of monolayer cells to respond to TSH, etc.

Effect of Serum

Concentrations ranging from 0.1 to 10% of fetal calf serum have been tried. While 0.1% had little effect on proliferation, concentrations as low as 0.3% and 1% when present throughout the culture period resulted in sustained proliferation (Table 1). The addition of 1% serum to the culture medium induced, within a few days, proliferation and the formation of a monolayer consisting of flattened and dedifferentiated cells. Iodide transport was completely inhibited and Tg mRNA levels were very low.

Cellular differentiation, induced by TSH or Forskolin, could be inhibited by increasing medium serum concentration (0.1% - 10%) (0.2% serum inhibited by 50% iodide trapping).

Effects of EGF

EGF had effects similar to those obtained with serum on calf thyroid cells. Added either with 0.1% or 1% serum 2 days after seeding and present throughout the culture period, it greatly enhanced DNA synthesis (Table 1)

Table 1. DNA Synthesis of Calf Thyroid Cells Induced by EGF of FGF

% Serum	% (^{3}H) thimidine-labeled nuclei Cont.	EGF	FGF
0.1	8.5	43.5	51
1	22	51	65

Cells were cultured 2 days after seeding in different medium: EGF (25 ng/ml) + 0.1% serum; EGF + 1% serum; FGF (100 ng/ml) + 0.1% serum: FGF + 1% serum, for 8 days. Tritiated thymidine (10 µCi/ml; 3.10^{-5} M) was added for the last 24 hours of incubation. The DNA synthesis is estimated by counting the percentage of labeled nuclei after autoradiography (6).

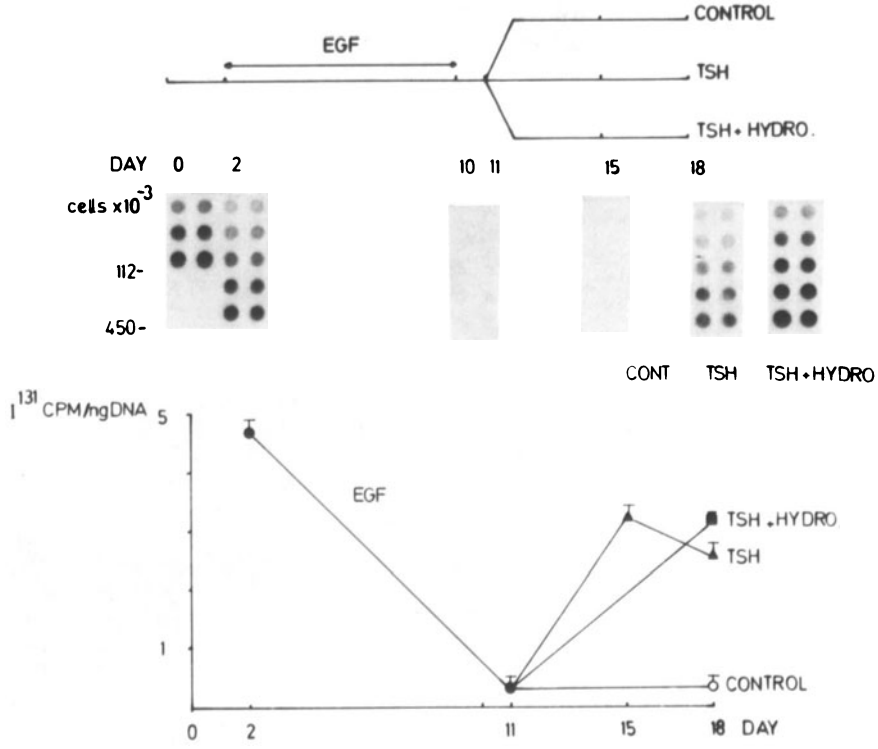

Fig. 2. Tg mRNA levels in cultured calf thyroid cells. Reinduction of Tg gene expression after culture with EGF. Cells were treated for 8 days with EGF (25 ng/ml) and then washed out for 24 hours before adding TSH (100 µU/ml) or TSH + hydrocortisone (100 ng/ml).

and cell proliferation. Cells reached confluency several days after EGF addition, but were completely dedifferentiated (iodide uptake was abolished and Tg mRNA level was undetectable) (Fig. 2). The effects on proliferation of EGF and 1% serum were almost additive. Calf thyroid cell proliferation was also stimulated by another growth factor, fibroblast growth factor (FGF) (Table 1) and by the tumor promoter, Tetradecanoyl Phorbol Acetate (TPA). Cells which are differentiated in the presence of TSH or Forskolin can be induced to dedifferentiate by washing-out the Forskolin or TSH and by adding EGF (25 ng/ml) for several days (Fig. 1). When added together with either TSH or Forskolin, EGF decreased the level or differentiation reached with these agents alone. The inhibitory action of EGF on differentiation was partially reversible. If EGF was removed from the medium and replaced by TSH or Forskolin, a progressive reinduction of iodide uptake and Tg gene expression was observed. Hydrocortisone (100 ng/ml) potentiates the TSH action on Tg gene expression (Fig. 2).

DISCUSSION

A primary cell culture system of calf thyroid cells has been developed. Calf thyroid tissue is readily available and allows experimentation with a large amount of cells. In Fig. 3 we have summarized the characteristics of this new thyroid cell model, comparing it to other thyroid cell culture systems from other species which also allow the long term in vitro study of proliferation and differentiation. Like dog thyroid cells, the calf thyroid

COMPARISON WITH OTHER SYSTEMS

MORPHOLOGY		FRTL$_5$ MONOLAYER	OVNI MONOLAYER FOLLICLES (TSH)	DOG MONOLAYER RARE MICROFOLLICLES (TSH)	SHEEP MONOLAYER FOLLICLES (TSH)	CALF MONOLAYER FOLLICLES DERIVED AGGREGATES (TSH)
Tg gene expression		+	+	+	+	+
TURN OFF/ON		-	?	+	+?	+
Iodide organification (PBI)		-	?	-/+	+	+
trapping		+	?	+	+	+
Effect of TSH	Morphology	+	+	+	+	+
	Proliferation	↗↗	↗	↗↗	0	0
	Differentiation					
	I⁻transport	↗	?	↗	↗	↗
	mRNA Tg	↗	Tg prod.	↗↗	?	↗↗
Effect of cAMP	Proliferation	↗	?	↗	0	?
	Differentiation	↗	?	↗	↗	↗
Effect of serum	Proliferation	↗	?	↗	↗	↗
	Differentiation	0	?	0	↘	↘
Effect of EGF	Proliferation	0	?	↗	↗	↗
	Differentiation	0	?	↘	↘	↘
Effect of TPA	Proliferation	↗	?	↗	↗	↗
	Differentiation	0?	?	↘	↘	?
Effect of FGF	Proliferation	0	?	↗	0	↗
	Differentiation	0	?	0	0	?
Immortal		yes	yes	no	no	no
Insulin required for proliferation		yes	yes	yes	yes	?

Fig. 3. Data summarized in this table are from references 12, 15, 17 (FRTL-5 rat cell line); 3 (OVNI ovine cell line); 4, 7, 11, 16 (dog primary cells); and 8, 12, 14, 15, 17 (sheep primary cells).

cell system shows that differentiation as measured by parameters such as Tg gene expression (here the cytoplasmic levels of Tg mRNA) can be either turned OFF/ON. High levels of Tg gene expression, as well as other differentiation markers such as iodide transport and organification, are strictly dependent on the presence of TSH or other agents which increase cAMP. The low but significant basal levels of these markers are further decreased by EGF which partly prevents the action of TSH. These data are in agreement with results obtained in dog and sheep thyroid models (4,5,7,8). However, the amplitude of these effects is particularly striking in calf cells. As in sheep (8,12), dog (4,5) and porcine (9) thyroid cells, EGF is a potent stimulator of the proliferation of calf thyroid cells, as shown in dog (5) and sheep cells (8). In addition, as observed in sheep thyroid cells (8), it leads to complete dedifferentiation. The bovine serum concentrations which are sufficcient to produce these effects on bovine thyroid cells are, however, strikingly low, suggesting the presence in serum of undefined species-specific factors. As bovine thyroglobulin gene promoters have been cloned (10), this new experimental system is now being used to study the molecular mechanism involved in thyroglobulin gene regulation.

REFERENCES

1. Ambesi-Impiombato FS, Parks LAM, and Coon HG. Proc Natl Acad Sci USA 77: 3455, 1980.
2. Ambesi-Impiombato FS, Picone R, and Tramontano D. In GH Sato, AB Pardee, and DA Sirbasku (eds), Cold Spring Harbor Conferences on Cell Proliferation, Vol. 9, Cold Spring Harbor, 1982, p 483.

3. Fayet G and Hovsepian S. In MC Eggo and GN Burrow (eds), Progress in Endocrine Research and Therapy, Vol. 2, Raven Press, New York, 1985, p 211.
4. Roger PP and Dumont JE. FEBS Lett 144: 209, 1982.
5. Roger PP and Dumont JE. Mol Cell Endocrinol 36: 79, 1984.
6. Roger PP, Servais P, and Dumont JE. FEBS Lett 157: 323, 1983.
7. Roger PP, Van Heuverswyn B, Lambert C, et al. Eur J Biochem, 1985 (in press).
8. Eggo MC, Bachrach LK, Fayet G, et al. Mol Cell Endocrinol 38: 141, 1984.
9. Westermark K, Karlsson FA, and Westermark B. Endocrinology 112: 1680, 1983.
10. Vassart G, Bacolla A, Brocas H, et al. Mol Cell Endocrinol 40: 89, 1985.
11. Rapoport B and Jones AL. Endocrinology 102: 175, 1978.
12. Westermark K and Westermark B. Exp Cell Res 138: 47, 1982.
13. Chambard M, Verrier B, Gabrion J, et al. J Cell Biol 96: 1172, 1983.
14. Errick JE, Eggo MC, and Burrow GN. In MC Eggo and GN Burrow, Progress in Endocrine Research and Therapy, Vol. 2, Raven Press, New York, 1985, p 271.
15. Eggo MC, Bachrach LK, Errick JE, et al. Ann Endocrinol (Paris) 45: 78, 1984 (abstract).
16. Roger PP, Reuse S, Servais P, et al. Cancer Res (in press).
17. Bachrach K, Eggo MC, Mak WW, et al. Endocrinology 116: 1603, 1985.

GROWTH FACTOR REQUIREMENT OF ISOLATED THYROID FOLLICLES

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INTRODUCTION

Our previous studies in intact animals (1,2) have demonstrated the existence of a growth desensitization mechanism which limits the proliferative response of the normal rat thyroid follicular cell to its trophic hormone, TSH, and which appears to be lost in early follicular cell tumors. To study this mechanism further we have established a tissue culture model using isolated thyroid follicles grown in suspension. We have now used this to define the responsiveness of normal follicular cells to purified growth factors in a serum-free medium.

METHODS

Primary Culture

Pooled thyroids from eight rats were digested in collagenase (200 U/ml) and dispase (2 mg/ml) in Hank's balanced salt solution and follicles released by periodic agitation. The washed follicle suspension was plated in agarose-coated microtiter plates at 5×10^4 cells per well in RPMI 1640 medium together with growth factors as appropriate.

Proliferation Assays

The response to growth factors was assessed (a) by measuring ^{3}H-thymidine (^{3}H TdR) incorporation over successive 24 hour periods of culture, and (b) by autoradiographic determination of the proportion of cells in S phase (^{3}H TdR labeling index) at 36 hour intervals.

RESULTS

Insulin (8 μg/ml) in the absence of other growth factors produced a $\sim$ tenfold stimulation of ^{3}H TdR incorporation, reaching a peak of 4222 $\pm$ 367 cpm per 5×10^4 cells 24-48 hours after addition, compared to a basal level without growth factors of 486 $\pm$ 18 cpm. In contrast, TSH when added alone had no significant effect.

Inclusion of insulin, even at a concentration (0.08 μg/ml) sufficient of itself to induce only a minimal response, permitted a marked response to TSH. The peak of TSH-induced incorporation occurred 48-72 hours after addition and rose approximately 7-fold from 1,089 ± 163 cpm in the presence of 0.08 μg/ml insulin alone to 7,548 ± 585 cpm with insulin plus 1 mU/ ml bovine TSH.

Epidermal growth factor (EGF) did not stimulate ^{3}H TdR uptake at any concentration tested (up to 100 ng/ml) either alone or in the presence of insulin.

The 36-72 hour autoradiographic labeling index correlated closely with ^{3}H TdR incorporation. Insulin alone at 8 μg/ml increased the LI from a basal value of 0.05 to 2.6%; TSH alone had no effect. TSH in the presence of 0.08 μg/ml insulin increased the LI from 0.14% to 6.2%.

DISCUSSION

We have used a defined medium and detailed "time course" analyses of ^{3}H TdR uptake and labeling index to define precisely the proliferative effects of pure growth factors on rat thyroid follicular cells in suspension culture. Firstly, we have shown that insulin stimulates ^{3}H TdR uptake at supraphysiological concentrations, an action which is likely to be mediated via its weak affinity for somatomedin receptors. Secondly, we have shown that while TSH has no effect alone in the presence of a permissive concentration of insulin, a marked stimulatory effect is observed, beginning at concentrations within the physiological range.

Our results demonstrate conclusively that TSH can act as a growth factor for rat thyroid follicular cells in the appropriate conditions, and should help to clarify the current controversy surrounding the role of TSH in thyroid growth.

REFERENCES

1. Wynford-Thomas D, Stringer BMJ, and Williams ED. Acta Endocrinol 101, 21, 1982.
2. Wynford-Thomas D, Stringer BMJ, and Williams ED. Virch Archiv (Cell Pathol) 40: 379, 1982.

PRODUCTION OF BOTH TYPES OF INSULIN-LIKE GROWTH FACTORS BY PRIMARY CULTURES OF OVINE THYROID CELLS

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INTODUCTION

One of the major tenets of thyroidology concerns TSH control of thyroid gland growth (1). The suppression of goiter by thyroid hormone is based on this premise. However, the effect of TSH on growth has been variable in different thyroid cell cultures in vitro. Recent advances in cell culture technique have allowed the culturing of ovine thyroid cells in chemically-defined, serum-free medium and, thus, the study on the role of TSH on thyroid growth and differentiation in the absence of serum factors. In primary cultures of ovine thyroid cells, TSH stimulates a number of differentiated aspects of thyroid function including follicle formation, iodide uptake and organification, cAMP production, thyroglobulin, thyroxine and triiodothyronine synthesis (2), as well as production of plasminogen activator (3). But, TSH does not stimulate growth in these ovine cells. The possibility existed that TSH did not stimulate growth directly. Conceivably, thyroid cells were producing growth factors as an aspect of differentiation.

RESULTS AND DISCUSSION

Primary cultures of ovine thyroid cells were prepared by collagenase digestion as described (4). The thyroid cells were maintained in serum-free medium containing insulin (10 μg/ml), transferrin (5 μg/ml), somatostatin (10 ng/ml), hydrocortisone (10 nM), glycyl-histidyl-lysine acetate (10 ng/ml) and TSH (0.5 mU/ml). Although these ovine thyroid cells can be maintained in the absence of TSH, differentiated aspects of thyroid function and morphology occur only in the presence of TSH.

To determine whether thyroid cells produce growth factors, conditioned media from primary cultures were compared to standard culture media for the ability to stimulate growth as measured by the incorporation of ^{3}H-thymidine into TCA-precipitable material in human foreskin fibroblasts. The addition of conditioned medium produced a threefold increase in thymidine incorporation. The addition of EGF at the optimal concentration of 10^{-9} M also produced a threefold increase in thymidine incorporation. Conditioned media and EGF had additive effect on growth stimulation, indicating that the thyroid growth factor was different from EGF.

Table 1. Growth Stimulation by Thyroid Cell Conditioned Media on (A) FRTL-5 and (B) NRK Cells

Treatment	% of control
(A) FRTL-5 cells	
Optimal growth (+TSH, +1% serum)	100 ± 9
-TSH, -serum: no addition	2 ± 1
" +GF1	18 ± 5
" +GF9	42 ± 10
(B) NRK cells	
(a) Optimal growth (+1% serum)	100 ± 11
-serum: no addition	17 ± 3
" +GF1	13 ± 3
" +GF9	42 ± 4
(b) Conditioned media collected on:	
Day 10	34 ± 10
Day 13	29 ± 7
Day 16	35 ± 6
Day 19	38 ± 12

Acid concentrates of standard culture medium (GF1), conditioned medium (GF9) and conditioned media collected at 3-day intervals were tested for growth promoting activity. Values are mean ± SD of quadruplicate measurements from two experiments.

The rat thyroid cell line, FRTL-5, is of particular interest because, unlike ovine thyroid cells in primary culture, these cells are dependent on TSH for growth (5). In the absence of TSH and serum, growth was almost completely suppressed (Table 1). The addition of acid concentrates of conditioned media (GF9) could restore growth on these FRTL-5 cells to 42% of the optimum despite a lack of TSH or serum. Acid concentrates of standard culture media had a small effect, 18% of optimum, indicating that the growth effect of the conditioned media was not simply due to concentrated TSH and insulin. Similarly, removal of serum reduced ^{3}H-thymidine incorporation into NRK fibroblasts to 17% of optimal growth. Acid concentrates of the conditioned media (GF9) again restored growth to 42% of the optimum (Table 1). The growth-promoting activity from the ovine thyroid cells thus stimulated both FRTL-5 cells and fibroblasts. The production of growth-promoting activity by ovine thyroid cells remained constant over a period of at least three weeks as shown by the constant growth-stimulating effect of the conditioned media collected at 3-day intervals (Table 1).

To identify the growth-promoting activity in the conditioned media, radioimmunoassays (6,7), and radioreceptor assays (8) were performed for insulin-like growth factors. Both IGF-I and IGF-II were present in a pooled 4-day collection of conditioned media at concentrations (ng/ml) of 15.2 ± 3.4 and 42.0 ± 8.2, respectively. Neither IGF-I for IGF-II were

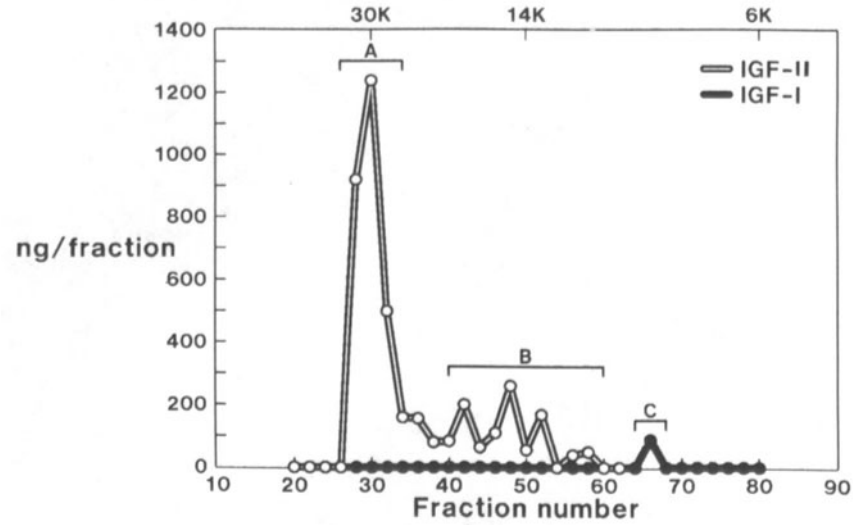

Fig. 1. Chromatography of an acid concentrate of conditioned media on a Bio-Gel P-60 column. Fractions were collected and assayed for IGF-I and -II reactivities. Markers used are carbonic anhydrase (30K M_r), ribonuclease (14K M_r), and insulin (6K M_r).

present in the standard culture media, nor was EGF, or transforming growth factors, found in the conditioned media.

An acid concentrate of conditioned media from ovine thyroid cells was chromatographed on a BioGel P-60 column (Fig. 1). Assay of the fractions for IGF-I reactivity by radioimmunoassay revealed one peak of IGF-I reactivity at Peak C (Fig. 1) between the markers of ribonuclease and insulin.

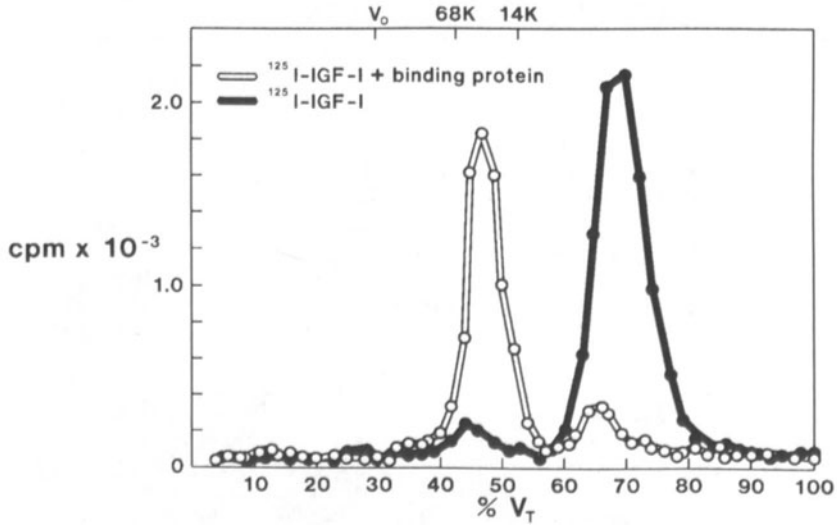

Fig. 2. Chromatography of ^{125}I-labeled IGF-I in the presence of albumin or Peak A material (see Fig. 1) on a Sephacryl S-200 column. Fractions were collected and counted in a gamma counter.

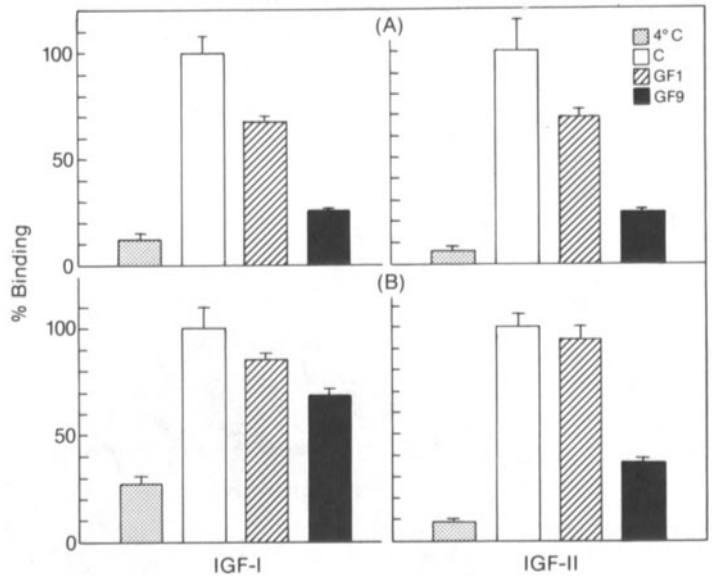

Fig. 3. Binding of labeled IGF-I and -II to (A) ovine thyroid cells and (B) FRTL-5 cells. Binding was carried out at 4°C and at 37°C (as control, C) with or without the addition of standard culture medium (GF1) or conditioned medium (GF9). Values are mean ± SD from triplicate determinations of two separate experiments.

Several peaks of IGF-II reactivity located at Peak B were identified by both radioreceptor assay and radioimmunoassay. A large peak of apparent IGF-II reactivity was also found at Peak A. Peak A had an estimated molecular weight greater than 20,000 M_r. Reported molecular weights of the various forms of IGF-II, including mature and precursor forms, range from 7,000 to 20,000 M_r (9).

To investigate the possibility that Peak A contained IGF binding proteins which have been shown to complex with IGF *in vivo* (10), the Peak A material was added to ^{125}I-IGF-I and analyzed on a Sephacryl S-200 column in phosphate buffer at pH 7.4 (Fig. 2). Addition of Peak A material resulted in a shift of the radioactivity peak to a higher molecular weight as compared to chromatography of the ^{125}I-IGF-I in the presence of albumin on a separate run. Similar results were found with ^{125}I-IGF-II. Binding of radiolabeled IGF to the Peak A material could be displaced with unlabeled, partially purified IGF. These data indicate that Peak A contains IGF binding proteins.

Since IGF receptors are widely distributed in various tissues and cultured cells (10,11), their presence on thyroid cells was investigated. Both ovine thyroid cells and the rat FRTL-5 cells were used to study the binding of ^{125}I-labeled IGF-I and -II (Fig. 3). Both radiolabeled IGF bound to both cell types and the binding could be displaced with the acid concentrate of conditioned media (GF9) from ovine thyroid cells. The acid concentrate of the standard media (GF1) contained no displacing activity on binding. Insulin at 2 μM did not displace any binding either. Finally, the binding of radiolabeled IGF to ovine thyroid cells was compared in the presence and absence of TSH. The addition of TSH resulted in a twofold increase in the binding of IGF.

In summary, growth-promoting activity was detected in conditioned media from ovine thyroid cell cultures, and the production of this activity was constant over a 3-week period. The activity was identified to be IGF-I and -II by radioimmunoassay and radioreceptor assay. In addition, IGF binding proteins were also found. Binding of IGF to thyroid cells could be displaced by the conditioned media from ovine thyroid cells. In light of the concept that growth factor my act through autocrine or paracrine mechanism, being produced at multiple sites and acting at or near their sites of production (12), these data suggest tht IGF may be important in thyroid cell growth.

REFERENCES

1. Dumont JE. In RS Harris, PL Manson, E Diczfalusy, et al. (eds), Vitamins and Hormones, Academic Press, New York, Vol. 29, 1971, p 287.
2. Eggo MC, Mak WW, Bachrach LK, et al. In MC Eggo and GN Burrow (eds), Thyroglobulin - The Prothyroid Hormone, Raven Press, New York, 1985, p 201.
3. Mak WW-N, Eggo MC, and Burrow GN. Biochem Biophys Res Comm 123: 633, 1984.
4. Eggo MC, Bachrach, LK, Fayet G, et al. Mol Cell Endocrinol 38: 141, 1984.
5. Ambesi-Impiombato FS, Parks LAM, and Coon HG. Proc Natl Acad Sci USA 77: 3455, 1980.
6. Bala RM and Bhaumick B. J Clin Endocrinol Metab 49: 770, 1979.
7. Bala RM, Bhaumick B, Armstrong GH, et al. In EM Spencer (ed), Insulin-like Growth Factors Somatomedins, Walter de Gruyter, Berlin, 1983, p 491.
8. Daughaday WH, Mariz IK, and Trivedi B. J Clin Endocrinol Metab 53: 282, 1981.
9. Yang YW-H, Acquaviva AM, Bruni CB, et al. In EM Spencer (ed), Insulin-like Growth Factors Somatomedins, Walter de Gruyter, Berlin, 1983, p 603.
10. Nissley SP and Rechler MM. Horm Prot Peptides 12: 127, 1985.
11. Froesch ER, Schmid C, Schwander J, et al. Ann Rev Physiol 47: 443, 1985.
12. D'Ercole AJ, Stiles AD, and Underwood LE. Proc Natl Acad Sci USA 81: 935, 1984.

THE EFFECT OF EPIDERMAL GROWTH FACTOR (EGF) ON THYROID FUNCTION IN THE SHEEP AND IN ISOLATED RAT HEPATOCYTES

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Evidence has accumulated in recent years showing that EGF is mitogenic in cultured thyroid cells while inhibiting ^{125}I uptake, transport, and organification (1-6). In thyroid follicles, EGF has specific receptors with K_a 1.2 x 10^{-9} M and capacity of 7.9 pmol/g tissue (1). The aim of this study was to extend previous investigations (1,2,7), describing in vivo of EGF on thyroid function and to investigate the effects of EGF of peripheral metabolism of thyroid hormones in isolated hepatocytes.

MATERIALS AND METHODS

EGF Infusion in Sheep

Six merino ewes, weighing between 40 and 45 kg, maintained under controlled conditions in metabolic cages, fed ad libitum, were infused via the jugular vein for 24 hours with 3.3 μg/kg/hr of mouse EGF (prepared according to the method of Cohen and Savage (8), equipotent with Collaborative Research Receptor Grade EGF in RIA, RRA, and bioassay). This dose gives EGF plasma levels of ∿10 ng/ml (2). Control animals were infused with saline. Blood samples (10 ml) were taken by a catheter in the contrajugular vein. Radioimmunoassays of plasma triiodothyronine (T_3), thyroxine (T_4), 3,3',5'-triiodothyronine (reverse T_3, rT_3), and 3,3'diiodothyronine (3,3'T_2) were performed using specific antisera raised in sheep. Crossreaction was negligible except T_4 with T_3 antiserum, ≦0.25%, distorted due to contamination of the Sigma T_4 with up to 0.5% T_3 (manufacturer's specifications). Plasma TSH was measured by a standard double antibody technique with ovine TSH antibody, bovine ^{125}I-TSH, and ovine TSH standard NIHS8.

Isolated Rat Hepatocytes

Hepatocytes were prepared by a modification of the method of Berry and Friend (9) from 200 g female Wistar rats using Worthington collagenase, 35 mg in 35 ml of recycled perfusate of Krebs-Ringer phosphate, pH 7.4, without calcium, contaiing 2% bovine serum albumin, 5.6 mM glucose, 20 mM HEPES and washed human erythrocytes (∿10% hematocrit) with bubbled air for oxygenation. The perfused liver was excised into incubation buffer, Basal Medium Eagles (BME), with the same additions as above and shaken for 10 min, strained, and washed three times, yielding between 180 and 230 million cells

with viability ~88%. They were incubated in 3 ml aliquots containing 4 x 10^6 cells/ml BME in silicon-coated Erlenmyer flasks with 10 ng/ml EGF shaken at 37°C for 5.5 - 6 hrs before washing and incubation for a further 3 hrs with more EGF, iodothyronine, and freshly desalted radioligand (5' labeled), to measure ^{125}I release (10). 5'deiodinase activity was assessed in preliminary experiments with T_4 in hepatocytes without radioligand by ethanol-extracted RIA.

RESULTS

EGF Infusion in Sheep

The results are shown in Figs. 1 and 2. Plasma T_3 and T_4 were very signifiantly depressed over the time course of the infusion. There was a

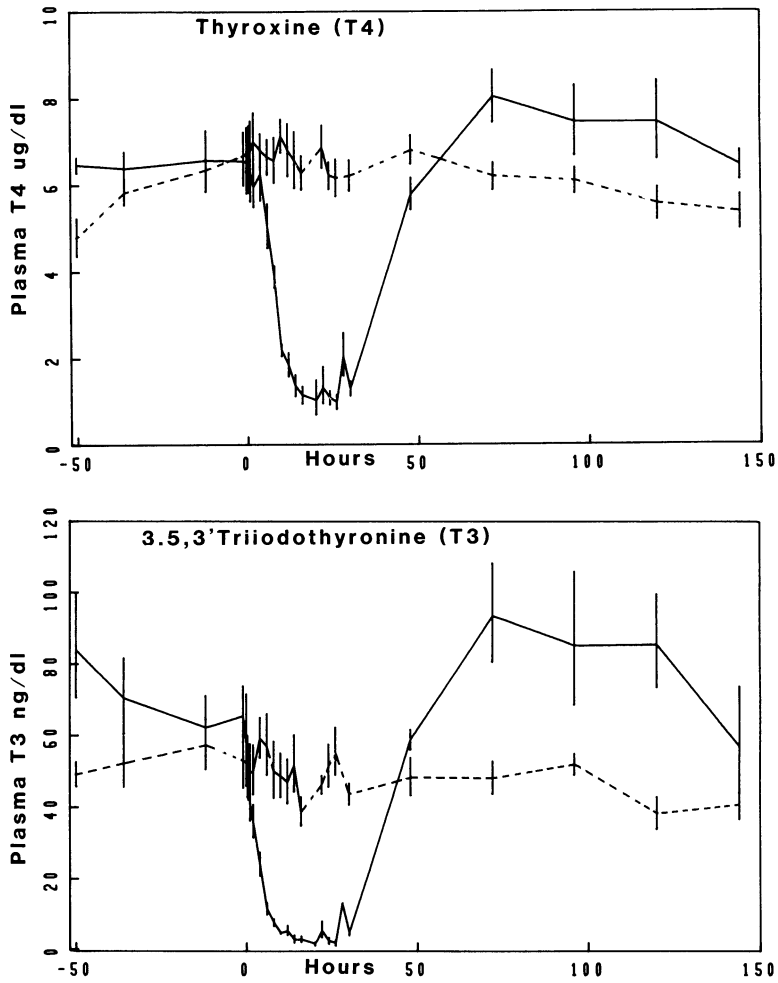

Fig. 1. The effect of 24 hour infusion of mEGF, starting at time zero, on ovine plasma T_4 and T_3 levels (mean ± standard error). EGF-treated animals (n=6) in solid lines; controls (n=6) in dotted lines. Changes were significant ($p<0.001$) in plasma T_3 after 4 hours and plasma T_4 after 10 hours.

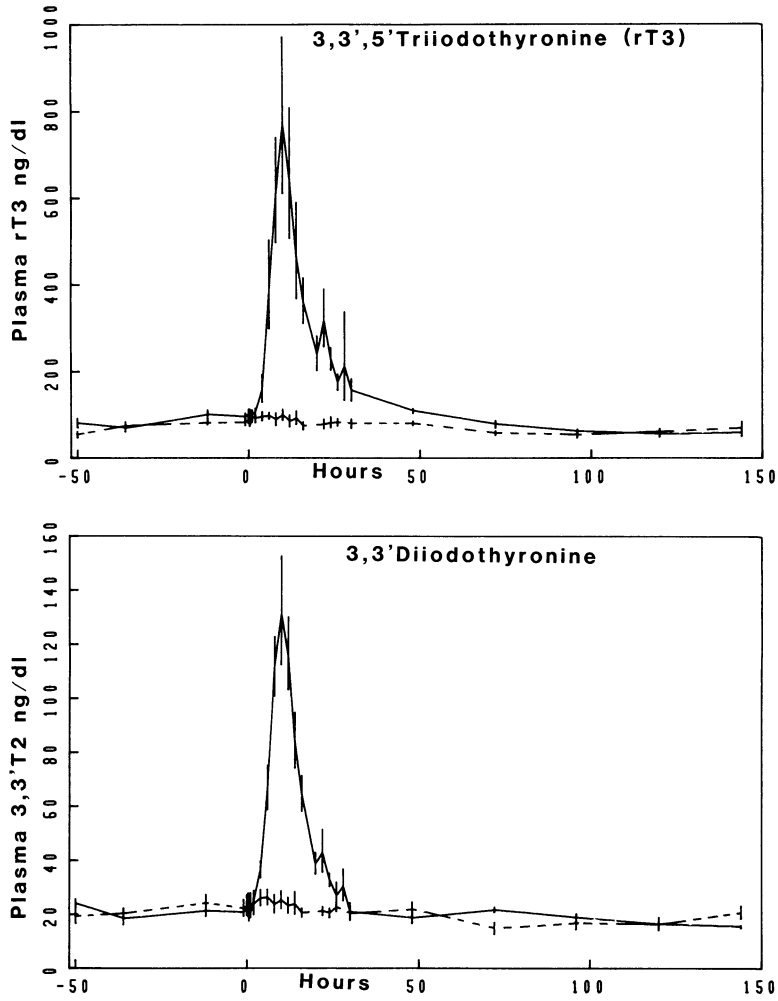

Fig. 2. The effect of 24 hour infusion of mEGF, starting at time zero, on ovine plasma rT_3 and $3,3'T_2$ levels (mean $\pm$ standard error). EGF-treated animals (n=6) in solid lines; controls (n=6) in dotted lines. Changes were significant ($p<0.001$) in plasma rT_3 and $3,3'T_2$ after 6 hours.

signifiant increase in plasma TSH presumably as a consequence of negative feedback from 48 to 96 hrs following the infusion, then plasma T_3 and T_4 returned to control values, and overshot. There were very highly significant increases in plasma rT_3 and $3,3'T_2$, though of shorter duration than the changes in T_3 and T_4.

Isolated Rat Hepatocytes

There was no change in iodine release and, therefore, in 5'deiodinase activity, when hepatocytes were preincubated with either 10 or 50 ng/ml EGF, whether the substrate was ^{125}I-T_3, ^{125}I-T_4, or ^{125}I-rT_3, incubated with non-radioactive ligand over concentrations from 10^{-6} M to 10^{-12} M. Data is given for T_3 in Fig. 3. There was also no change when a crude homogenate of rat liver and T_4 were preincubated under the same conditions with EGF. In investigating 5 deiodinase activity, unconfirmed preliminary RIA results

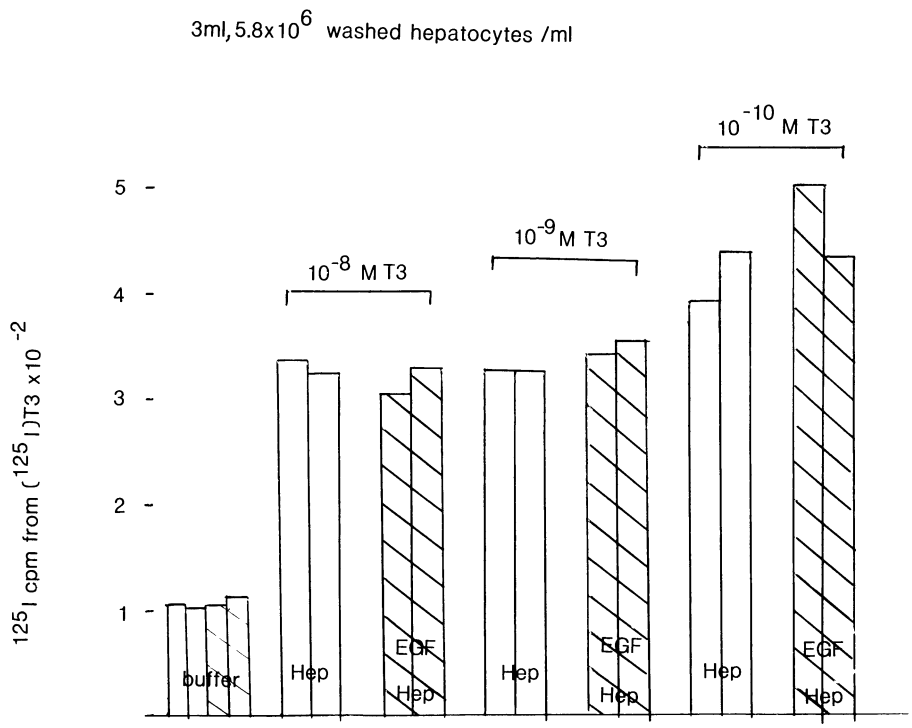

Fig. 3. ^{125}I release from 5' ^{125}I T_3 incubated with isolated rat hepatocytes + 10 ng/ml mEGF with doses of non-radioactive ligand as shown. ^{125}I release from all flasks containing hepatocytes was significantly greater ($p<0.001$) than buffer and from dose 10^{-10} M T_3 compared with 10^{-8} M T_3. No EGF-treated flask was significantly different from its respective control flask.

with T_4 substrate show that EGF-treated hepatocytes metabolize more T_4 than untreated heptocytes, over three hours.

DISCUSSION

This study confirms earlier results showing a profound effect of EGF, at defleecing doses, consistent with inhibition of T_3 and T_4 secretion from the thyroid gland. However, the mean rate of disappearance of both T_4 and T_3 from the circulation during EGF infusion suggests half-lives of about four hours, much shorter than reported previously of 1.1 days for T_4 (11) and 7 hours for T_3 (12). It is possible that EGF enhanced T_4 5 deiodination to rT_3, increasing plasma levels to a remarkable mean peak value of 771 ng/dl. With T_3 as substrate, enhanced 5 deiodinase activity would cause the observed rise in $3,3'T_2$, However, since thyroidal T_4 and T_3 secretion are inhibited by 10 ng/ml of EGF, lack of substrate would subsequently lead to a fall in circulating rT_3 and $3,3'T_2$, as was observed. It was postulated that rapid removal of T_3 and T_4 might also be attributable to an increase in 5'deiodinase, however, this was not confirmed by experiments using isolated hepatocytes and 5'-labeled iodothyronines. EGF is known to bind to cell surface receptors with internalization before exerting biological action. That such an intact cell system is necessary is supported by the negative findings when crude homogenate was incubated with T_4 and EGF.

In summary, we have confirmed that EGF in vivo has a remarkable effect on thyroidal secretion in the intact sheep. It is postulated that this is achieved both by an inhibitory action on the thyroid and by enhancing 5 deiodination of T_4 and T_3. Since EGF is present as a normal constituent of bodily fluids, it is likely that EGF in physiological concentrations has a modulating effect on thyroid function.

REFERENCES

1. Waters MJ, Whip TA, McGrath P, et al. Proc Endocr Soc Aus 26: 39, 1983.
2. Waters MJ, Corcoran JM, Tennison M, et al. Proc Endocr Soc Aus 25: 34, 1982.
3. Roger RP and Dumont JE. FEBS Lett 144 (2): 209, 1982.
4. Westermark K and Westermark B. Exp Cell Res 138: 47, 1982.
5. Karlsson FA, Westermark K, and Westermark B. Mol Cell Endocr 28: 99, 1982.
6. Humphries H, MacNiel S, Munro DS, et al. J Endocr 102: 57, 1984.
7. Corcoran JM, Waters MJ, Jorgensen G, et al. (submitted to Endocrinol.).
8. Cohen S and Savage Jr CR. Meth Enzymol 36: 424, 1974.
9. Berry MN and Friend DS. J Cell Biol 43: 506, 1969.
10. Cutten AE, Smith HC, Corcoran JM, et al. (in preparation)
11. Dussault JH, Hobel CJ, and Fisher DA. Endocr 88: 47, 1971.
12. Dussault JH, Hobel CJ, DiStefano JJ, et al. Endocr 90: 1301, 1972.

FORMATION OF INTRACYTOPLASMIC MICROFOLLICULAR LUMINA IN DISPERSED THYROID CELLS*

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Dispersed thyroid cells have been used as a convenient experimental system to study thyroid cell activities in the absence of normal tissue architecture and colloid stores. Iodination inside the cells was demonstrated to be dependent on active iodide transport and regulated by thyrotropin (1,2). Electron microscopic autoradiographic studies revealed intracytoplasmic lumina (ICL) which represent a site of iodination inside dispersed thyroid cells (3). The aim of the present work was to answer the following questions: a) which are the conditions determining the formation of ICL? and b) do thyroid hormone synthesis and secretion by dispersed thyroid cells depend upon iodination reactions in ICL?

MATERIAL AND METHODS

Freshly dispersed hog thyroid cells prepared by a discontinuous trypsinization procedure (4) were incubated in Earle's balanced salt solution in the presence or absence of TSH added to tested agents. The latter were puromycine, cycloheximide, vinblastine, methimazole, dibutyryl-3',5'cyclic AMP, and monensin. Incubation took place in the presence of ^{125}I for the examination of cell pellets by autoradiography with an electron microscope and for the measurement of organic iodine formation. ICL, labeled (i.e., containing silver grains) or unlabeled, were counted in a minimal sample of 50 cells in each experimental condition.

RESULTS

Cytoplasmic vacuoles identifed as ICL were recognizable by the presence of microvilli protruding into the vacuoles. Completely absent in freshly dispersed cells, ICL were present in 15 to 20% of the cells, not only associated in follicle fragments, but also in isolated cells without apparent cell polarity after 60 min of incubation at 37°C. ICL were not observed in cells incubated at 4°C. The addition of TSH at the beginning of the incubation induced an increase of ICL formation; after four hours, their number was threefold higher in TSH-treated cells than in control

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cells. Dibutyryl- cyclic AMP (2 mM) mimicked the effect of TSH on ICL formation. Cycloheximide (0.5 mM) and puromycine (0.5 mM) did not affect the TSH-stimulated formation of ICL, whereas vinblastine (30 µM) inhibited their formation in both unstimulated and TSH-stimulated cells. Monensin (30 µM), a carboxyclic acid ionophore, caused the formation and accumulation of vesicular bodies and completely suppressed ICL formation in control and TSH-stimulated cells.

The time course of the appearance of radioiodinated material in ICL, assessed by labeled ICL counting, was very similar to the time course of ICL formation both in control and TSH-stimulated cells. After 2 min of incubation, no radioactivity was evidenced by autoradiography. At 10 min, grains could be clearly identified but were located only in ICL. It is only after 30 min of incubation with radioiodide that ICL were substantially labeled and that the labeling outside ICL, i.e., over the cytoplasm, became obvious. Pulse-chase experiments have been performed in order to examine whether iodinated products generated in ICL could be released from from ICL. Thyroid cells prelabeled with ^{125}I for 60 min were incubated in a radioiodide-free medium in the presence of methimazole. The number of labeled ICL observed at the end of the labeling period did not change significantly during the incubation in basal conditions. In contrast, in the presence of TSH, there was a progressive decrease of the number of prelabeled ICL, whereas silver grains were detected over the cytoplasm from the beginning of the incubation. After 210 min of incubation in the presence of TSH, very few autoradiographic grains were observed over ICL and cytoplasm.

DISCUSSION AND CONCLUSIONS

Dispersed thyroid cells undergo a morphological transformation conducive to the formation of ICL, which could represent functional intracytoplasmic equivalents of colloid lumen. The autoradiographic analysis revealed that the newly formed iodinated products are located in ICL, suggesting that ICL represent the site of iodide organification in the dispersed thyroid cells. Since iodinated products were first observed in ICL, then in the cytoplasm, and were more abundant in ICL than outside ICL whatever the labeling time, it seems likely that labeled material encountered over the cytoplasm at later stages of incubation correspond to iodinated products, which had translocated from the ICL to the cytoplasm.

The formation of the ICL appears to be a time- and temperature-dependent process enhanced by TSH, the action of which is mediated through the adenylate cyclase-cyclic AMP system. The formation of ICL does not require newly formed proteins as it is not inhibited by cycloheximide and puromycine, but it requires the integrity of the microtubular system as it is inhibited by vinblastine. Thus, the material necessary to the formation of ICL preexists in the dispersed thyroid cells. ICL probably originate from the confluence of Golgi vesicles, as their formation is inhibited by monensin, which impedes the transit of membrane vesicles from the Golgi apparatus to the plasma membrane causing the intracellular ac- cumulation of large vacuoles (5).

TSH stimulates the release of thyroid hormones from prelabeled, dispersed thyroid cells (4). This hormone secretion does not seem to be related to a previous step of phagocytosis or macropinocytosis. Indeed, in the present study, no pseudopod phagocytizing the content of ICL and nearly no colloid droplets were observed after TSH stimulation. A large part of the silver grains encountered over the cytoplasm outside of the ICL could, therefore, correspond to iodinated material coming out of the ICL by fluid pinocytosis (6), a mechanism already postulated in the hormone secretion from hyperactive dog thyroids (7).

REFERENCES

1. Rodesch F and Jortay A. Experientia 24: 268, 1968.
2. Rousset B, Poncet A, Dumont JE, et al. Biochem J 192: 801, 1980.
3. Neve P and Rousset B. Ann Endocrinol 43: 48A, 1982.
4. Rousset B, Poncet C, and Mornex R. Biochim Biophys Acta 437: 543, 1976.
5. Stein BS, Bensch KG, and Sussman HH. J Biol Chem 259: 14762, 1984.
6. Van den Hove-Vandenbroucke MF. In M De Visscher (ed), The Thyroid Gland, Raven Press, New York, 1980, p 61.
7. Rocmans PA, Ketelbant-Balasse P, Dumont JE, et al. Endocrinology 103: 1834, 1978.

DIFFERENT CALCIUM REQUIREMENTS FOR LOW IODINE DIET (LID) INDUCED AND DEVELOPMENTAL GROWTH OF THE RAT THYROID: EVIDENCE FROM IN CULTURE EXPERIMENTS

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In vivo, in post-embryonic life, most mammalian cells are growth arrested. Considered a few years ago as the main target of extracellular Ca^{2+} on the cell cycle, initiation to division is not believed to be extracellular Ca^{2+}-dependent (1). Initiation covers one quarter to one third of G_1, the phase of the cycle that precedes DNA synthesis (2). Presence of the cation is required in the incubating media over the second half of G_1 (3). Once initiation is accomplished, each phase of the cycle proceeds within a determined time and, if adequate Ca^{2+} concentration is not provided over the Ca^{2+}-sensitive period, initiation vanishes. No DNA synthesis at all occurred with BALBc/3T3 mouse cells, initiated in very low Ca^{2+}, when mMolar Ca^{2+} was given past three lengths of G_1 (3). That in vivo hypocalcemia suppressed cell proliferation in some instances and not in others (4) could be interpreted in two different ways: either that Ca^{2+} requirements for cell division are different for divisions induced by different mitogens, since it was shown that epidermal growth factor (EGF) reduced Ca^{2+} requirements for growth of the normal epithelial cell line NP-2s by two orders of magnitude (5); or that alteration of other parameters, such as permeability of the plasma membrane (PM) to the ion and/or cell geometry, affected the rate of Ca^{2+} collection.

In the in vivo experiments mentioned above (4), nutritional techniques were used to obtain both hypocalcemia and excess cell proliferation of the thyroid. The diets were the Triantaphyllidis low iodine - low Ca^{2+} set of diets (Institut National Production Industrielle (INPI) license no. 1601899) supplemented or not with KI, 0.1 mg/liter of drinking water, and/or Ca^{2+} combined with different anions (6). Cell counts were performed using morphometric techniques described (4,7).

Developmental growth of the thyroid consisted of three rounds of cell divisions from age one month (at weaning) to age nine months, when both iodine and Ca^{2+} intake were adequate; number of cells was multiplied by four between one and three months and again by two between three and nine months (Fig. 1). In hypocalcemic rats fed iodine-supplemented diets, the size of the glands was significantly smaller (4), but the number of cells did not differ from that of the controls at the time points examined. Size of cells made the difference as it appears from Fig. 2.

LID-induced, TSH-mediated cell proliferation. As shown in previous publications, thyroid weight increased after as little as one week on LID.

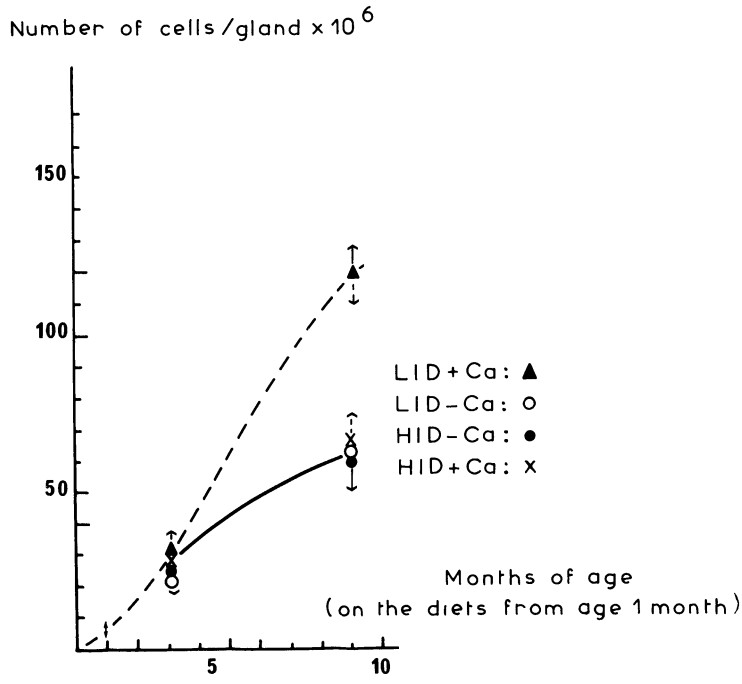

Fig. 1. Developmental growth of the thyroid as indicated by number of cells under different iodine and calcium diets.

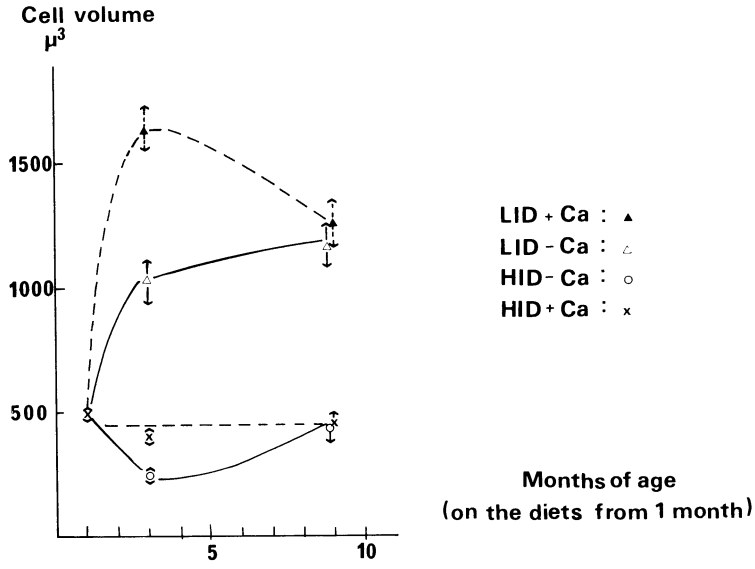

Fig. 2. Changes in thyroid cell size under different iodine and calcium diets.

Nonetheless, more than two months elapsed before LID had any impact on the number of cells (Fig. 1). A threefold increase in thyroid weight, at the end of the second month on the diet, was exclusively accounted for by a fourfold increase in cell size (Fig. 2). The difference in the two increases comes from diminished size of lumens in LID fed rats, from 30 or 35% of histologic sections on high iodine diet (HID) to less than 1% after two months on LID (7). Size of cells was significantly smaller after eight months than after two months on LID. Increased size of cells is not related to variations of size over the cell cycle, since most of the cells were growth arrested; duration of cell cycle is 2-5 days, frequency of cell doublings over the period starting at the end of the second month on LID was one per 90 days (2 in 6 months), which means that less than 5% of the cell population was cycling at the time of the measure. The twofold increase of the modal volume of cultured cells, from early G_1 to mitosis (8), occurs in all dividing cells independently of their initial size.

After eight months on LID and adequate Ca^{2+} intake, the number of cells was only twice that of HID $+Ca^{2+}$ rats of the same age, though the weight of the glands was six times larger. The single TSH-mediated cell doubling of the LID $+Ca^{2+}$ was suppressed by hypocalcemia (Fig. 1); number of cells in thyroids of LID $-Ca^{2+}$ fed rats was identical to that of HID fed animals, + or $-Ca^{2+}$.

It is well established that TSH is not involved in developmental growth of the thyroid. Developmental growth, in general, is believed to be initiated by serum-contained growth factors. The above mentioned information on EGF altering Ca^{2+} requirements for growth, as EGF was shown to be a growth factor for thyroid cells in culture (9), prompted me to perform experiments aimed at examining Ca^{2+} requirements for growth initiated by the mixture of growth factors contained in serum vs TSH-induced growth. The established thyroid cell line, FRTL (10), was suitable for the experiments because: 1) it is dependent both on TSH and serum for growth, 2) it can be grown in very low serum and in higher serum concentrations, and 3) when grown in different serum concentrations and equal TSH, growth rates differ enough to ensure that involvement of serum-contained growth factors was, indeed, different.

In sets of cultures grown in 1 mMolar Ca^{2+} and 10^{-9} TSH, doubling times were 2 days in 5% serum and 3.5 days in 0.5% (11). Rate of growth was reduced in 20 µMolar Ca^{2+} (11); ^{3}H-thymidine incorporation per µg DNA in 20 µM, as percent of controls in 1 mMolar Ca^{2+}, was the same in 5% and 0.5% serum, 66 and 58%, respectively (11). This is evidence that serum-contained growth factors did not alter Ca^{2+} requirements for growth of this normal rat thyroid cell line.

Alteration of geometric parameters, resulting from long-term metabolic TSH stimulation, is liable to affect Ca^{2+} collection over the second half of G_1; increased cell size implies decreased ratio of plasma membrane/cell volume (PM/V), i.e., decreased surface of entry of Ca^{2+} into each volume unit of cytoplasm. Comparing, in Table 1, PM/V ratios for the two mono-deficient groups, LID $+Ca^{2+}$ and HID $-Ca^{2+}$, with their serum Ca concentrations, brings into sight a striking similarity; the ratio of the figures representing PM/V equals the reciprocal of the ratio of the figures representing serum Ca (and extracellular fluid Ca) concentrations. The thyroids of the two groups had accomplished, at the same time point (two months on diet), the two rounds of developmental growth, those of the controls HID $+Ca^{2+}$, and this is evidence that they collected enough Ca^{2+} over the second half of G_1 for the programmed cell divisions to proceed, despite:

A) a 45% reduced extracellular Ca concentration in the HID $-Ca^{2+}$, and this was certainly accomplished thanks to increased PM permeability to Ca^{2+}, as

Table 1. Serum Calcium and Ratio of Plasma Membrane to Cell Volume of Thyroid Cells at the End of Two Months on Each of the Four Diets

	HID $+Ca^{2+}$	LID $+Ca^{2+}$	HID $-Ca^{2+}$	LID $-Ca^{2+}$
Serum calcium (mg/liter)	119.56 ± 2.38		66.35 ± 3.17	
PM/V	0.816 ± 0.043	0.508 ± 0.023	0.951 ± 0.063	0.590 ± 0.030

Means ± SD.

was shown in vitro (12) and in culture (11), through removal of Ca bound on the PM (11,13). Evidence that removal of Ca took place, also, in vivo in the $-Ca^{2+}$ rats was given by experiments (personal, unpublished) in which animals were injected with $^{47}Ca^{2+}$ and the ratio of tissue/serum was measured 24 and 48 hours later; ratios of tissue/serum were equal in hypocalcemic rats and in normocalcemic controls, demonstrating that tissue Ca was reduced as much as that of serum in the $-Ca^{2+}$ rats. As 90% of tissue Ca is bound on the cell coat, when extracellular Ca^{2+} is in the mMolar range (11, 13), the data demonstrate that Ca of the cell coat of the hypocalcemic rats was reduced. Elevated parathormone, secondary to hypocalcemia (14), and high phosphate of extracellular fluids, also helped collection of Ca^{2+}.

B) a 45% reduced surface for Ca^{2+} entry into the volume unit of cytoplasm, in the LID $+Ca^{2+}$, and this was performed thanks to increased Ca^{2+} uptake of the thyroid induced by TSH through its metabolic spectrum of actions, since increased $^{45}Ca^{2+}$ uptake in rats fed LID (or injected with TSH) occurred as early as one week on diet (15), a time at which no mitogenic effect of TSH could be detected, as shown here.

The LID $-Ca^{2+}$ rats, past the second month on diet PM/V, became nearly as unfavorable to Ca^{2+} collection as in the LID $+Ca^{2+}$. The easing effects of low Ca^{2+} environment, permitting sufficient Ca^{2+} collection by increasing PM permeability through removal of PM Ca (as in A) and that of TSH stimulation (as in B), did not appear to be additive, since from the two rounds of division between three and nine months, one of developmental growth and one LID-induced, only one took place. Indeed, the in-culture described increased Ca^{2+} uptake on the second half of G_1 of TSH-induced divisions, and involved overloading of the PM with Ca (11). This apparently counteracted the Ca^{2+} collection easing effect of removal of Ca from the PM, operated by hypocalcemia.

That only one out of the two initiated divisions proceeded in the LID $-Ca^{2+}$ rats, poses the problem of the selection. Did cells in areas of better blood supply carry on more than one round vs none in other areas? Or was the preference initiator dependent? Does increased Ca^{2+} collection in late G_1 of cell cycles induced by serum-contained growth factors involve overloading with Ca of the PM as in cycles in which TSH is involved? Parathormone, for instance, was found to increase intracellular Ca of HeLa and Rat Kidney cell lines without affecting Ca associated with the cell coat (13). In 3T3 cells, with serum initiator, cell surface Ca appeared (Fig. 3) (16) to be much less affected by the pre-DNA synthetic increased Ca uptake than intracellular Ca. This highly seductive hypothesis will have to be tested with thyroid cells other than the FRTL, since TSH is an obligatory link to this cell line in the sequence of events leading to cell division (10).

In conclusion, division initiated cells must collect extra Ca^{2+} for the cell cycle to proceed. The hypothesis of different Ca^{2+} requirements for growth induced by different initiators in vivo was rejected after considering information from in-culture experiments. In addition to the extracellular concentration of the cation, geometry of the cells and the Ca load of the PM affect the rate of Ca^{2+} collection. Both hypocalcemia and TSH ease Ca^{2+} collection, but their effects are not additive. This, together with the impact of metabolic effects of TSH on cell geometry, may account for the preferential inhibition by hypocalcemia of LID-induced, TSH-mediated, cell proliferation over cell divisions of developmental growth.

REFERENCES

1. Hesketh TR, Smith GA, Houslay MD, et al. Nature 267: 490, 1977.
2. Novogrodsky A and Katchalski E. BBA 228: 579, 1971.
3. Boynton AL, Whitfield JF, and Isaaks RJ. In Vitro 12: 120, 1976.
4. Triantaphyllidis H. In M Andreoli, F Monaco, and J Robbins (eds), Advances in Thyroid Neoplasia, Field Educational Italia, Rome and Chicago, 1981, p 109.
5. Lechner JF and Kaighn ME. Exp Cell Res 121: 432, 1979.
6. Triantaphyllidis H. Ann Endocrinol (Paris) 40: 385, 1979.
7. Triantaphyllidis H and Guichard C. In SR Stockight and S Nagataki (eds), Thyroid Research VIII, Pergamon Press, London and New York, 1980, p 149.
8. Meishrich ML, Meyn RE, and Barlogie B. Exp Cell Res 105: 169, 1977.
9. Westermark K and Westermark B. Exp Cell Res 138: 47, 1982.
10. Ambesi-Impiombato FS, Parks LAM, and Coon HG. PNAS 77: 3455, 1980.
11. Triantaphyllidis H, Tramontano D, and Ingbar SH. In preparation.
12. Douglas WW and Rubin RP. J Physiol (London) 159: 40, 1961.
13. Borle AB. J Cell Biol 36: 567, 1968.
14. Triantaphyllidis H. In N Ui, K Torizuka, S Nagataki, et al. (eds), Current Problems in Thyroid Research, Excerpta Medica, Amsterdam-London-Princeton, 1982, p 69.
15. Hachiya T, Miyazaki T, Kaimasu I, et al. In J Robbins and LE Braverman (eds), Thyroid Research, Excerpta Medica - American Elsevier, Amsterdam-Oxford-New York, 1976, p 89.
16. Tupper JT, Del Rosso M, Hazelton B, et al. J Cell Physiol 95: 71, 1978.

EFFECTS OF THE CHRONIC AND ACUTE TREATMENTS OF PIG THYROID CELLS WITH FORSKOLIN ON THE CYCLIC AMP ACCUMULATION, THE PHOSPHOLIPID TURNOVER, AND IODINATION

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INTRODUCTION

Thyrotropin (TSH) exerts its physiological effects on thyroid cells via two pathways. The one involving the stimulation of adenylate cyclase and of cyclic AMP-dependent protein kinase (kinase A) is well documented. The other mechanism is associated with the increased turnover of phosphoinositides and activation of the Ca^{2+}-phospholipid-dependent protein kinase (kinase C). It is very difficult to understand the respective role of both pathways. Until now, the physiological parameters modified by dibutyryl cyclic AMP (iodine metabolism) were considered to be under the control of the cyclic AMP cascade. While it is true that cyclic AMP can regulate the iodination of thyroglobulin, it is difficult to show a regulatory role for the phosphatidylinositol (PI) pathway in this event, because of the absence of partial agonists and antagonists of TSH.

Freshly isolated cells present poorly reproducible responses to TSH. This is due to the heterogeneity of tissue obtained from the slaughtered animals and the uncontrolled destruction of hormone receptors by proteolysis during dispersing treatment. The culture of isolated cells allows the recovery of receptors but leads to a functional dedifferentiation of the cells unless TSH is present in the media. The concentration of the hormone is critical: below 0.2 mU/ml, the presence of TSH (during the culture) promotes an increased sensitivity of adenylate cyclase to an acute TSH challenge ("positive regulation") (1), whereas with higher concentrations of adenylate cyclase, activity becomes refractory to a second TSH stimulation (2). The response of phosphatidylinositol metabolism does not coincide with that of adenylate cyclase sensitivity (3). The chronic treatment of cells with 0.1 mU/ml TSH promotes an increase of PI turnover that we named "chronic phospholipid effect" to distinguish it from the "acute phospholipid effect" obtained after 2 hrs in the presence of higher hormonal concentration (10-40 mU/ml). The acute challenge of these cells with 40 mU/ml TSH is without effect or produces a "reverse phospholipid effect," i.e., a relative decrease of PI labeling. In contrast to adenylate cyclase activity, the effects observed on PI metabolism are unchanged, even with high TSH concentrations (10 mU/ml) during chronic treatment. The organifying capacity of the cells (PBI) is also increased by their chronic treatment with 0.1 mU/ml TSH (4).

In order to discriminate between the cyclic AMP dependent and independent effects of TSH, we have previously examined and reported the effects of PAF-acether (5) and the phorbol ester TPA (6) on cyclic AMP accumulation, phospholipid metabolism, and PBI in cultured pig thyroid cells. We further study the relationship between these pathways by examining the effect of forskolin, a selective stimulator of adenylate cyclase, on the same parameters.

MATERIALS AND METHODS

Preparation, Culture, and Washings of Thyroid cells

The preparation, culture and washings of thyroid cells were as in (3). The "chronic treatment" was applied during the culture period of the cells. The "acute treatment" took place during the incubation of the cells after the washings.

Cyclic-AMP Assay

Aliquots (250 µl, 0.1-0.2 mg protein) of washed thyroid cell suspension were incubated for 5 min at 37°C in a final volume of 350 µl containing Earle's Hepes buffer (pH 7.2) with mM isobutylmethylxanthine (IBMX), with or without TSH (40 mU/ml) and/or forskolin (0.1 mM). The incubation was terminated by addition of 39 µl 10 N $HClO_4$. The cyclic AMP was assayed by a radioimmunological method as in (3).

Phospholipid Assay

Aliquots (250 µl, 0.1-0.2 mg protein) of washed thyroid cell suspension were incubated for 2 hrs at 37°C in a final volume of 350 µl containing Earle's Hepes buffer (pH 7.2) and ^{32}P-orthophosphate (10 µCi), in the presence or absence of TSH (40 mU/ml) and/or forskolin (0.1 mM). The phospholipids were extracted and analyzed as previously described (3). The phosphatidylinositol (PI) (cpm/mg protein)/phosphatidylcholine (cpm/mg protein) ratio is calculated as an index of the phospholipid effect.

Thyroid Protein Iodination

Aliquots (250 µl, 0.1-0.2 mg protein) of washed cell suspension were incubated for 1 hr at 37°C in a final volume of 350 µl containing Earle's Hepes buffer (pH 7.2) and $Na^{125}I$ (1 µCi) in the presence or absence of TSH or forskolin. At the end of the incubation, 650 µl Earle's Hepes buffer containing 0.1 mM KI and bovine serum albumin fraction V (5 mg/ml), then 1 ml cold 20% trichloracetic acid were added. After centrifugation and washings, the pellet was counted as PB ^{125}I (protein-bound iodine).

RESULTS AND DISCUSSION

As expected from the results obtained with prostaglandin E2 and dibutyryl cyclic AMP (1), 1 µM forskolin reproduced the chronic effects of TSH. Indeed, the "positive regulation" of adenylate cyclase, the "chronic pshopholipid effect," and the increased PBI are cyclic AMP-dependent processes. They appear between day 2 and day 3 of the culture period and reach a maximum at day 4. Although these pleiotypic effects are produced by adenylate cyclase stimulants, the concentration of cyclic AMP during the culture period is not significantly increased. Their induction is accompanied by the synthesis of distinct protein factors. Indeed, a 24-hr treatment with cycloheximide at the end of the culture period is necessary to prevent the "positive regulation" of adenylate cyclase (1) while a 12-hr

treatment is sufficient to prevent the appearance of the "chronic phospholipid effect" (3). Another difference concerns the concentration response of both effects depending on the agonist used. For example, the "positive regulation" of adenylate cyclase results in refractoriness beyond 0.2 mU/ml TSH; however, a progressive increase in adenylate cyclase responsiveness is observed with forskolin between 0.1 and 10 μM. Moreover, acute challenge by forskolin is able to relieve the desensitization due to TSH (10 mU/ml) chronic treatment.

In comparison, the "chronic phospholipid effect" produced by 0.1 or 10 mU/ml TSH are equivalent. Forskolin is also able to produce this effect, but less efficiently than TSH. When TSH-treated cells showing the "chronic phospholipid effect" are challenged with 10 μM forskolin, they exhibit the "reverse phospholipid effect" more intensely than when they are stimulated by 40 mU/ml TSH. Forskolin-treated cells, however, showing the "chronic phospholipid effect," respond to an acute forskolin challenge by a "reverse phospholipid effect" and to an acute TSH challenge by a "normal phospholipid effect."

PBI, in control cells, is regularly decreased in parallel with the duration of the culture, between 1 and 4 days, although the basal cyclic AMP level is unchanged. After day 4 in culture, the acute challenge of control cells with 40 mU/ml TSH or 10 μM forskolin produces a moderate increase in cyclic AMP accumulation without signifiantly altering PBI levels. In cells cultured in the presence of TSH (0.1 mU/ml) or forskolin (10 μM), basal PBI increased progressively during this period, reaching at least a doubling by day 4. The acute challenge of TSH or forskolin-treated cells by TSH or forskolin produces in all the situations a concentration-dependent, biphasic response of PBI level. However, the maxima are variable and are not directly related to the level of cyclic AMP. The lower maximal PBI is obtained with TSH-treated cells stimulated by low TSH concentration (0.1 mU/ml) and is associated with low cyclic AMP levels. The higher maximal PBI is obtained with forskolin-treated cells challenged with higher (x10) concentration of TSH and corresponds to high cyclic AMP levels. The bidirectional control of PBI is, therefore, likely to be due to the level of cyclic AMP as it is observed with forskolin-treated cells, stimulated with forskolin, i.e., cells never submitted to another intracellular mediator. The descending limb of the concentration response curve of TSH decreases below the control value. The difference in the curves obtained after forskolin challenge may be due to the involvement of cyclic AMP independent effects induced by TSH (i.e., increased PI turnover) but not by forskolin.

The present report suggests that both the cyclic AMP dependent and independent cascades promoted by TSH interact at the level of iodide organification. The cyclic AMP independent pathway involves the activation of kinase C and of phospholipase A_2 and the release of arachidonate which, in turn, may control the phosphoinositides cycle and adenylate cyclase activity. Numerous arguments support this interpretation. The stimulation of thyroid cells with TSH produces a transient rise of diacylglycerol (DG) (7). DG is the endogenous stimulator of kinase C, which is present in thyroid tissue (8). TSH treatment probably results in a decreaed phospholipase A_2 activity in these cells, attested by the decreased ability to synthesize prostaglandin E_2 (9). The very high PBI level due to the chronic treatment of the cells with TSH is decreased by an acute treatment with exogenous arachidonate (10), as well as by PAF-acether, which behaves like a partial agnoist of TSH (5), and by the phorbol ester TPA which directly stimulates kinase C activity (6).

Thus, the chronic treatment of isolated thyroid cells with moderate amounts of TSH or with selective stimulators of adenylate cyclase modifies the balance between the cyclic AMP cascade and the phosphoinositides cascade

in favor of the former. This shift, probably linked to a decreased phospholipase A_2 activity, is also linked to a very important increase of GTP cellular level (11) - resulting perhaps from the endogenous ADP ribosylation of Ni and the correlative inhibition of its GTPase activity.

REFERENCES

1. Takasu N, Charrier B, Mauchamp J, et al. Eur J Biochem 90: 139, 1978.
2. Takasu N, Charrier B, Mauchamp J, et al. Eur J Biochem 90: 131, 1978.
3. Gerard C, Haye B, Jacquemin C, et al. Biochim Biophys Acta 710: 359, 1982.
4. Mauchamp J, Margotat A, Chambard M, et al. Cell Tissue Res 204: 417, 1979.
5. Haye B, Aublin JL, Champion S, et al. Eur J Pharmacol 104: 125, 1984.
6. Haye B, Aublin JL, Champion S, et al. Biochem Pharmacol (in press), 1985.
7. Igarashi Y and Kondo V. Biochem Biophys Res Comm 97: 759, 1980.
8. Omri B and Pavlovic Hournac M. Mol Cell Endocrinol 40: 175, 1985.
9. Margotat A, Rolland PH, Charrier B, et al. FEBS Lett 95: 347, 1978.
10. Gerard C, Haye B, and Jacquemin C. FEBS Lett 132: 23, 1981.
11. Aublin JL, Champion S, Lambert B, et al. (unpublished data).

REGULATORY NETWORKS IN THE ACUTE CONTROL OF THYROID FUNCTION

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Two main regulatory pathways are involved in the acute control of dog thyroid cell function: TSH activates thyroid adenylate cyclase and, through cyclic AMP, stimulates both thyroid hormone synthesis and secretion. TSI, E prostaglandins, and norepinephrine, through β receptors, all act similarly. Norepinephrine, through α receptors and the inhibitory pathway of cyclase (NI), directly inhibits adenylate cyclase and the cyclic AMP-mediated effects (Fig. 1). Acetylcholine, through muscarinic receptors, enhances calcium influx and phosphatidylinositol (PI) turnover. The latter process releases in the cell two other intracellular signal molecules, diacylglycerol (DAG) and myoinositol 1,4,5-phosphate (IP_3). IP_3 induces the intracellular release of Ca^{++}, while DAG activates protein kinase C (Fig. 2). Increased cytosolic calcium enhances H_2O_2 generation and iodination, cyclic GMP accumulation, and arachidonate release; it activates calmodulin-dependent cyclic nucleotide phosphodiesterase and, thus, lowers cyclic AMP levels and thyroid hormone secretion (Fig. 1). These effects are mimicked by high extracellular calcium and ionophore A23187.

The phorbol esters tumor promoters tetradecanoyl-phorbolacetate (TPA), phorbol-12,13-diacetate (PDA), and phorbol-dibutyrate (PDBu), reproducing the action of DAG probably through activation of protein kinase C, also stimulate iodide organification (Fig. 3) and inhibit thyroid hormone secretion (Fig. 4). These actions do not require the presence of extracellular calcium and occur without modifications of basal cyclic nucleotide levels, nor of PI turnover (Fig. 5,6). The stimulation of adenylate cyclase by TSH or forskolin is, however, unaffected (Fig. 5).

Phorbol esters also exert a negative feedback at the level of the muscarinic receptor and of the α type, cAMP-independent effects of TSH: they block the increased PI turnover resulting from carbamylcholine (Cchol) and TSH actions (Fig. 7), decrease basal ^{45}Ca efflux from ^{45}Ca preloaded slices, and inhibit the TSH and Cchol-induced increased ^{45}Ca efflux (Fig. 8). These actions of phorbol esters (through protein kinase C activation) possibly explain the mechanism of cholinergic desensitization.

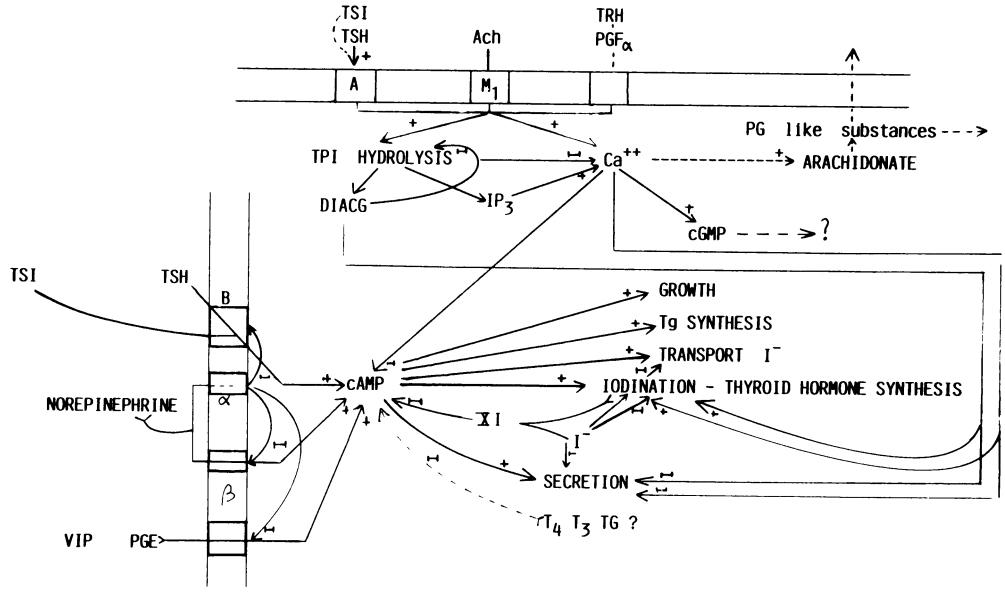

Fig. 1. General regulatory networks of dog thyroid metabolism in vitro. TSI: thyroid-stimulating immunoglobulins; Ach: acetylcholine; PG: prostaglandins; TPI: phosphatidylinositol-4,5-bisphosphate; DIACG: diacylglycerol.

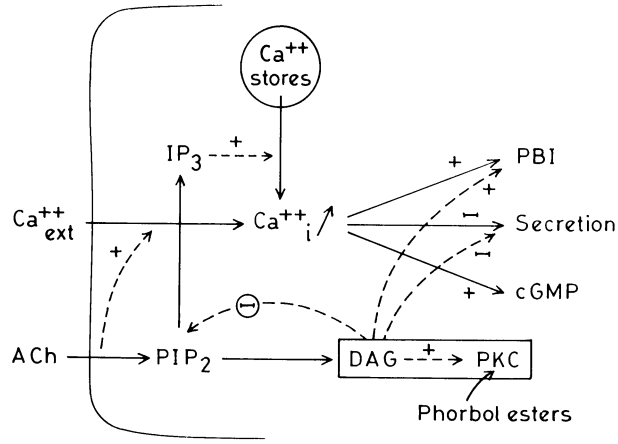

Fig. 2. Effects of protein kinase C activation on dog thyroid metabolism.

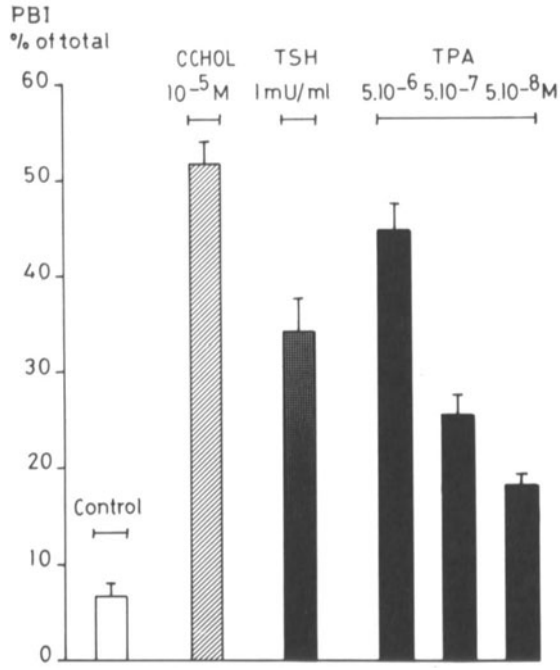

Fig. 3. Effects of TPA stimulation of iodide organification (PBI). Dashed column: carbamylcholine (Cchol 10^{-5} M); checked column: TSH, 1 mU/ml; and solid columns: TPA, 5.10^{-6} M to 5.10^{-8} M on iodide organification. Each point represents the mean ± SEM of triplicate determinations.

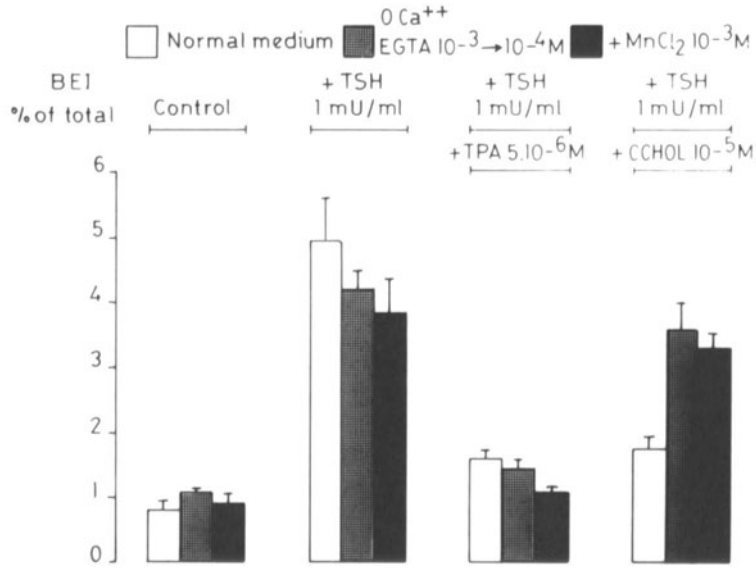

Fig. 4. Inhibition by TPA (5.10^{-6} M) and carbamylcholine (Cchol 10^{-5} M) of TSH (1 mU/ml) stimulated hormone secretion (expressed as BEI % of total - see methods). Ca^{++} depletion, as $MnCl_2$ addition, does not relieve the inhibition exerted by TPA, as opposed to what is observed with Cchol. Open columns: normal, 1.45 mM Ca^{++} medium; checked columns: 1 hour preincubation in a $0^{-}Ca^{++}$ medium, containing EGTA, 10^{-3} M; and solid columns: preincubation and incubation in a $0^{-}Ca^{++}$ medium containing $MnCl_2$, 1 mM. Each point represents the mean ± SEM of triplicate determinations.

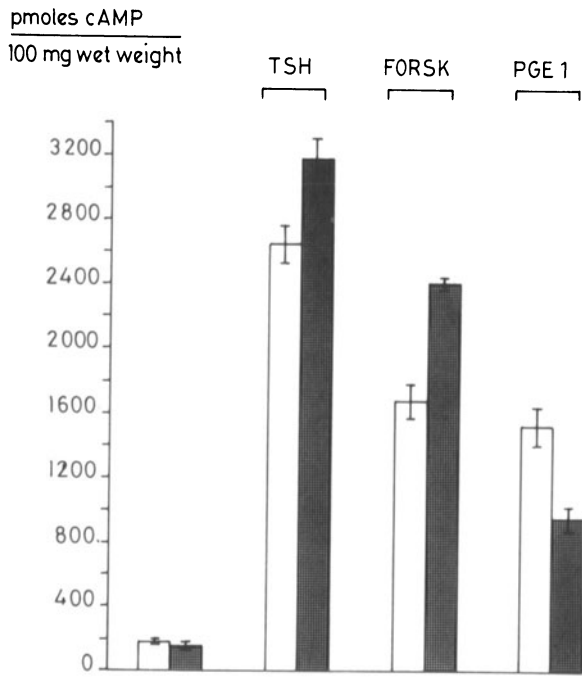

Fig. 5. Effect of TPA (5.10^{-6} M) on cyclic AMP accumulation in thyroid slices stimulated by TSH (1 mU/ml) or forskolin (forsk: 2.5 µM) or PGE_1 (5 µg/ml). TPA was present during the whole 60 min preincubation and the 30 min incubation. TSH, forskolin, and PGE_1 were only added in the test incubation. Open columns: control; checked columns: TPA. Results are expressed as pmoles cAMP ± SEM per 100 mg wet weight tissue in one typical experiment.

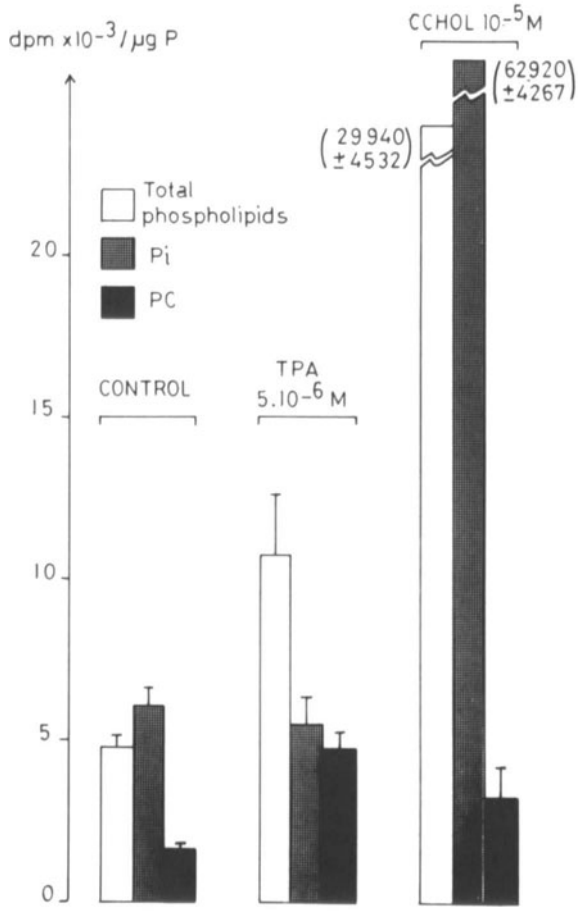

Fig. 6. Effect of TPA (5.10^{-6} M) and carbamylcholine (Cchol) on ^{32}P incorporation into: total phospholipids (open columns); phosphatidylinositol (PI) (checked columns); and phosphatidylcholine (PC) (solid columns). TPA had no effect on P_1 turnover. Each point represents the mean ± SEM of triplicate determinations.

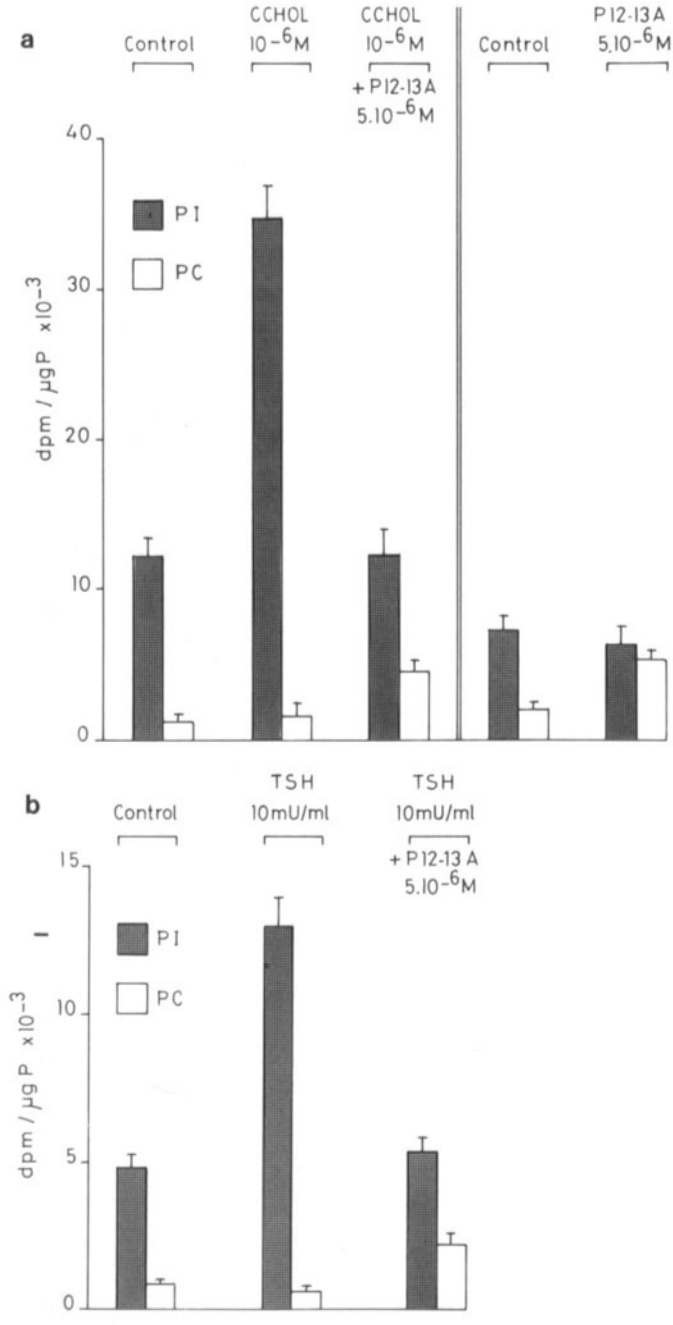

Fig. 7. P12, 13 A inhibits the increased PI turnover induced by carbamylcholine (Cchol and TSH.

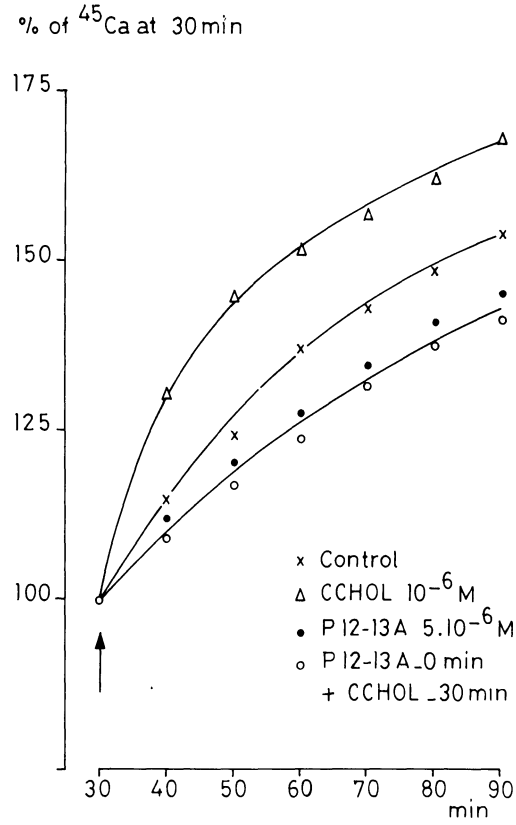

Fig. 8. Phorbol-12,13-diacetate (PDA) decreases basal ^{45}Ca efflux and inhibits Cchol-induced increased efflux.

STIMULATION OF THYROID ADENYLATE CYCLASE ACTIVITY IN SERA OF PATIENTS WITH NONTHYROIDAL ILLNESS

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INTRODUCTION

In assessing serum bioactive TSH, as measured by thyroid adenylate cyclase (TAC) stimulating activity, using the FRTL-5 cell line, we observed that sera from hypothyroid patients stimulated TAC. However, in our earlier studies, no correlation between TAC-stimulating activity and serum TSH or IgG was found. In the present study, TAC-stimulating activities were investigated in sera of patients with nonthyroidal illness (NTI).

MATERIALS AND METHODS

Patients

Blood samples were collected from 89 patients with NTI, including 10 with hepatocellular carcinoma, 14 with liver cirrhosis, 13 with hepatitis, 6 with gastric cancer, 12 with collagen disease, 24 with neurological disease, and 10 with other diseases. Thyroid-stimulating activities were measured in all 89 patients using FRTL-5 cell line. However, in the analysis of thyroid-stimulating activity, the results from only 36 of the patients were used, because the other patients had been treated with drugs which may have affected serum thyroid hormone concentrations.

Methods

The FRTL-5 rat thyroid cell culture system. FRTL-5 cells were grown in Coon's modified Ham's F-12K medium supplemented with 5% calf serum and a six hormone mixture consisting of TSH, insulin, hydrocortisone, transferrin, somatostatin, and glycyl-L-histidyl-L-lysine acetate, and were transferred biweekly.

For the studies, 1 x 10^5 cells were seeded in each well of Nunc 24-well plates, and cultured for three days. The media were then removed and replaced by a five hormone medium (without TSH), and cultured for an additional four days. The media were removed by suction, and 80 µl of serum or TSH solution of various concentrations and 80 µl Hank's balanced salt solution (HBSS) containing 0.4% FBS were added. Incubations were continued for 60 min at 37°C in the presence of 0.4 mM methyl-3-isobutyl-xanthine (MIBX).

Cyclic AMP concentrations in media were determined by cAMP RIA kits (Yamasa). Sera of 10 normal individuals were run for each assay as controls (1-3).

The porcine thyroid cell culture system. Porcine thyroid cells were cultured by usual methods. For the studies, 5 x 10^5 thyroid cells per 350 µl of cell suspension were seeded in each well of 24-well culture plates. The assay for thyroid stimulating activity was usually performed after 18-24 hours of culture. After removing the medium, thyroid cells in monolayer culture were incubated for two hours at 37°C with 120 µl serum or TSH of various concentrations in the assay buffer. Cyclic AMP concentrations in media were determined by cAMP RIA kits (Yamasa) and sera of 10 normal individuals were run for each assay as controls (4).

Measurements of serum thyroid hormone concentrations. Serum concentrations of T_4, free T_4 (FT_4), T_3, and TSH by RIA were measured.

Testing of various known adenylate cyclase activators for thyroid-stimulating activity using FRTL-5 cells. Various known adenylate cyclase activators such as glucagon, norepinephrine, epinephrine, ACTH, ADH, prostaglandin E_1, and TSH were used. The concentrations of the above activators were those of normal serum concentrations for each activator, or 10, 10^2, 10^3, and 10^4 times normal dissolved in HBSS, and the volume used was 80 µl.

RESULTS

Thyroid-stimulating activities of the patients are shown in Fig. 1. Activities were higher than those of normal subjects in greater than half of the patients with each disease except in those with neurological disease in whom approximately one quarter had elevated values. No disease specificity was observed in thyroid-stimulating activities. Cyclic AMP concentrations were measured in media before and 60 min after incubation with sera from 10 normal subjects and 10 patients. Four patients showed clearly increased cAMP concentrations at 60 min compared to those of normal subjects. There was no difference between the two groups before incubation. The porcine thyroid cell culture systems were employed to measure thyroid-stimulating activities and the results were compared to those of FRTL-5 cells.

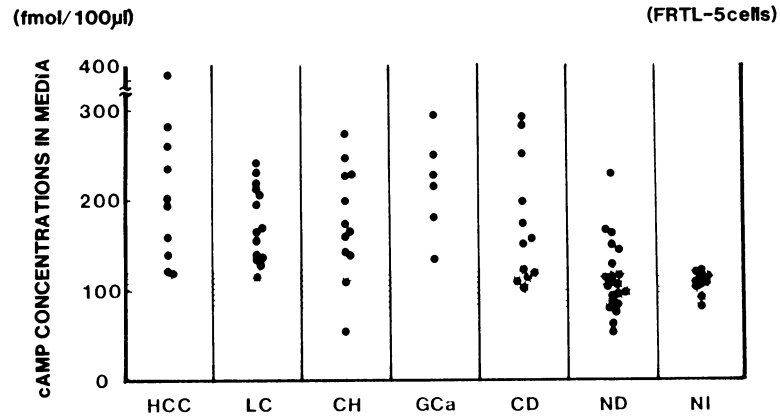

Fig. 1. Thyroid-stimulating activity in sera of patients with nonthyroidal illness. HCC: hepatocellular carcinoma, CH: chronic hepatitis, GCa: gastric cancer, CD: collagen disease, ND: neurological disease, Nl: normal.

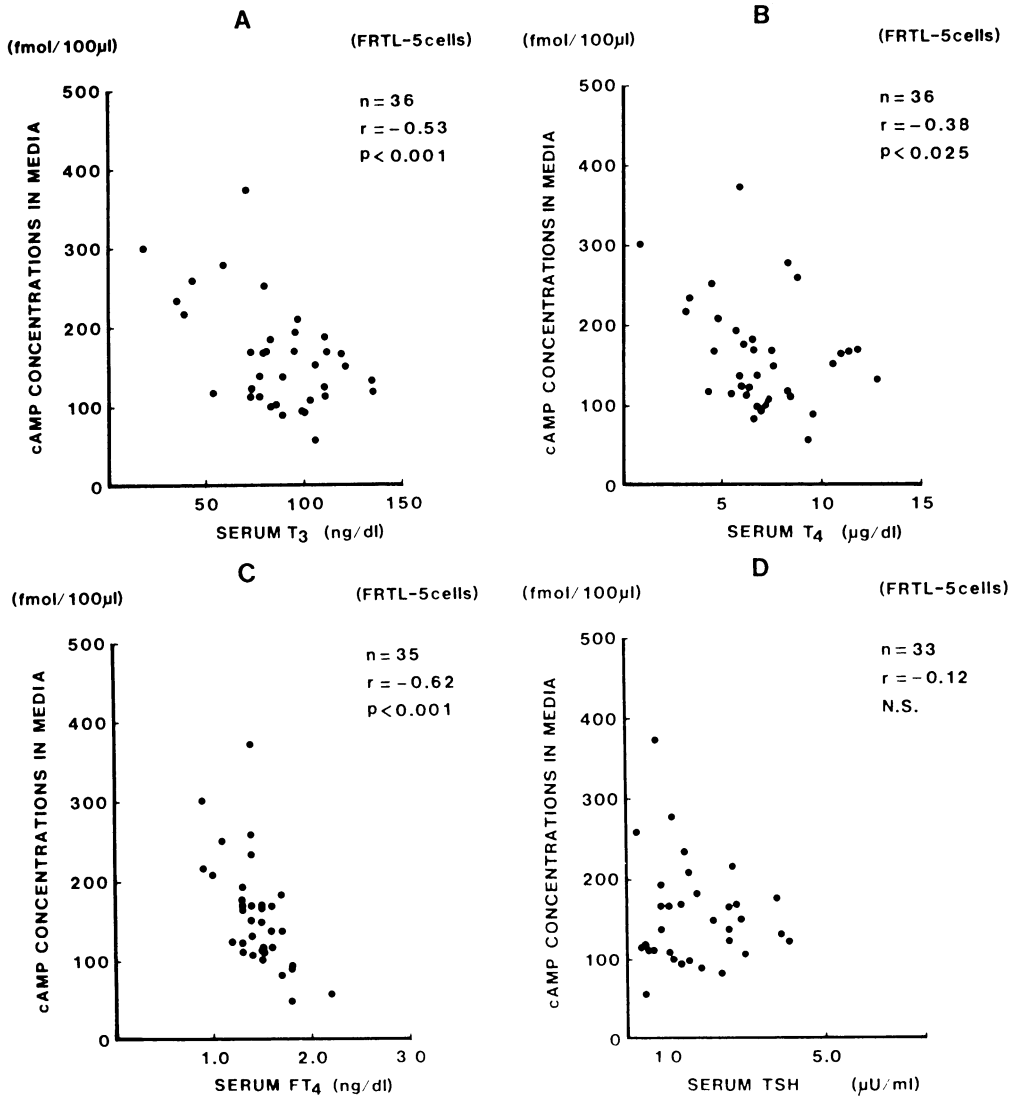

Fig. 2. Correlations of cAMP concentrations in media at 60 min with serum T_3 (A), T_4 (B), FT_4 (C), and TSH (D) concentrations.

A strongly positive correlation between thyroid-stimulating activities in the porcine thyroid cell culture systems and the FRTL-5 cell culture system was found, demonstrating that both porcine thyroid cells and rat thyroid cells are stimulated by sera of patients with nonthyroidal illness. Correlations of cAMP concentrations in media at 60 min with serum T_3, T_4, FT_4, and TSH concentrations are shown in Fig. 2. Highly significant inverse correlations of cAMP concentrations with serum T_3 (α=0.53, $p<0.001$), T_4 (α=0.38, $p<0.025$), and FT_4 (α=-0.62, $p<0.001$) concentrations were found. However, cAMP concentrations did not correlate with serum TSH concentrations. Various known adenylate cyclase activators were added to FRTL-5 cell culture systems. Adenylate cyclase activators such as glucagon, norepinephrine, epinephrine, ACTH, ADH, prostaglandin E_1, and TRH did not elevate cAMP production even at concentrations as high as 10^4 times that of normal serum concentrations.

DISCUSSION

In this study, sera obtained from patients with various NTI increased cAMP concentrations in culture media of FRTL-5 cells.

The stimulating effects of sera were also observed in porcine thyroid cells in primary culture, indicating that the stimulatory effects were not specific for FRTL-5 cells.

Furthermore, the thyroid-stimulating effects of sera were not disease specific and significantly correlated inversely with serum T_3 and T_4 concentrations. These results suggest that in low T_3 or in low T_3 and low T_4 syndrome, sera of patients stimulate the adenylate cyclase activity in thyroid cells *in vitro*.

In the present study, sera of patients with low T_3 syndrome clearly stimulated the production of cAMP in thyroid cells, and this TAC-stimulating activity was not due to the known substances, such as glucagon, norepinephrine, epinephrine, ACTH, ADH, prostaglandin E_1, or TRH, since thousands of times more normal serum concentrations of these substances did not stimulate cAMP production in the present study.

Although the nature of substance(s) present in sera of low T_3 syndrome is not entirely known, it is conceivable that there exists some mechanism to stimulate thyroids in order to compensate the decreased serum T_3 levels in low T_3 syndrome (5).

However, it is also likely that adenylate cyclase-stimulating activity of sera in these patients is completely independent of metabolic changes in thyroid hormone and simply represents a nonspecific metabolic response to the severe illness.

In conclusion, these results indicate that sera of patients with low T_3 syndrome stimulates TAC activity. It is conceivable that there exists some mechanism independent of TSH to compensate for the decreased serum T_3 levels in low T_3 syndrome.

SUMMARY

Sera of many patients with NTI stimulate TAC activity and the stimulating activity inversely correlated with serum T_3 concentrations. Although the nature of such substance(s) present in sera of patients with low T_3 syndrome is not entirely known, it is conceivable that there exists some mechanism independent of TSH to compensate for the decreased serum T_3 levels in low T_3 syndrome.

ACKNOWLEDGMENT

The author is indebted to Dr. Shigenobu Nagataki and Dr. Lindy F. Kumagai for their revision and to Misses Keiko Hakugawa and Keiko Takahashi for their excellent secretarial service.

REFERENCES

1. Ambesi-Impiombato FS, Parks LAM, and Coon HG. Proc Natl Acad Sci 77: 3455, 1980.
2. Vitti P, Rotella CM, Valente WA, et al. J Clin Endocrinol Metab 57: 782, 1983.

3. Valente WA, Vitti P, Rotella CM, et al. New Engl J Med 27: 1028, 1982.
4. Kasagi K, Konishi J, Iida Y, et al. J Clin Endocrinol Metab 54: 108, 1982.
5. Wartofsky L and Burman KD. Endocr Rev 3: 164, 1982.

HORMONAL REGULATION OF DNA POLYMERASE β ACTIVITY IN RAT THYROID GLAND

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Our previous studies provided the evidence that rat adrenal gland and testis contain three species of DNA polymerase, α, β, and γ (1,2). Among them, the β-enzyme activity in the endocrine organs was modulated by pituitary trophic hormones. Namely, the manipulation of hypophysectomy reduced the level of β-enzyme activity and changed its molecular size. These changes were reversed by the administration of the pituitary trophic hormone to the hypophysectomized rat. Therefore, DNA polymerase β activity may be strongly regulated by the feedback mechanism in these endocrine organs. Here we extended our work to another endocrine organ, the thyroid gland, which is regulated by TSH and shows some extent of autoregulation under the depletion of TSH.

In this paper, we confirmed the existence of three molecular sizes of DNA polymerase β and the decrease in the activity of DNA polymerase β of the small molecular size, accompanied by a concomitant shifting of the molecular size to the larger form.

MATERIALS AND METHODS

Sham-operated and hypophysectomized male Wistar rats weighing about 150 g fed with regular diet and saline were obtained from Imamichi Animal Laboratory, Co., Ohmiya, Japan. Thyroidal regeneration was induced by daily intraperitoneal injections of a thyroid-stimulating hormone (TSH, Armour Pharmaceutical Co., IL, USA) to hypophysectomized rats immediately after operation (for 7 days). The thyroid glands were removed at 1, 2, 3 and 4 weeks after hypophysectomy and were weighed. Ten thyroid glands were collected and homogenized as described previously (1).

Sucrose gradient centrifugation was performed using 5 to 20% (W/V) linear gradient sucrose in 50 mM Tris-HCl buffer, pH 7.5, containing 0.2 M KCl, 2 mM 2-mercaptoethanol, and 0.5% Nonidet P-40. Enzyme samples (0.2 ml) were layered onto the 4.8 ml gradient and centrifuged in a Hitachi RPS 50 rotor (Hitachi Koki Co., Ltd., Katsuta, Japan) at 45,000 rpm at 4°C for 16 h.

The assays for DNA polymerase α, β, and γ were performed by the methods reported previously (2). One unit of the enzyme (u) is defined as an amount which catalyzes the incorporation of 1 nmol of deoxynucleotide in 60 min under the assay conditions. The reaction showed a near-linearity with respect to the enzyme dose up to 30 μl.

RESULTS

Rat thyroid contained a relatively high level of DNA polymerase β activity (215 u/g tissue) when compared to the level of DNA polymerase α (58 u/g tissue) and γ (12 u/g tissue). The level of thyroidal DNA polymerase β was similar to those in liver, thymus, and brain and lower than that in adrenal and testicular extracts (1,2).

Hypophysectomy induced no remarkable changes in the activity of polymerase α, β, and γ species in rat thyroid extract, but only a slight reduction of DNA polymerase β activity 4 weeks after hypophysectomy (Table 1). The β-enzyme activity per gland was slightly reduced as compared to the control thyroid at 1 and 4 weeks after hypophysectomy. The contents of protein and DNA per wet weight of sham-operated and hypophysectomized rat thyroid were unchanged. Therefore, DNA polymerase β activity per DNA (per cell) showed no remarkable changes during 4 weeks after hypophysectomy when the extracted samples were used for measuring the activity (Table 1). The extracts from sham-operated or hypophysectomized rat thyroid glands were centrifuged through sucrose gradient (Fig. 1). The sedimentation profiles

Table 1. DNA Polymerase Activities in Thyroid Extracts from Hypophysectomized Rats

DNA polymerase activities (cpm)		Sham-operated 1W	Sham-operated 4W	Hypophysectomized (hypox) 1W	Hypophysectomized (hypox) 2W	Hypophysectomized (hypox) 3W	Hypophysectomized (hypox) 4W	Hypox + TSH
α	a	3263	4410	2029	2857	2572	2114	2477
	b	251	294	205	304	289	271	292
	c	8700	9483	7884	10218	9665	9033	10812
	d	139	147	104	160	145	135	126
β	a (x 10^3)	36.1	37.8	21.2	25.4	31.1	15.4	22.7
	b	2775	2520	2140	2700	3495	1475	2904
	c (x 10^3)	96.4	86.9	76.5	90.8	109.2	75.9	90.8
	d	1542	1326	1085	1421	1519	1039	1452
γ	a	2665	3600	2228	2265	1869	1669	1833
	b	205	240	225	241	210	214	235
	c	7105	8040	7758	8100	7500	7379	8392
	d	114	120	102	126	116	107	117

a: per gland, b: per mg wet weight, c: per mg protein, d: per μg DNA, w: weeks. The values are the mean of triplicate experiments.

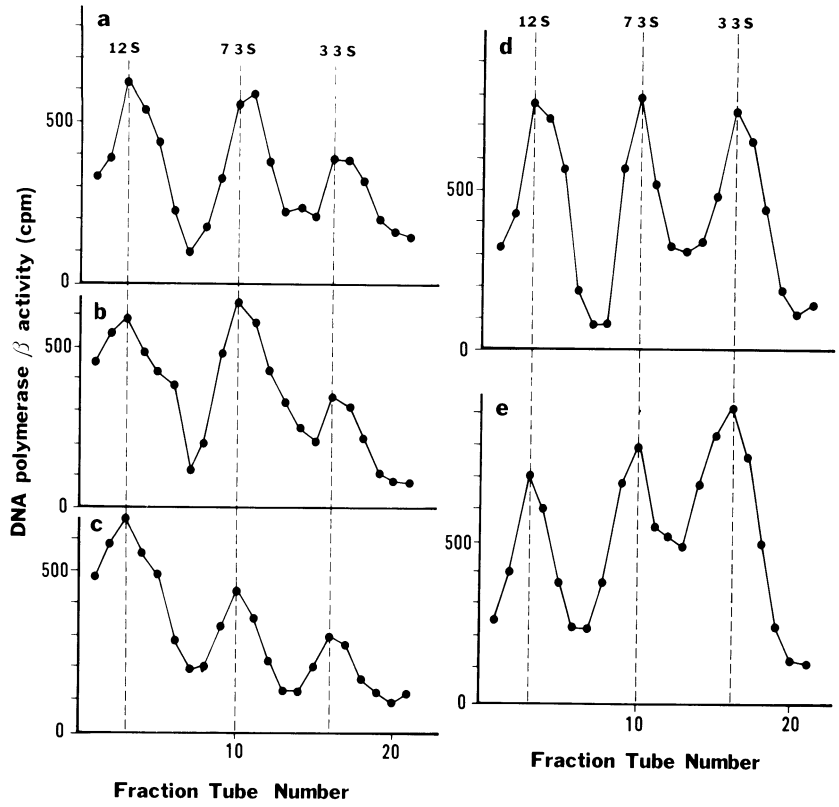

Fig. 1. Effect of hypophysectomy on sedimentation profile of DNA polymerase β activity in rat thyroid gland. (a) one week after hypophysectomy; (b) three weeks after hypophysectomy; (c) four weeks after hypophysectomy; (d) sham operation; (e) two units of TSH were administered to the hypophysectomized rat for seven days.

revealed three peaks of DNA polymerase β activity in the rat thyroid, which sedimented at 3.3S, 7.3S, and 12S. After hypophysectomy, 3.3S DNA polymerase β decreased gradually and 7.3S or 12S DNA polymerase β increased slightly, suggesting the shifting of molecular size from 3.3S to 7.3S or 12S by hypophysectomy. The α- and γ-enzyme activities were undetectable in the samples collected after sucrose gradient centrifugation.

Enzymological characteristics of DNA polymerase β from hypophysectomized rat thyroids were examined with respect to the sensitivities to aphidicolin, 2', 3'-dideoxy TTP and potassium phosphate (Table 2). The preparations of 3.3S, 7.3S, and 12S DNA polymerases from sham-operated or hypophysectomized rats were similarly resistant to aphidicolin and were sensitive to inhibition by 2', 3'-dideoxy TTP and potassium phosphate. Therefore, all of these molecular species can be classified as DNA polymerase β.

The changes in the activity and the molecular size of DNA polymerase β after hypophysectomy were reversed by TSH. Namely, 3.3S β-enzyme activity returned to the level in sham-operated rat thyroid, and the reversed shift of the molecular size from 12S or 7.3S to 3.3S resulted (Fig. 1).

Table 2. Effect of d_2TTP, KPO_4 Buffer and Aphidicolin on Thyroidal Polymerase β Activity (cpm)

β-enzyme fraction		Control	d_2TTP	KPO_4	Aphidicolin
Sham	12S	550	60	55	485
	7.3S	635	45	65	685
	3.3S	725	70	95	855
Hypox 1W	12S	430	80	70	495
	7.3S	415	65	130	405
	3.3S	195	75	25	160
Hypox 3W	12S	605	90	60	520
	7.3S	630	80	115	530
	3.3S	205	65	75	345
Hypox 4W	12S	445	50	60	375
	7.3S	290	95	50	430
	3.3S	235	85	75	225

5 μM d_2TTP, 20 mM KPO_4 or 20 μM aphidicolin was added to the reaction medium. 25 μl of β-enzyme fraction was used for the assay.

Thyroid hormone administered to hypophysectomized rats through the drinking water did not reverse these changes in DNA polymerase β activity.

DISCUSSION

We have shown that there are three species of DNA polymerase α, β, and γ in the thyroid, and the β-enzyme is most abundant among them. Sucrose gradient centrifugation revealed three peaks of DNA polymerase β activity, sedimenting at 3.3S, 7.3S, and 12S. 3.3S β-enzyme is sensitive for TSH regulation. We confirmed that these peaks obviously have the characteristics of DNA polymerase β, using inhibitors, i.e., d_2TTP, aphidicolin and phosphate buffer. A putative conversion of the low molecular weight form (3.3S) to the higher molecular weight form (7.3 and 12S) was observed after hypophysectomy. Similar high molecular weight forms of β enzyme have also been observed in the extracts from adrenal glands or testes, and the ratio of the high to the low molecular weight form has been changed by hypophysectomy and reversed by pituitary trophic hormones (1,2). Since purified DNA polymerase β showed a single peak at 3.3S (3), the high molecular weight forms of β-enzyme may represent the modified form associated with other protein factors and may play an unknown function.

In adrenal gland and testis, the depletion of pituitary trophic hormone by hypophysectomy induced a drastic decrease in the total DNA polymerase β

activity measured with crude extract or fractions of sucrose gradient centrifugation. In contrast, TSH depletion by hypophysectomy did not influence the total activity of DNA polymerase β in the thyroid gland. The sustaining of the total activity of β-enzyme and the shifting of β-enzyme to the larger to the larger molecular form in the thyroid after hypophysectomy may relate to the fact that the thyroid is less dependent on pituitary trophic hormone than are adrenal gland or testis and that the thyroid shows some autoregulation of function.

ACKNOWLEDGMENTS

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REFERENCES

1. Nagasaka A and Yoshida S. Endocrinology 111: 1345, 1982.
2. Nagasaka A and Yoshida S. Endocrinology 115: 1110, 1984.
3. Weissbach A. Annu Rev Biochem 46: 25, 1977.

NOREPINEPHRINE AND TSH STIMULATION ON IODIDE EFFLUX IN FRTL-5 THYROID CELLS INVOLVES METABOLITES OF ARACHIDONIC ACID AND IS ASSOCIATED WITH THE IODINATION OF THYROGLOBULIN

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Previous studies in FRTL-5 rat thyroid cells have indicated that TSH and norepinephrine (NE) stimulation of iodide efflux is mediated by a calcium signal, rather than a cAMP signal (1,2). The mechanism by which calcium mobilization signals iodide efflux is unknown in the thyroid or in FRTL-5 thyroid cells. In several systems, calcium mobilization has been associated with an increased breakdown of polyphosphoinositides and subsequent release of arachidonic acid (3). The possible involvement of this biochemical pathway in TSH- and NE-stimulated iodide efflux in FRTL-5 cells is supported by the observation that arachidonic acid stimulates iodide efflux in these cells (1).

In the present report, we have further investigated the role of arachidonic acid or its metabolites in the TSH and NE stimulation of iodide efflux in FRTL-5 cells and have directly linked this process to the iodination of thyroglobulin.

MATERIALS AND METHODS

Materials

Purified TSH was the same as a preparation previously described (4). NE, arachidonic acid, quinacrine, trifluoroperazine, indomethacin, and nordihydroguaiaretic acid (NDGA) were from Sigma Chemical Co. (St. Louis, MO). 5,8,11,14-eicosatetraynoic acid (ETYA) was kindly supplied by Dr. F. Hirata. Piperonil butoxide and 2-diethylaminoethyl-2,2 diphenyl valerate (SKF 525A) were a generous gift of Dr. J. Gillette. All other chemicals were of reagent of higher grade.

Cells

The isolation, growth, and basic characteristics of FRTL-5 rat thyroid cells have been previously described (5).

Iodide Efflux

Iodide efflux was measured as previously described (1). In brief, cells grown in 35 mm Costar dishes (Cambridge, MA) were incubated for 40 min at 37°C with 2 ml of 10 mM Hepes-buffered Hank's Balanced Salt Solution (buffered HBSS), containing approximately 1 μCi carrier-free ^{125}I and 10 μM NaI. At the end of the incubation, the medium was gently removed and replaced every 2 min with 2 ml fresh nonradioactive buffered HBSS, containing 10 μM NaI and other additions when indicated. After the last medium removal, the cells were extracted with ethanol for counting along with the previously collected medium samples. Efflux data are presented as rate coefficients based on the counts per min remaining (as percentage of total) at the indicated times and were calculated using the following formula:

$$Kn = \frac{(\ln n_{t1} - \ln n_{t2})}{(t2 - t1)} \times 100\%$$

where Kn is the rate coefficient (as percentage per min) and n is the percentage of radioactivity remaining in the cells at time t.

Measurement of TSH or NE Stimulation of Iodination of Thyroglobulin

FRTL-5 thyroid cells released from culture plates by gentle trypsinization (5) were resuspended in 10 mM Hepes-buffered (pH 7.3) growth medium containing 0.5% albumin. Aliquots of the cells were incubated with radioiodine and either no hormone, 0.1 μM TSH plus or minus 10 μM ETYA, 1 μM NE plus or minus 10 μM ETYA, or 10 μM arachidonic acid. After 4 h at 37°C, cells were collected by centrifugation, washed once with HBSS, solubilized in 200 1 of electrophoresis sample buffer (0.062 M Tris-HCl, pH 6.8; 1% SDS; 10% glycerol; 5% 2-mercaptoethanol; and 0.01% bromophenol blue), and heated at 100°C for 10 min. Aliquots were subjected to 10% polyacrylamide slab gel electrophoresis, as described by Laemmli (6), stained, and then subjected to autoradiography.

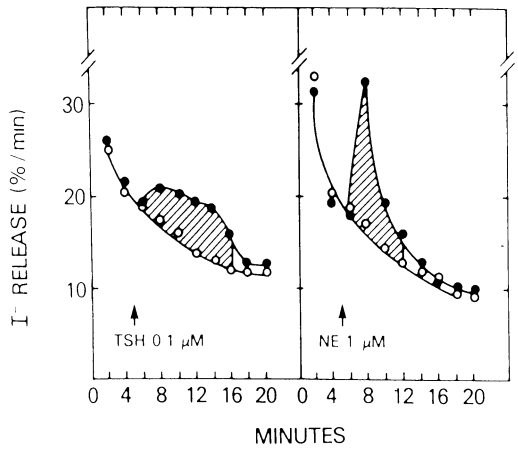

Fig. 1. Effect of TSH and NE on the rate of iodide efflux in FRTL-5 cells. Experiments were performed and data presented as described in Materials and Methods. Iodide efflux was measured in separate dishes where agents (●) were added for the final 14 min at the indicated final concentrations, with duplicate dishes serving as controls (○). The stimulation of iodide efflux induced by each agent was quantified (Δ_1 iodide release, % per min) by comparing the difference in the rate of iodide release at each time point with respect to the control (shaded area).

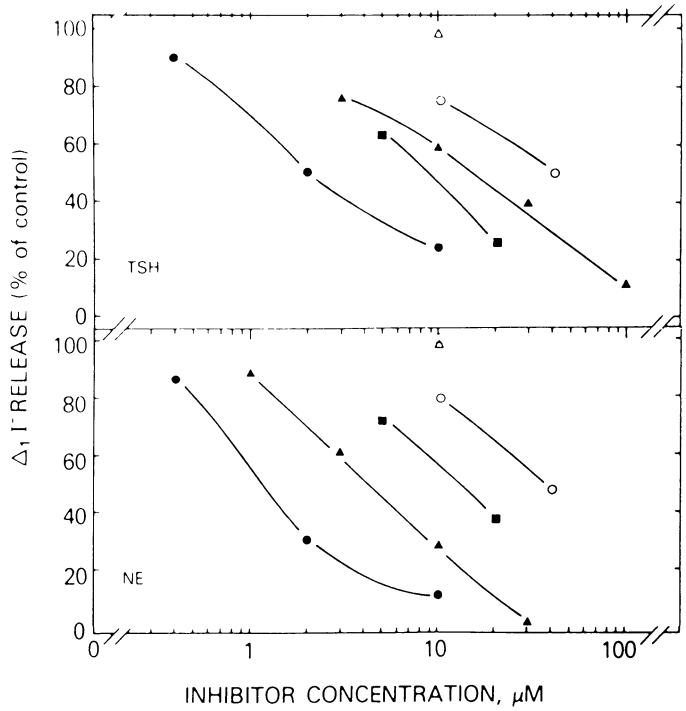

Fig. 2. Effect of different inhibitors of arachidonic acid metabolism on TSH- and NE-stimulated iodide efflux in FRTL-5 cells. Experiments were performed as detailed in Materials and Methods and in the legend of Fig. 1. The inhibitors were included in the medium from the beginning of the efflux experiment at the indicated final concentrations. Results are expressed as Δ_1 iodide release (% of control), which was derived by comparing the difference at each time point in the rate of iodide release induced by the agents tested in the absence or in the presence of each inhibitor. Time intervals appropriate to each stimulatory agent (shaded areas in Fig. 1) were used for comparison. ●——● = ETYA; ■——■ = NDGA; ▲——▲ = piperonyl butoxyde; ○——○ = SKF 525A; △——△ = indomethacin.

RESULTS AND DISCUSSION

The effects of TSH and NE on the rate of iodide efflux in FRTL-5 cells are summarized in Fig. 1. The stimulation of iodide efflux can be quantitated by comparing the differences in the rate of iodide release at each time point in the presence or in the absence of a stimulator, either in experiments performed in control medium (buffered HBSS) or in medium containing the inhibitors to be tested below. This difference (Δ, iodide release) is readily represented in Fig. 1 by the shaded areas between the two curves.

To further substantiate the involvement of arachidonic acid in the stimulation of iodide efflux in FRTL-5 cells, we have studied the effect

of two inhibitors of the phospholipase A2, which releases arachidonic acid from membrane phospholipids (3), on NE- or TSH-stimulated iodide efflux in these cells. Both quinacrine (1 mM) (7) and trifluoroperazine (20 μM) (8), at concentration similar to those blocking this enzyme in platelets (9), markedly inhibited (∿90%) the stimulation of iodide efflux induced by either hormone.

In several mammalian cells, arachidonic acid is metabolized via three enzymatic pathways; the ciclooxygenase which generates prostaglandins and thromboxanes, the lipoxygenase which forms leukotrienes, and a NADPH-dependent cytocrome P450 (epoxygenase) which produces epoxide metabolites (10). The effects of various inhibitors of arachidonic acid metabolism on TSH- and NE-stimulated iodide efflux are shown in Fig. 2. Inhibitors of the lipoxygenase, such as ETYA and NDGA, at concentrations comparable to those required to inhibit arachidonic acid metabolism in beta pancreatic cells (11) and in human platelets (12), suppressed the stimulation of iodide efflux by TSH and NE, while indomethacin, at a concentration that specifically inhibits the ciclooxygenase (13), was without effect. Piperonyl butoxide and SKF 525A, two inhibitors of the epoxygenase (14), also blocked TSH- and NE-stimulated iodide release in a dose-dependent manner, at concentrations similar to those used to inhibit this enzyme in other systems (14, 15).

Although the site of the membrane where iodide efflux occurs in FRTL-5 cells cannot be identified because these cells are not polarized and do not form follicles in culture, it can be representative of the *in vivo* process wherein iodide is lost from the cell into the follicular lumen prior to the iodination of thyroglobulin. TSH, as well as NE and arachidonic acid, has been shown to stimulate the iodination of thyroglobulin in thyroid slices (16) or lobes (17). The possibility must, therefore, be considered that iodide efflux induced by TSH and NE in FRTL-5 cells is an *in vitro* model of the release of iodide into the follicular lumen. This hypothesis is supported by the demonstration that TSH and NE, in addition to stimulating iodide efflux, increased the iodination of thyroglobulin in FRTL-5 cells. This activity was duplicated by arachidonic acid and blocked by ETYA (data not shown).

In conclusion, the results of the present study indicate that TSH and NE stimulation of iodide efflux in FRTL-5 cells involves metabolites of arachidonic acid produced through the lipoxygenase and/or epoxygenase pathways and is linked to the iodination of thyroglobulin.

REFERENCES

1. Weiss SJ, Philp NJ, and Grollman EF. Endocrinology 114: 1108, 1984.
2. Corda D, Marcocci C, Kohn LD, et al. J Biol Chem 260: 9230, 1985.
3. Berridge MJ. Biochem J 220: 345, 1984.
4. Winand RJ and Kohn LD. J Biol Chem 245: 967, 1970.
5. Ambesi-Impiombato FS, Parks LAM, and Coons HG. Proc Natl Acad Sci USA 77: 3455, 1980.
6. Laemmli UK. Nature 227: 680, 1970.
7. Blackwell GL, Duncombe WG, Flower RJ, et al. Br J Pharmacol 59: 353, 1977.
8. Walenga RW, Opas EE, and Feinstein MB. J Biol Chem 256: 12523, 1981.
9. Lapetina EG, Billah MM, and Cuatrecasas PJ. J Biol Chem 256: 5037, 1981.
10. Capdevila J, Chacos N, Werringloer J, et al. Proc Natl Acad Sci USA 78: 5362, 1981.
11. Metz S, Van Rollins M, Strife R, et al. J Clin Invest 71: 1191, 1983.
12. Van Wauwe J and Goossens J. Prostaglandins 25: 725, 1983.

13. Salari H, Braquet P, and Borgeat P. Prostaglandins Leukotriens Med 13: 53, 1984.
14. Testa B and Jenner P. Drug Metab Rev 12: 1, 1981.
15. Capdevila J, Chacos N, Falck JR, et al. Endocrinology 113: 421, 1983.
16. Boeynaems JM, Van Sande J, Decoster C, et al. Prostaglandins 4: 537, 1980.
17. Maayan ML, Volpert EM, and From A. Endocrinology 109: 930, 1981.

EFFECT OF TSH AND THYROID-STIMULATING ANTIBODY (TSAb) ON THE DESENSITIZATION OF THE ADENYLATE CYCLASE ACTIVITY IN FRTL-5 CELLS

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INTRODUCTION

Thyroid-stimulating hormone (TSH) is known to stimulate the thyroid adenylate cyclase-cAMP system (1). Hormonal stimulation of adenylate cyclase produces an immediate response followed by a reduced responsiveness to a subsequent challenge with the hormone itself. This phenomenon is referred to as desensitization of refractoriness and is believed to be dependent on new protein synthesis (2,3). Furthermore, iodide in the thyroid gland, independently from TSH, can modulate thyroid function by inhibiting several metabolic steps (4). Thus, thyroid function is regulated in a complex manner, where pituitary and autoregulatory mechanisms can be distinguished.

Previous studies show that a continuous line of rat thyroid cells (FRTL-5) cultured in the presence of TSH are desensitized to the acute stimulation by the hormone, and that this refractoriness can be reversed by the removal of TSH from the culture medium (5). FRTL-5 cells are also responsive to thyroid-stimulating antibody (TSAb) present in sera of patients with Graves' disease (6).

In the present study, we investigated the mechanisms by which TSH induces refractoriness in FRTL-5 cells, and questioned whether TSAb could mimic TSH in the induction of refractoriness.

MATERIALS AND METHODS

The FRTL-5 cell line used in this study is a cloned normal rat thyroid cell line previously shown to maintain the functional characteristics of iodide uptake and thyroglobulin synthesis over prolonged periods of culture (7). Cells are grown in medium as previously described (7) in the presence of 0.3 mU/ml of TSH.

The TSAb IgG preparation was prepared from a serum of a patient with hyperthyroid Graves' disease by DEAE Sephadex separation. It was microsomal antibody negative and strongly positive for adenylate cyclase-stimulating activity in both human thyroid plasma membrane and FRTL-5 cells.

After splitting as previously described (7), FRTL-5 cells were seeded in 24 well Costar plates (Costar, Cambridge, MA), cultured in the presence of TSH for 2-3 days and, after washing, fed with the same medium deprived of TSH every two days. After 4-5 days of culture in the absence of TSH, cells were washed twice with Hank's Balanced Salt Solution (HBSS), and a 24 hr preincubation was performed with medium alone, or medium supplemented with 100 pM TSH or 1 mg/ml TSAb.

At the end of the preincubation period, cells were washed twice with HBSS and an acute adenylate cyclase stimulation assay was performed as previously described (6), measuring intracellular or total cAMP produced after 1 hr incubation in the presence of 0.5 mM isobutyl-methyl-xanthine. cAMP was measured using a RIA and DNA content was measured in the pellet. Results were usually expressed as picomoles/dish, since, under the conditions described, the DNA content varied less than 5% from well to well. Experiments were performed in duplicate, as well as cAMP measurements in each well. The results were the average of these and the standard deviation was less than 5% in all the experiments.

RESULTS

As previously reported, TSH withdrawal from culture medium restores the responsiveness of FRTL-5 cells to an acute TSH stimulation (5). On the basis of this observation, the ability of TSH in desensitizing cells maintained for 4-5 days in medium deprived of TSH was evaluated. A 24 hr preincubation with TSH clearly reduces the ability of FRTL-5 cells to increase intracellular cAMP production following a new acute challenge with TSH. This indicates that TSH induces refractoriness in FRTL-5 cells within 24 hours.

A 24 hr preincubation with medium containing TSH and supplemented with 10 μM cycloheximide showed that this inhibitor of protein synthesis partially prevented the desensitization induced by TSH preincubation.

Indeed, at the highest dose employed in the acute stimulation assay, i.e., 1 nM, cells pretreated with TSH had a 46% reduction of cAMP production with respect to unpretreated cells, while in the presence of cycloheximide, the responsiveness of TSH-pretreated cells decreased by only 14%.

Table 1. Adenylate Cyclase Assay Performed on Cells Preincubated with 100 μM KI or 100 μM Methimazole Together with TSH for 24 hr

24 hr preincubation	None	TSH	TSH + KI	TSH + MMI
Basal	1.2	3.0	2.3	2.9
TSH 50 pM	2.1	1.8	2.0	2.5
200 pM	3.4	3.4	2.8	3.6
800 pM	7.6	3.3	3.4	2.4
3 nM	16.8	12.1	12.5	10.6

Numbers represent pmoles of cAMP/50,000 cells.

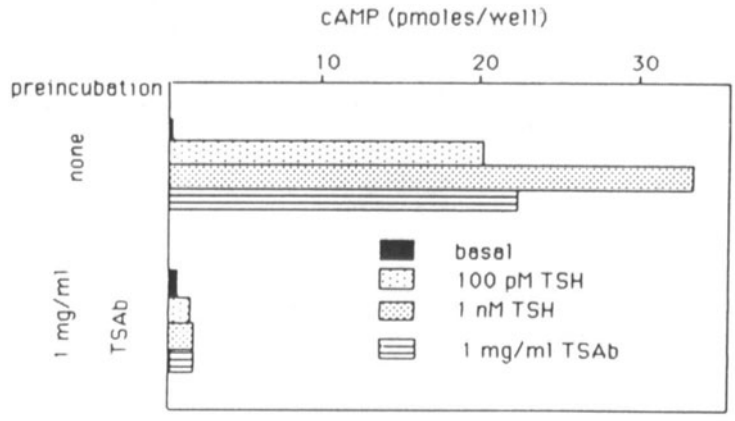

Fig. 1. Adenylate cyclase stimulation assay performed in FRTL-5 cells preincubated for 24 hr with 1 mg/ml TSAb, or medium alone, as control. Results are expressed as picomoles of cAMP/ well.

Preincubation for 24 hours with 100 µM KI or 100 µM methimazole did not alter the cAMP response to TSH (Table 1).

It has been previously reported that FRTL-5 cells are responsive to TSAb in terms of adenylate cyclase activity (6). We, therefore, questioned whether TSAb could mimic TSH in the induction of refractoriness. To this purpose, cells were preincubated with 1 mg/ml of a TSAb preparation. Fig. 1 shows that cells pretreated with TSAb have a reduced ability to respond to a subsequent acute stimulation by TSH.

DISCUSSION

This study deals with mechanisms by which TSH can induce desensitization in FRTL-5 thyroid cell line that, with respect to thyroid cell primary cultures or slices, have the advantage of absence of contamination with nonthyroid cells. Refractoriness is elicited by an exposure to the hormone for a period of at least 24 hours. This lag period suggests that single receptor occupancy or down-regulation are not major mechanisms for loss of responsiveness. Furthermore, an inhibitor of protein synthesis, cycloheximide, could partially prevent the induction of refractoriness by TSH, thus suggesting that cyclase is inhibited by a protein, the synthesis of which is under the regulation of the hormone.

In addition to TSH, iodine can modulate the thyroid cell response to the hormone (4). In dog thyroid cells in primary culture, methimazole or propylthiouracil, which block iodine organification, prevent the inhibitory action of iodine, indicating that iodine must be in an organic form to exert its inhibitory effect (7). In FRTL-5 cells, a 24 hr preincubation with KI did not increase the desensitization elicited by TSH and, accordingly, methimazole was also ineffective. These results may be explained by the observation that FRTL-5 cells, in the presence of TSH, are able to actively concentrate iodide from the culture medium, but are unable to incorporate it into proteins (8).

Immunoglobulins present in sera of patients of Graves' disease have been shown to influence many aspects of thyroid cell metabolism in a manner

similar to TSH (9). FRTL-5 cells are responsive to TSAb in terms of adenylate cyclase and growth stimulation (7,10). Thus, we investigated whether TSAb could mimic TSH in its ability to produce cyclase desensitization in FRTL-5 cells. This TSAb preparation, after a preincubation period similar to that described above for TSH, was able to mimic TSH in the induction of refractoriness to the acute stimulation by the hormone itself. Noteworthy is that refractoriness induced by TSAb or TSH was not specific, but once desensitized, cells showed reduced adenylate cyclase responsiveness to both stimulators.

In this paper we described that TSAb directed to TSH receptor (9) is able to desensitize cells after a 24 hr preincubation period. The mechanisms involved in this process are still unknown, although, as discussed above for TSH-induced desensitization, the lag period necessary to observe refractoriness suggests that TSH regulator occupancy or down-regulation are not major mechanisms of this action of TSAb.

Further studies using either TSAb or other antibodies to TSH receptor with different spectra of activities are necessary to clarify this problem.

REFERENCES

1. Pastan J and Katzen R. Biochem Biophys Res Commun 29: 792, 1967.
2. Lefkowitz RJ, Wessels MR, and Stadel JM. Curr Top Cell Reg 17: 205, 1980.
3. Rapoport B and Adams RJ. J Biol Chem 251: 6653, 1976.
4. Ingbar SH. Mayo Clin Proc 47: 814, 1972.
5. Vitti P, Valente WA, Ambesi-Impiombato FS, et al. J Endocrinol Invest 5: 179, 1982.
6. Vitti P, Rotella CM, Valente WA, et al. J Clin Endocrinol Metab 57: 782, 1983.
7. Ambesi-Impiombato FS, Parks LAM, and Coon HG. Proc Natl Acad Sci USA 77: 3455, 1980.
8. Weiss SJ, Philp NJ, and Grollman EF. Endocrinology 114: 1099, 1984.
9. Fenzi GF, Mariotti S, Bartalena L, et al. Special Topics in Endocrinology and Metabolism 2: 43, 1981.
10. Valente WA, Vitti P, Rotella CM, et al. N Engl J Med 309: 1028, 1983.

REGULATION OF HLA-DR GENE EXPRESSION IN CULTURED HUMAN THYROID CELLS: A ROLE FOR LECTIN, GAMMA INTERFERON, AND CYCLOSPORINE*

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INTRODUCTION

HLA Class II antigens (DR, DQ, and DP) are expressed primarily on the surface of the lymphocytes (B and activated T cells) and cells of the monocyte-macrophage lineage, but may also be expressed in a variety of tissues, in particular those which contain immune infiltration with local release of lymphokines (i.e., gamma interferon), which are potent inducers of Class II antigen expression. Immunofluorescence studies have demonstrated that although normal human thyroid tissue is negative for DR antigen, HLA-DR antigen is present in human thyroid cells in cases of autoimmune thyroid disease such as Graves' hyperthyroidism (1,2). Furthermore, Class II HLA-DR antigen in normal thyroid cells is detected after exposure in vitro to lectin or gamma-interferon (1,3). Since Class II antigens are important in T cell amplification, antigen presentation and self-recognition (4), such quantitative differences in DR antigen expression in the thyroid may be involved in the control of thyroid autoimmunity. In order to further elucidate the role of Class II antigens in thyroid immunoregulation, we have studied HLA-DR antigen gene expression in a variety of cultured human thyroid cells.

METHODOLOGY

Tissue and Cell Culture

Postoperative human tissue was quick-frozen and stored at -70°C prior to RNA preparation. Thyroid cell cultures were prepared by collagenase digestion (1.25 mg/ml) of thyroid tissue followed by culture for five days ± lectin (leucoagglutinin, 10 µg/ml), human gamma-interferon (E. coli-derived, Genetech, 100 units/ml), and/or Cyclosporine A (Sandoz, 5 µg/ml).

Detection of DR Antigen

Thyroid cell cultures were harvested using trypsin-EDTA and stained for DR antigen by indirect immunofluorescence using anti-DR monoclonal antibody L-243 (Becton Dickinson, CA).

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Total Cellular RNA Analysis

Total cellular RNA was prepared from tissue and cells according to quanidinium isothiocyanate extraction and cesium chloride centrifugation (5). RNA preparations were transferred to Gene Screen (NEN) membrane filters for Northern blot analysis or dot blot analysis, both according to the method of Thomas (6).

Cytoplasmic RNA Analysis

Cytoplasmic extracts were prepared from cells according to the method of White and Bancroft (7) before application to Gene Screen using a Minifold dot or slot blot apparatus.

Northern, Dot, and Slot Blot Hybridization

Blots were baked, prehybridized under standard conditions, and hybridized to an HLA-DR alpha chain-specific cDNA probe (DR alpha-10) cloned

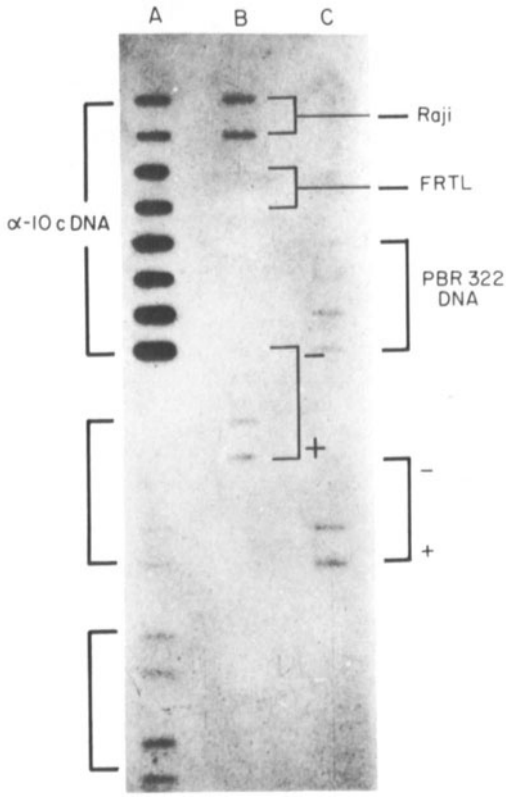

Fig. 1. Cytoplasmic slot blot analysis of HLA-DR alpha chain transcripts in human thyroid cells. Normal human thyrocyte cultures were established from thyroid tissue obtained at surgery, as described previously (8) and grown with (+) or without (-) lectin (leucoagglutinin, 10 µg/ml) for 5 days. Cytoplasmic extracts to be used in DR alpha chain transcript analysis were prepared according to the method of White and Bancroft (7). Briefly, cells were lysed in the presence of 1% Nonidet P 40 and nuclei were removed by centrifugation (13,000 x g, 2.5 min) in a Fisher Model 235B centrifuge. Cytoplasmic RNA in the supernatants was denatured at 60°C for 15 min in 7% formaldehyde and applied to Gene Screen filters (New England Nuclear) using a Minifold II Slot Blot apparatus (Schleicher and Schuell). Prehybridization, hybridization, and autoradiography were performed as described elsewhere (8). Cytoplasmic extracts or known amounts of DNA standards were applied in duplicate slots as follows: Lane A: DR alpha cDNA-Rows 1-4, 10, 25, 50, 100 pg, respectively; normal thyroid cell: Patient D (1×10^5 cells per slot)-Row 5, control, Row 6, +lectin; Patient E (2×10^5 cells per slot)-Row 7, control, Row 8, +lectin. Lane B: (3.5×10^5 cells per slot) Raji cell line-Row 1; Fisher rat thyroid cells line-Row 2; normal thyroid cell: Patient F-Row 4, control, Row 5, +lectin. Lane C: plasmid pBR322 DNA-Rows 1-4, 10, 25, 50, 100 pg, respectively; normal thyroid cell: Patient G (7×10^4 cells per slot)-Row 5, control, Row 6, +lectin.

into the Pst 1 site of pBR322 by GC tailing (from E. Long, NIAID). The cDNA probe was ^{32}P-labeled by nick-translation to a specific activity of 5-8 x 10^8 cpm per µg of DNA.

RESULTS

Northern blot analysis of total cellular RNA revealed a single DR alpha chain transcript less than 2 kb in size in all human thyroid tissues examined, including thyroid adenomata, Graves', and normal thyroid tissue (8). The thyroid DR alpha chain transcript was indistinguishable in size from the DR alpha chain mRNA species present in the Raji human B lymphoblastoid cell line, which constitutively expresses HLA-DR antigen. Dot blot analysis indicated that basal mRNA-DR alpha levels were variable among normal and abnormal thyroid tissues, as well as thyroid cells in culture, with the highest levels of DR alpha chain mRNA found in cases of Graves' hyperthyroidism (8).

Cytoplasmic blot analysis of cell extracts prepared from 5-day thyroid cell cultures indicated that normal thyroid cells contain detectable amounts of DR alpha chain-specific mRNA (Fig. 1). Lectin treatment resulted in a mean 5-fold increase in DR alpha chain transcript levels in normal thyroid cells (8). Both lectin and gamma-interferon treatment of normal, as well as Graves' thyroid cells in culture, resulted in enhanced levels of HLA-DR alpha chain transcripts (Fig. 2A). Such increases at the level of DR alpha chain gene transcription correlated with increased levels of DR antigen demonstrated in thyrocyte cultures by indirect immunofluorescence (Fig. 2B).

Cyclosporine A, which is known to suppress lymphokine production by T cells (9), was used to treat Graves' thyroid cell cultures in order to eliminate the possible contribution of T cell-derived gamma interferon to thyroid DR antigen levels. Cyclosporine treatment resulted in a 40-60% reduction in DR alpha chain transcript levels in untreated, as well as lectin-treated Graves' thyroid cells (data not illustrated). Cyclosporine A treatment of Graves' thyroid cell cultures grown in the presence of gamma

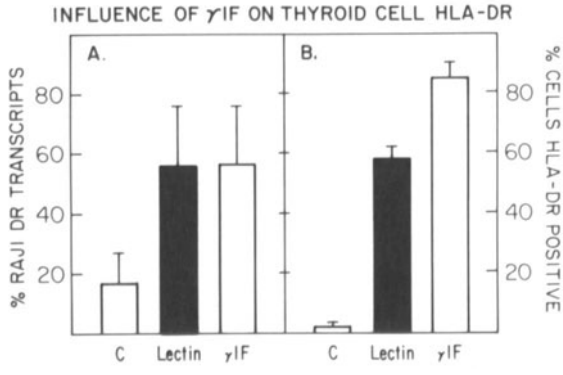

Fig. 2. Comparison of the influence of gamma-interferon on DR alpha chain transcript levels in normal human thyrocytes with numbers of DR-positive cells. 2a: Mean levels of DR alpha chain transcripts in cultured normal or Graves' human thyrocytes. Densitometric scans of slot blots of normal human thyrocytes cultured for 5 days with or without lectin (10 µg/ml) or gamma- interferon (100 units/ ml) were performed after hybridization to the DR alpha-10 probe as described in Fig. 1. Values were reported as % of DR-positive Raji cell line and normalized for cell number. 2b: Quantitation of DR antigen-positive cells in normal human thyrocyte cultures. DR antigen-positive cells were enumerated using indirect immunofluorescence. Thyroid cell cultures were harvested using trypsin-EDTA and reacted with anti-DR monoclonal antibody L-243 (Becton Dickinson), followed by fluorescein-conjugated F(ab')2 fragment of goat anti-mouse IgG (Cappel).

interferon did not result in decreased levels of DR alpha chain transcripts. Similar results were observed using indirect immunofluorescence to detect DR antigen in Graves' thyroid cell cultures (Fig. 2B).

DISCUSSION

Normal human thyroid tissue, as well as thyroid adenomata, express low levels of DR alpha chain transcripts in contrast to the high levels detected in Graves' thyroid tissue. Such variation in DR alpha chain gene expression is also detected in cultured human thyrocytes. Detectable levels of DR antigen gene expression in all human thyroid cultures examined to date in our laboratory suggest the capacity of thyroid cells to act directly in antigen presentation.

Normal and Graves' thyroid cell DR alpha chain transcript levels are regulated in vitro by lectin. The mechanism of action of lectin on DR antigen gene expression in the thyroid cell has been explored in studies using Cyclosporine A, which does not directly inhibit the action of gamma interferon enhancement of DR alpha chain gene expression in the thyrocyte. Cyclosporine A significantly reduces lectin stimulation of DR gene expression, suggesting that lectin may regulate DR antigen gene activity indirectly by enhancing gamma interferon synthesis by "passenger" lymphocytes present in thyrocyte cultures. Cyclosporine A may also inhibit lectin action directly at the level of the thyrocyte.

The role of gamma interferon in the expression of HLA-DR antigen in the human thyroid may prove to be an important mechanism in the etiology and amplification of autoimmune thyroid disease.

REFERENCES

1. Davies TF. J Clin Endocrinol Metab (in press), 1985.
2. Hanafusa T, Chiovato L, Doniach D, et al. Lancet II: 1111, 1983.
3. Pujol-Borrell R, Hanafusa T, Chiovato L, et al. Nature 304: 71, 1983.
4. Benacerraf B. Science 212: 1229, 1981.
5. Chirgwin JM, Przybyla AE, Macdonald RJ, et al. Biochemistry 18: 5294, 1979.
6. Thomas PS. Proc Natl Acad Sci 77: 5201, 1980.
7. White BA and Bancroft FC. J Biol Chem 257: 8569, 1982.
8. Piccinini LA, Schachter BS, and Davies TF. New York Academy of Sciences: Conference on Autoimmunity, June 17-19, 1985 (in press).
9. Borel JF, et al. Immunology 32: 1017, 1977.

THYROID HORMONE STRUCTURE-ACTIVITY RELATIONSHIPS: AN UNUSUAL CONFORMATION FOR 3,5,3'-TRIIODOTHYROPROPIONIC ACID*

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Although the major metabolic reactions affecting the thyroid hormone alanine side chain are deamination and decarboxylation, there is a paucity of data concerning the physiological function of these reaction products, in particular the thyropropionic acid metabolites. In man it has been shown that 44% of labeled thyroxine is excreted as thyroacetic acids whose plasma half-times are considerably shorter than those of the parent compounds (1). This is in contrast to the observation that, despite their low hormonal potencies, the acetic acid metabolites are bound at least as strongly to rat liver nuclei and more so to the plasma proteins (2,3). However, at present there is no clear evidence for the formation of thyropropionic acids in human or animal tissues (4). The acid metabolites (formic, acetic, propionic) have been shown to bind more tightly to thyroxine-binding prealbumin and the type I deiodinase enzyme, than does the thyroid hormone thyroxine (T_4) (5,6).

While the function of the thyropropionic acid metabolites in hormone action is not yet understood, various activities have been ascribed to them. For example, 3,5,3'-triiodothyropropionic acid (T_3P), while having weak calorigenic and goiter prevention activities (28% of T_4 activity) (7, 8), has an inhibitory effect on mammary growth comparable to T_4 or T_3 (9). Also, T_3P has been shown to inhibit mitochondrial glutamate dehydrogenase activity (10), as well as other cellular processes (11).

Since different aspects of the thyroid hormone structure are recognized by specific hormone-binding proteins, knowledge of hormone conformational flexibility is important. A conformational analysis of thyroid hormone structures in different environments can delineate characteristic patterns of molecular conformation and geometry. Therefore, the crystal structure of T_3P, crystallized as a (1:1) N-diethanolamine salt, was undertaken and the results are compared with those of the thyroid hormones and their acid metabolites.

As illustrated (Fig. 1), T_3P has an overall transoid conformation, (i.e., the side chain and the phenolic ring are oriented on the opposite sides of the tyrosyl ring plane) (12), the 3'-iodine atom is distal to the tyrosyl ring, and the propionic acid side chain is perpendicular to the tyrosyl ring (X^2, Table 1), features observed in many other thyroid hormone

*Supported by the Kroc Foundation and NIH AM15051.

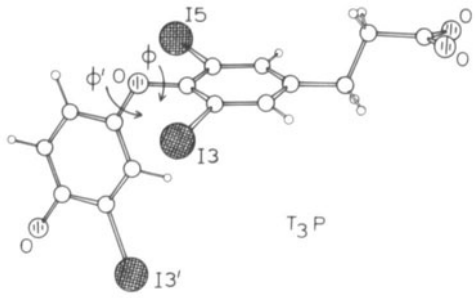

Fig. 1. Molecular conformation of 3,5,3'-triiodothyropropionic acid.

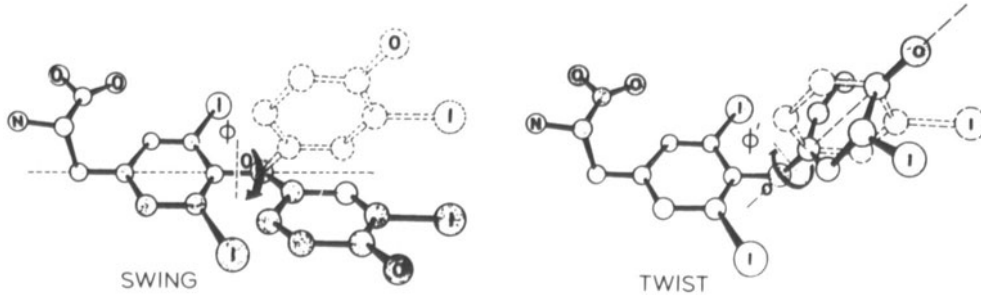

Fig. 2. Definition of the movements about the diphenyl ether bonds.

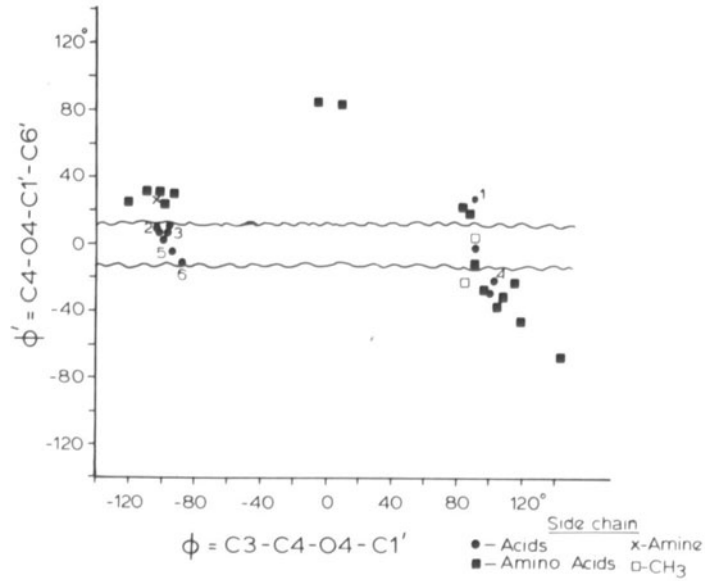

Fig. 3. Plot of diphenyl ether torsion angles for all thyroactive structures (13).

Table 1. Thyropropionic Acid Conformations

No.	Structure	ϕ	ϕ'	3'I	X^2	Conform	Ref
1	T_3P	90°	28°	Proximal	106°	transoid	this paper
2	T_3P2	-105	10	Distal	-16	cisoid	13
3	T_4P	-98	8		100	cisoid	13
4	MeT_3Pme	103	-21	Proximal	99	transoid	15
5	T_2P	-100	4		101	cisoid	16
6	T_3Pee	-89	-10	Proximal	46	cisoid	17

structures (13), as well as in other thyropropionic acid structures. The diphenyl ether conformation, as described by the two torsion angles about the ether bridge (Fig. 1), is twist-skewed, an unusual conformation for acid metabolites (13,14).

Analysis of the diphenyl ether torsional parameters shows that the two motions about the phenyl ether bonds are correlated and take place in a concerted manner, i.e., as the phenolic ring swings about its axis past the ideal skewed position, there is a concurrent twist of the phenolic ring about its axis in such a way that the steric repulsion between the tyrosyl ring iodine atoms and the phenolic ring hydrogen atoms is minimized (Fig. 2) (14).

A comparison of the crystal structure of the thyroid hormones with their acid metabolites indicates a specific influence of the side chain composition, i.e., the presence or absence of the amine function rather than side chain length, upon diphenyl ether conformation (13). Further analysis of these data (Fig. 3) shows that other, more subtle, correlations exist. Within the transoid and cisoid classifications, there is a separation into subsets described by the deviation of the torsion angles from the ideal skewed values of $\phi/\phi' = 90°/0°$. As seen (Table 2), the acid metabolites (formic, acetic, propionic) fall within a 10° range of the skewed conformation, whereas the full amino acid structures cluster on either side of these values and adopt a twist-skewed conformation.

Table 2. Average Diphenyl Ether Conformational Parameters

Conformation	ϕ	ϕ'	Conformation	ϕ	ϕ'
Skewed -transoid	91°	-2°	Acids	94°	1°
Twist-skewed	104	-18	Amino acids	108	-27
Skewed -cisoid	-98	4	Acids	-98	4
Twist-skewed	-107	29	Amino acids	-107	30

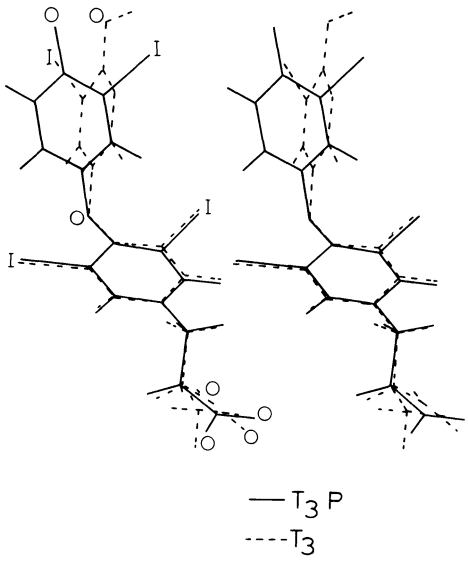

Fig. 4. Superposition of T_3P (——) on T_3 (---).

Comparison of the diphenyl ether conformations of these thyropropionic acid structures with those of the active hormones (Fig. 4) shows that the phenolic ring 6'-position is relatively constant for divergent conformations. These data show also that the phenolic ring functional groups occupy different regions of conformational space as a result of a skewed or twist-skewed conformational preference, and that this may play an important role in determining the selectivity, and therefore the subsequent activity of these analogues.

While these data show that the conformation of T_3P is more flexible than previously observed, further study is required to understand the relevance of the unusual conformation features to its observed inhibitory properties.

REFERENCES

1. Gershengorn MC, Glinoer D, and Robbins J. In M DeVisscher (ed), The Thyroid Gland, Raven Press, New York, 1980, p 81.
2. Goslings B, Schwartz HL, Dillman W, et al. Endocrinology 98: 666, 1976.
3. Snyder SM, Cavalieri RR, Goldfine ID, et al. J Biol Chem 251: 6489, 1976.
4. Nakamura N, Choppra IJ, and Solomon DH. J Clin Endocrinol Metab 46: 91, 1978.
5. Andrea TA, Cavalieri RR, Goldfine ID, et al. Biochem 19: 55, 1980.
6. Kohrle J, Auf'mkolk M, and Hesch RD. 7th Internat Endocrine Cong, Quebec City, July 1984.
7. Mittra I. Experientia 31: 1218, 1975.
8. Money WL, Meltzer RI, Feldman D, et al. Endocrinology 64: 123, 1959.
9. Stasilli NR, Kroc RL, and Meltzer RI. Endocrinology 64: 62, 1959.
10. Goudonnet H, Truehot R, and Michel R. Biochem Pharmacol 24: 1679, 1975.
11. Pittman CS and Barker SB. Amer J Physiol 197: 1271, 1959.
12. Cody V, Hazel J, Langs DA, et al. J Med Chem 20: 1628, 1977.
13. Cody V. Endocrine Rev 1: 140, 1980.
14. Cody V. In IJ Chopra (ed), Triiodothyronines in Health and Disease, Springer-Verlag, New York, 1981, p 15.

15. Cody V, Hazel J, and Osawa Y. Acta Crystallog B34: 3407, 1978.
16. Cody V, Erman M, and DeJarnette E. J Chem Res 5: 126, 1977.
17. Camerman N and Camerman A. Can J Chem 52: 3048, 1974.

CHROMATOGRAPHIC PROPERTIES AND PROBABLE STRUCTURE OF THE URINARY CONJUGATE OF AN ETHANE-1,2-DIOL FORMED IN VIVO FROM 3,5,3'-TRIIODOTHYRONINE (T_3)

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INTRODUCTION

In earlier communications we have suggested that the diverse actions of thyroid hormones could be explained in one coherent framework if thyroxine were viewed as an amino acid (1,2). Both thyroxine (T_4) and its monodeiodination product, 3,5,3'-triiodothyronine (T_3), are aromatic amino acids not only derived from, but similar in structure to tyrosine. In view of the adrenergic effects of thyroid hormones, it seemed reasonable to propose that T_4, like tyrosine, would be taken into the nervous system where it and its metabolites could become substrates for the same enzymes which take part in the transformation of tyrosine to the adrenergic neurotransmitters, as well as to their major catabolic products.

In the course of studying this hypothesis, we have shown that both T_4 and T_3 are concentrated in neurons of specific brain regions (3) and, more recently, have demonstrated that the hormone, as T_3, is axonally transported in the fiber tracts of the upper motor neuron and in other selected neural pathways (4). With such strong evidence for the functioning of thyroid hormones as precursors of neurotransmitters or neuromodulators in the central nervous system, we asked ourselves where to look for neurally relevant iodothyronine metabolites and what properties such compounds would have which would aid in their detection and isolation. Knowing that the adrenergic neurotransmitters are recovered from urine in the form of conjugates, as well as in the form of conjugates of their stable side chain oxidation products, we began to collect and analyze urine from ^{125}I-T_3-treated rats. If thyroid hormones were to be metabolized along adrenergic neurotransmitter pathways, the products, owing to ring and/or side chain hydroxylation, as well as probable sulphate conjugation, should be more polar and less lypophilic than T_4 and T_3 or their phenolic sulphates. Since the mechanism of reversed phase HPLC is essentially a matter of retention times paralleling lypophilicity, we anticipated that HPLC would lend itself well to the search for such metabolites.

When the first organic extract of rat urine, obtained three hours after administration of ^{125}I-T_3 to a hypothyroid thyroidectomized (T_x) rat, was subjected to gradient analysis by HPLC, we observed a prominent labeled peak which eluted at only 55% of the retention time of T_3 itself and well before other known iodothyronines and their phenolic conjugates. The unknown accounted for 16% of all organically-bound ^{125}I in the urine sample, while

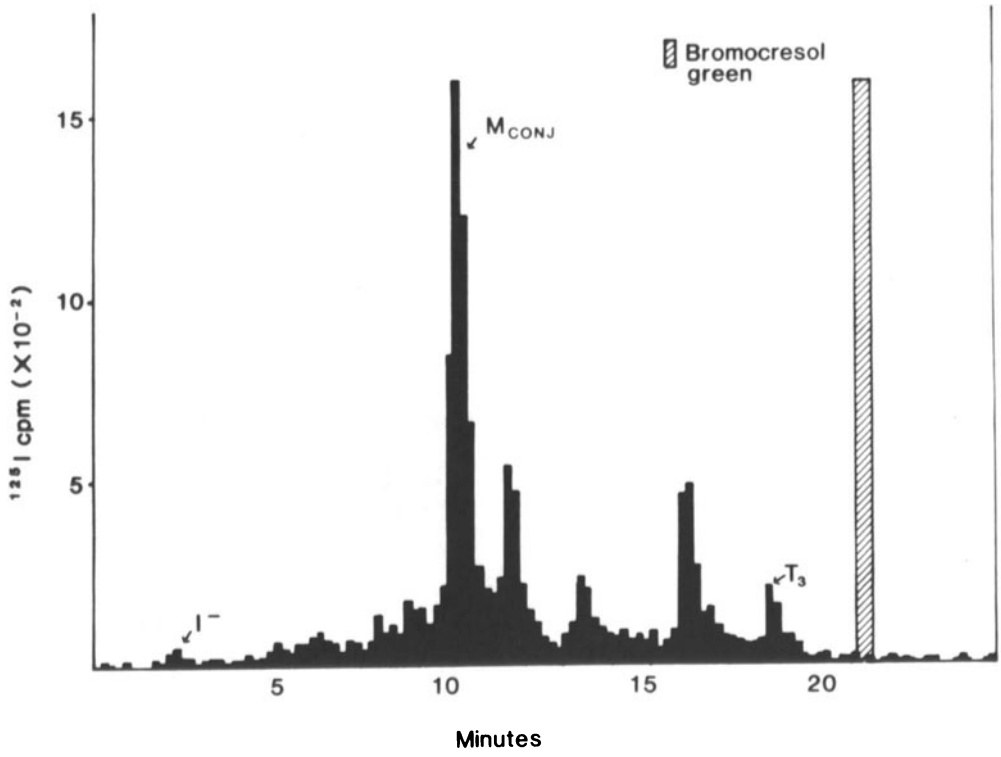


Fig. 1. Result of HPLC analysis of an organic extract of urine collected from 2 T_x rats for 5 hours following i.v. ^{125}I-T_3.

in other experiments it has represented as much as 50% of organically-bound urinary radioiodine (Fig. 1). Hydrolysis of the unknown produced another new compound with a longer HPLC retention time, though still only 80% of that of T_3. Further experiments showed that the product, M, has neither amine nor carboxylic acid functions.

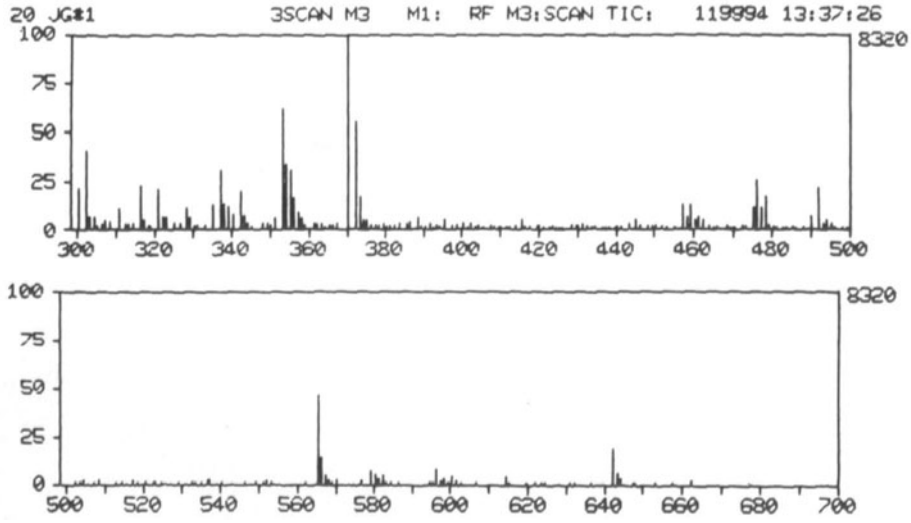
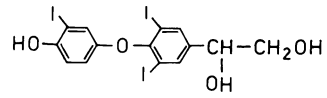


Fig. 2. Desorption-chemical ionization mass spectrum of 3,5,3'-triiodothyroethylene glycol, recorded by Noel Whittaker, NIH, Bethesda, MD.

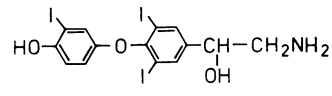
STRUCTURE OF NEW T_3 METABOLITE:



4-(4-Hydroxy-3-iodophenoxy)-3,5-diiodophenylethylene glycol

or 3,5,3'-Triiodothyroethylene glycol

ITS IMPLIED NEUROACTIVE PRECURSOR:



2-Hydroxy-2-[4-(4-hydroxy-3-iodo)phenoxy-3,5-diiodophenyl]ethyl amine

or β-Hydroxy-T_3-amine

Fig. 3. Structure of new T_3 metabolite and its implied neuroactive precursor.

To obtain sufficient mass of M for structure determination by mass spectrometry (MS), labeled urine was added to urine collected from intact rats. Organic compounds were concentrated, hydrolyzed by 4 M HCl, and separated by HPLC. ^{125}I-labeled M, recovered after further chromatographic purification, was subjected to desorption - chemical ionization MS (Fig. 2). Formation of the molecular ion - NH_3 adduct indicated M to be a compound of 624 molecular weight. Analysis of the fragment ions confirmed this assignment, showing M to be 4-(4-hydroxy-3-iodophenoxy)-3,5-diiodophenylethylene glycol (5). To emphasize its in vivo production from T_3, we have also called the new compound 3,5,3'-triiodothyroethylene glycol. Its molecular structure and that of its presumed neuroactive precursor, the T_3 analogue of octopamine, are shown together in Fig. 3. Fig. 4 outlines the proposed role of adrenergic enzymes in their biosynthesis.

In this communication, we describe methods used in isolating, characterizing, and identifying the urinary metabolite, Mconj, from which M is obtained by hydrolysis.

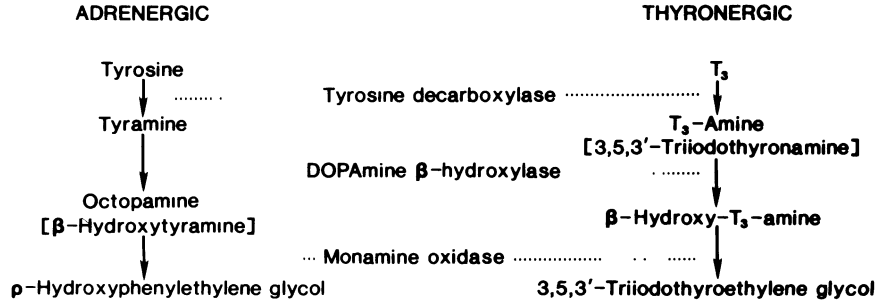


Fig. 4. Parallel biosynthetic pathways suggested by the authors and supported by the elucidation of structure of 3,5,3'-triiodothyroethylene glycol.

EXPERIMENTAL

Urine was removed from the bladders of hypothyroid T_x rats (Zivic Miller ∿200 g) at time of decapitation 3, 48, and 72 hours after intravenous administration of high specific activity ^{125}I-T_3 (Abbott Diagnostic Laboratories). Samples were acidified to pH 3.5 with 6 M HCl and processed on C_{18} sample preparation columns (J. T. Baker Co.). Radioactive I^- passed straight though and was discarded. Retained organic compounds, accounting for less than 5% of urine radioactivity, were eluted with EtOH containing 0.1% 1 M HCl, concentrated by evaporation under N_2 and analyzed by HPLC on μ-Bondapak C_{18}. A 4% per min solvent gradient from 0.1% H_3PO_4 to 0.1% H_3PO_4 in 80:20::MeOH:H_2O and a flow rate of 2 ml/min were used. Mconj, eluting at 11.2 min compared to T_3 at 20 min, represented from 10-20% of organically-bound ^{125}I in each case. On a new C_{18} column, Mconj elutes at 16.0 min and T_3 at 22.1 min. The disproportionate decrease in Mconj R_t with column use is a clue to its highly polar character.

In subsequent experiments, T_x or intact rats, acclimated to and housed in pairs in metabolism cages, were injected with ^{125}I-T_3; urine was collected at 5, 24, and 48 hours and processed as above. In 5 and 24 hour urines from young rats (150-200 g), the Mconj peak often represented from 35-50% of organically bound urinary radioiodine.

For identification of Mconj, HPLC eluates from the appropriate regions were neutralized with 0.1% conc. NH_4OH in MeOH and reduced in volume for paper chromatography. Descending in the upper phase of t-AmOH:hexane:2 M NH_4OH::10:1:12 (AHA), the Mconj:T_3 R_f ratio is 1.5. T_3 and Mconj R_fs are similar in n-BuOH:EtOH:0.5 M NH_4OH::5:1:6 (BEA), and n-BuOH:gl HOAc:H_2O:: 4:1:1 (BAC). Typical migration distances are 28-33 cm after 17 1/2 hours in AHA, 24 cm after 18 hours in BEA, and 19 cm after 18 hours in BAC. Following partial evaporation to reduce MeOH content, Mconj eluates from HPLC were also recovered on C_{18} separation columns, and after evaporation of the solvent, redissolved for further characterization. Dissolved in 10^{-3} M HCl, Mconj was not retained by an aryl sulfonate separation column, but dissolved in 0.1 M NaOAc it was retained by a quaternary amine column from which it was quantitatively eluted by 0.2 M Na_2CO_3, disclosing its acidic character. For hydrolysis, Mconj was dissolved in 4 M HCl and digested at 37°. Hydrolysis to M was usually complete in three hours but can be carried out overnight with little apparent loss of product. Mconj was also hydrolyzed by aryl sulfatase from Aerobacter aerogenes but was impervious to the sulfatase and glucuronidase action of active preparations of crude glucuronidase from Patella vulgata.

Urine collected for 24 hours after i.v. ^{125}I-T_3 given to an intact rat was processed to obtain endogenous Mconj for structure determination. Mconj was separated by HPLC and purified by paper chromatography (BEA and BAC). After a final analysis by HPLC, Mconj was recovered on C_{18} and eluted with MeOH; analysis by ^{252}Cf-plasma desorption MS gave negative ions of masses 96 and 97 ($SO_4^=$, HSO_4^-). Another sample was subjected to thermospray MS. Detection of a positive ion of mass 802 (784 + NH_4^+) suggests that Mconj is a disulphate of M.

CONCLUSION

We have described the isolation and characterization of sulfate conjugate of 3,5,3'-triiodothyroethylene glycol, a newly isolated thyroid hormone metabolite. Because an enzyme preparation which is active in hydrolyzing phenolic conjugates of iodothyronines is ineffective with Mconj, we propose that the side chain hydroxyl groups are the ones conjugated. The dissimilarity of Mconj R_f values and those of phenolic conjugates of iodothyronines in paper chromatographic systems (AHA and BEA) also supports this

view. The discovery of these new triiodothyronine metabolites introduces the probability that a significant amount of endogenous thyroid hormone is metabolized through heretofore undisclosed pathways. As in the case of tyrosine metabolism, processing of thyroid hormones in the nervous system may lead to a family of related metabolites of which this thyroethane-1,2-diol and its disulphate may only be examples.

REFERENCES

1. Dratman MB. J Theoret Biol 46: 255, 1974.
2. Dratman MB. In CH Li (ed), Hormonal Proteins and Peptides, Academic Press, New York 1978, p 205.
3. Dratman MB, Futaesaku Y, Crutchfield FL, et al. Science 215: 309, 1982.
4. Dratman MB, Crutchfield FL, Futaesaku Y, et al. Submitted to Neuroscience.
5. Gordon JT, Dratman MB, and Wassel MS. Abstract #847, 67th Annual Meeting of the Endocrine Society, 1985.

EFFECTS OF d-PROPRANOLOL TREATMENT ON TRANSPORT OF THYROID HORMONES INTO TISSUE CELLS

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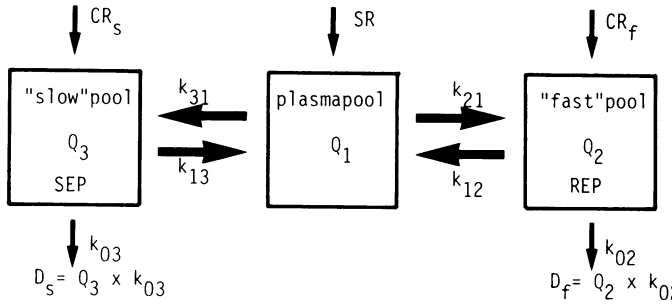
INTRODUCTION

It is well known that treatment with d,l-propranolol elicits a low T_3 syndrome in man (1,2). This has been explained by the assumption of an inhibition of 5'-deiodinase in the liver by d,l-propranolol, leading to a diminished production of T_3 from T_4 and a diminished breakdown of rT_3. However, the K_i of d,l-propranolol on this enzyme *in vitro* is about 0.8 mM (3), (3), while therapeutic levels of this compound are between 0.5 and 1 µM in serum (4), and, at least in the rat, the liver does not concentrate propranolol (5). Recently, it has been shown that a derivative, 4-OH-propranolol, markedly inhibits 5'-deiodinase activity *in vitro* at a concentration of about 50 µM (6), but the concentration in serum is below 50 nM (6). Inhibition of the enzyme *in vivo* seems, therefore, questionable. On the other hand, we have found that transport of iodothyronines into rat liver hepatocytes in primary culture is strongly inhibited by propranolol in a concentration of only 1 µM (7). Furthermore, we have shown that at least part of the low T_3 syndrome, elicited by caloric deprivation, can be attributed to inhibition of transport of T_4 into the liver (8). Therefore, we have performed tracer T_4, T_3, and rT_3 kinetic studies to investigate if the low T_3 syndrome elicited by propranolol is caused by transport inhibition of T_4 and rT_3 into the liver, or by inhibition of conversion of T_4 to T_3 and rT_3 to T_2.

SUBJECTS

Six normal, healthy male subjects, age 22-24 years, volunteered in this study. All had normal thyroid function as measured by serum T_4, T_3-resin uptake, and TSH responses to TRH. The study and protocols were approved by the Ethical Committee of the medical centre and informed consent was obtained from all participants. During the study, subjects were kept on a normal, weight-maintaining diet. L-thyroxine substitution (200 µg daily) was started five weeks before the first experiment and continued throughout the study. Two weeks after the start of the first (control) experiment, an oral d-propranolol (40 mg tablets of d-propranolol were a generous gift of ICI-Pharma, Holland B.V.) regimen of 80 mg three times daily was started. After 14 days the second experiment was performed.



Computable from blood-borne data

$-k_{11} = k_{21} + k_{31} > 0$ $\quad c_1 = k_{12} \times k_{21} > 0$

$-k_{22} = k_{12} + k_{02} > 0$ $\quad c_2 = k_{13} \times k_{31} > 0$

$-k_{33} = k_{13} + k_{03} > 0$

Fig. 1. Three pool model of iodothyronine distribution and metabolism. K_{ij} values ($i \neq j$; i,j= 1,2,3; all $K_{ij} > 0$) are fractional transport rates from pool j to pool i. K_{ii} (i= 1,2,3) are fractional turnover rates of pool Q_i. In the T_4-model only, secretion (SR) of T_4 into the plasma pool should be considered. In case of the other iodothyronines, also production by conversion both in the slow pool (CR_S) as in the fast pool (CR_F) must be taken into account. D_S = disposal in the slow pool; D_F = disposal in the fast pool.

METHODS

25 µCi ^{125}I-T_4 (Radiochemical Centre Amersham) and 30 µCi ^{131}I-rT_3 (prepared by iodination of 3,3'-T_2 with $^{131}I^-$ using chloramine-T) (9) were injected intravenously in 2 ml of 2% human serum albumin in phosphate buffered saline. Three days later, 30 µCi ^{131}I-T_3 (prepared by iodination of 3,5-T_2 with $^{131}I^-$ using chloramine-T) (9) was given in a similar way. Blood samples were collected at regular intervals during 24 hours for rT_3, 3 days for T_3, and 9 days for T_4. Tracer T_4 was isolated from serum by TCA-precipitation, tracer T_3 or rT_3 by adsorption of the iodothyronines to Sephadex G-25 at high pH, and subsequent elutions of T_3 or rT_3 with specific T_3 or rT_3-antibodies (10). During the kinetic studies, 10 drops of lugol's solution three times daily were used to prevent thyroidal utilization of radioactive iodide liberated during the study.

CALCULATIONS

Serum radioactivity of each iodothyronine was expressed as % dose per liter. For each iodothyronine, the change of the activity as % dose/l as a function of time could be described as the sum of three exponentials,

$$y(t) = \sum_{i=1}^{3} A_i e^{-\lambda_i t}$$

From the A_i and λ_i obtained, the parameters as depicted in Fig. 1 of a three pool model of iodothyronine distribution and metabolism (11,12) could

be calculated. Rearrangements of these terms gives us the following formula for K_{21},

$$-C_1/(K_{22} + K_{02}) = K_{21} = C_2/(K_{33} + K_{03}) - K_{11}$$

A minimal value of K_{21} can be found if $K_{02} = 0$, i.e., no disposal in the rapidly equilibrating (REP) pool. K_{21} is maximal if $K_{03} = 0$, i.e., no disposal in the slowly equilibrating pool (SEP). If K_{21} is known, all other parameters can be calculated.

RESULTS

In Table 1, the minimal and maximal values of K_{21} for T_4, T_3, and rT_3 are depicted together with the difference between the maximal and minimal values in percent of the mean value. For T_4 and T_3, the difference between the maximal and minimal value for K_{21} is only small, therefore, the mean value of K_{21} was used to calculate all other parameters. However, this assumption will divide the disposal of the hormone equally between the REP and the SEP, although we have no indication that this is actually the case *in vivo*. The difference between the upper and lower value of K_{21} for rT_3 is too large to use the means of these values as a reliable estimate of this parameter. Additional information is needed about the site of the rT_3 disposal to be able to calculate K_{21}. Recently, it has been found (13) by direct blood sampling in the hepatic vein and the hepatic artery in humans that the clearance of rT_3 by the liver is equal to the total body disposal of rT_3. Because the liver is part of the REP (11,12), we have assumed that the disposal of rT_3 is located in the REP, and, therefore, we have used the highest value for K_{21} to calculate all other parameters for rT_3. A summary of the results can be found in Table 2.

DISCUSSION

d-Propranolol induced a low T_3 syndrome in all subjects, as can be concluded from the significantly lower serum T_3 concentrations and elevated rT_3 concentrations (Table 2). We have chosen d-propranolol as the experimental drug because of its proven ability to induce a low T_3 syndrome without side effects due to β-blocking activity (14-16). Serum T_3, free T_3, plasma pool, mass transfer rates to the REP and SEP, and pool sizes were all decreased ($p<0.001$) to a similar extent as the production rate. It seems, therefore, that all changes can be explained by the diminished production rate, without any transport inhibition of T_3 into the REP or SEP. During propranolol treatment, serum T_4 and free T_4 were elevated, while the disposal rate and

Table 1. Upper and Lower Values for K_{21} (Fractional Transfer Rate from Plasma to the REP) h^{-1}

	T_4	T_3	rT_3
Lowest value	0.508 (0.060)	1.338 (0.169)	0.809 (0.029)
Highest value	0.521 (0.061)	1.528 (0.173)	2.591 (0.094)
Difference	2.8 % (0.3)	14.0 % (1.5)	104.6 % (3.3)

n=6; mean (SEM)

Table 2. T_4, T_3, and rT_3 Kinetics in T_4 Substituted Volunteers Before and During Propranolol Treatment

		Control			d-Propranolol		
		T_4	T_3	rT_3	T_4	T_3	rT_3
Production rate	nmol/d	149.5	66.8	61.3	126[c]	41.2[b]	69.4
MCR	l/d	1.53	37.2	186.2	1.13[a]	32.1[e]	84.0[a]
Plasma compartment							
Free hormone conc.	pmol/l	25.2	5.3	0.29	28.6[c]	3.9[a]	0.77[a]
Total hormone conc.	nmol/l	99.3	1.8	0.33	113[b]	1.28[a]	0.85[a]
Pool	nmol	463	14.7	1.45	510	10.2[b]	3.4[b]
REP							
Pool	nmol	661	8.3	4.9	814[d]	6.0[d]	12.2
Mass transfer rate from plasma	nmol/h	229	20.7	3.7	266	12.2[a]	8.4[e]
SEP							
Pool	nmol	341	76.7	32.5	302	53.7[b]	20.6[b]
Mass transfer rate from plasma	nmol/h	15.8	7.5	0.43	7.5	5.0[a]	1.28

[a]=$p<0.001$; [b]=$p<0.005$; [c]=$p<0.01$; [d]=$p<0.025$; [e]=$p<0.05$; (paired t test; n=6)

MCR were lower ($p<0.01$). Mass transfer to the REP was elevated leading to an increased pool (<0.01). Similarly, serum rT_3, free rT_3, and plasma pool were elevated ($p<0.001$), MCR was lower ($p<0.001$), while disposal rate did not change. Mass transfer rate to the REP was higher, leading to a greater pool ($p<0.001$). Thus, no transport inhibition of T_4 and rT_3 into the REP could be demonstrated. The elevated pools of T_4 and rT_3 in the REP (which contains liver and kidney (11,12)) indicate that limited substrate availability to 5'-deiodinase could not be the explanation for the diminished T_3 production and rT_3 MCR.

Therefore, two explanations remain, either a diminished concentration of enzyme during d-propranolol treatment, or an inhibition of the enzyme by an (as yet unknown) metabolite of propranolol, assuming that the mechanism of inhibition of the enzyme by propranolol in vivo is similar to that observed in vitro.

REFERENCES

1. Lumholtz IB, Siersbaek-Nielsen K, Faber J, et al. J Clin Endocrinol Metab 47: 587, 1978.
2. Verhoeven RP, Visser TJ, Docter R, et al. J Clin Endocrinol Metab 44: 1002, 1977.
3. Fekkes D, Hennemann G, and Visser TJ. Bioch Pharmacol 31: 1705, 1982.
4. Shand DG, Nuckolls EM, and Dates JA. Clin Pharmacol Therp 11: 112, 1970.
5. Bianchetti G, Elghozi JL, Gomeni R, et al. J Pharmacol Exp Therap 214: 682, 1980.
6. Jorgensen PH, Lumholtz IB, Faber J, et al. Acta Endocrinol (khhn) 105: 205, 1984.
7. Krenning EP, Docter R, Visser TJ, et al. J Endocrinol Invest 6: 59, 1983.
8. Docter R, Van der Heijden JTM, Krenning EP, et al. Annal d'Endocrinol 45: 76A, 1984.
9. Greenwood FC, Hunter WM, and Glover JS. Biochem J 89: 144, 1963.
10. Bianchi R, Zucchelli GC, Giannessik D, et al. J Clin Endocrinol Metab 46: 203, 1978.
11. DiStefano III JJ, Jang M, Malone TK, et al. Endocrinology 110: 198, 1982.
12. DiStefano III JJ, Malone TK, and Jang M. Endocrinology 111: 108, 1982.
13. Bauer ACG, Wilson JHP, Lamberts SWJ, et al. Submitted for publication, 1985.
14. Nies AS, Evans GH, and Shand DG. J Pharmacol Exp Therap 184: 716, 1973.
15. Nies AS, Evans GH, and Shand DG. Am Heart J 85: 97, 1973.
16. Shand DG. Postgrad Med J 52 (suppl. 4): 22, 1976.

IMPAIRED RELEASE OF HEPATIC TRIIODOTHYRONINE (T_3) IN THE DIABETIC LOW T_3 SYNDROME

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INTRODUCTION

Previous studies have shown that experimental diabetes causes a significant decrease in serum thyroxine (T_4) and 3,5,3'-triiodothyronine (T_3) concentrations and reduces hepatic generation of T_3 from T_4 (1-4). Since hypothyroidism per se induces a reduction in 5'-deiodinase activity (5,6), some studies have suggested that the abnormalities seen in diabetes could be a consequence of this low serum T_4 level (7,8). In fact, administration of T_4 prevents the decrease in serum T_4 concentration and corrects the abnormal T_3 production in diabetic animals, but fails to normalize the serum T_3 levels (9). Thus, the low T_3 syndrome in this situation cannot be fully explained by decreased peripheral conversion of T_4 to T_3 consequent to a reduction in 5'-deiodinase activity. The present study explores the hypothesis that an alteration of T_3 release from intracellular to extracellular space may be an additional factor contributing to the low T_3 syndrome observed in diabetes.

MATERIALS AND METHODS

Materials. T_4, T_3, and serum bovine albumin (BSA) were obtained from Sigma Chemical Co., St. Louis, MO. All other chemical products were provided by Merck S.A. Industrias Quimicas, Rio de Janeiro, Brazil. $[^{125}I]T_3$ and $[^{125}I]T_4$ were prepared by iodination of 3,5 T_2 and T_3, respectively, using previously described techniques (10). Streptozotocin (STZ) was purchased from Calbiochem Co., La Jolla, CA.

Animals. Male Wistar rats weighing 200-250 g were used in all experiments. The animals were maintained on an ad libitum intake of tap water and Purina rodent laboratory chow. Diabetes was induced by a single intravenous STZ injection of 40 mg/kg b.w. An intravenous injection of the vehicle (citrate buffer, pH 4.5) was given to the control animals. T_4 replacement was obtained by daily intraperitoneal injection of 1.5 µg/100 mg b.w., during six days. At the end of the experimental period all animals were submitted to liver perfusion.

Procedure of rat liver perfusion. The technique of rat liver perfusion used in this study was described by Borges et al. (11). After induction of

anesthesia with intraperitoneal sodium pentobarbital (40 mg/kg b.w.), an intratracheal tube was inserted and connected with a volume respirator. Then, the chest and abdomen were opened and ligatures placed around the inferior vena cava above the right renal vein, the splenic vein and the portal vein. Outflow cannulas were rapidly inserted into the portal vein and thorough the right atrium into the thoracic vena cava. Finally, the ligatures around all the veins were tied and the perfusion started. The perfusate leaving the liver flowed to a reservoir and was subsequently recirculated. The perfusion medium consisted of Krebs-Henseleit bicarbonate buffer containing BSA (3 g/dl), T_4 (10 µg/dl), and glucose (100 mg/dl). The medium was equilibrated in the apparatus with 95% O_2-5% CO_2, and maintained at constant temperature and pH (37°C and 7.4). The final volume in the system was 30 ml. All livers were perfused for one hour. At the end of the perfusion, the pump was stopped and the medium was pumped out of the apparatus and the volume determined. After centrifugation to separate the red blood cells, the perfusion medium was stored at -20°C for further determinations. At that time, a portion of the liver (approximately 1 g) was rapidly placed in a preweighed grinding vessel containing 1 ml of 0.01 M PO_4, 0.15 M NaCl, pH 7.4 at 4°C. The portion of the liver was then weighed and processed.

Processing liver samples. The liver sample processing was similar to that described by Jennings et al. (12) and consisted of a double alcoholic extraction with 100% ethanol. Using this method, $[^{125}I)T_3$ was extracted with an efficiency that was inversely proportional to liver weight according to the following equation: extraction efficiency (%) = -11.7 x liver weight (g) + 85.2 ($r = 0.870$, $p<0.01$). The efficiency of extraction of T_3 was unaffected by diabetes.

T_4 and T_3 determinations. All samples were assayed in triplicate using radioimmunoassay methods similar to those previously described (12, 13).

Calculations. The appearance of T_3 in the perfusion medium was calculated by subtracting the initial T_3 perfusate concentration from the final T_3 perfusate concentration and multiplying this difference by the perfusion volume. The appearance of T_3 in the liver was obtained by subtracting from the observed liver T_3 concentration, the mean liver T_3 concentration in a comparable group of rats following perfusion with T_4-free medium, which did not alter the endogenous T_3 liver concentration in either control or diabetic animals. Thus, total T_3 production was calculated as the sum of the perfusate and the liver T_3 productions. The T_3 production was expressed relative to the weight of liver in grams.

Table 1. Effects of Diabetes and T_4 Replacement on Serum T_4 and T_3 Concentrations

	Control (n=10)	Diabetic (n=9)	Diabetic + T_4 (n=10)
T_4 (µg/dl)	3.9 ± 1.1†	1.1 ± 0.6*	4.0 ± 1.6
T_3 (ng/dl)	121 ± 16	49 ± 13*	85 ± 22*

†Mean ± SD; *$p<0.05$ vs control.

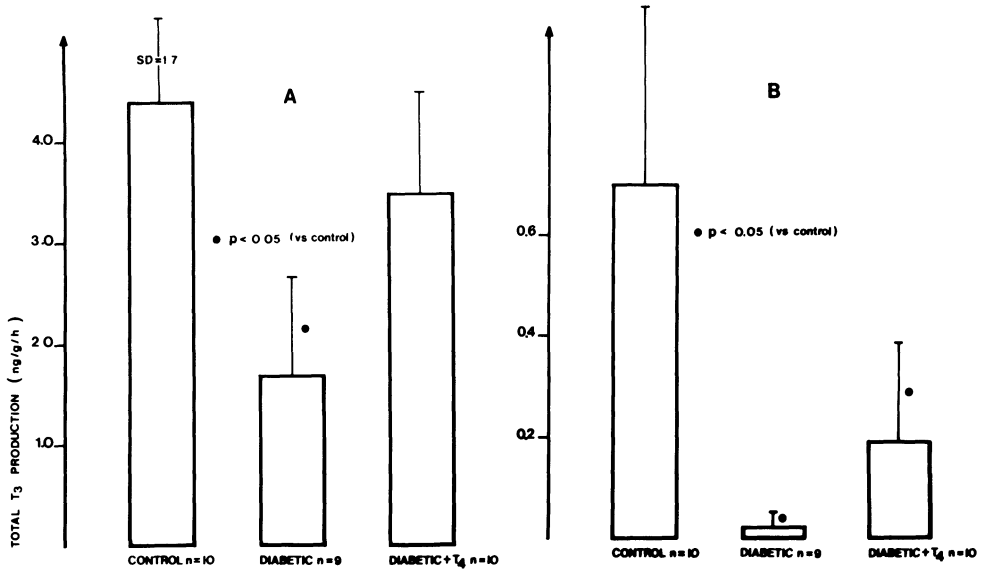

Fig. 1. Effects of diabetes and T_4 replacement on total (A) and perfusate (B) T_3 production after 1 hour liver perfusion. Values shown are mean $\pm$ SD.

Statistical methods. Statistical analysis was performed using a one-way analysis of variance, followed by Duncan's multiple range test to identify significant differences among specific groups (14).

RESULTS

Both mean serum T_4 and T_3 values were reduced by the diabetic state, as is shown in Table 1. The administration of T_4 prevented the decrease in serum T_4 levels in diabetic animals, indicating that the rats were physiologically replaced with T_4. In contrast, the mean serum T_3 concentration remained lower in the T_4-treated diabetic animals when compared with controls.

Figure 1 shows that the total and perfusate T_3 production was reduced in diabetic rats. On the other hand, there was no difference in the total T_3 production between T_4-treated diabetic animals and the controls. However the T_4-treated diabetic rats still had significantly lower levels of T_3 in the perfusate compared with controls.

DISCUSSION

Our results confirm the findings from Jennings (9) that T_4 replacement in STZ-induced diabetic rats normalized serum T_4 levels, but did not significantly affect serum T_3 concentration. From our perfusion system we were able to show that administration of T_4 normalized hepatic T_3 production. However, this increase in the total production rate did not reflect an increase in perfusate T_3 levels. This finding suggests that the low T_3 syndrome seen in STZ-induced diabetic rats might be due in part to an impairment in liver ability to release the newly generated T_3. This could be an adaptative mechanism to maintain physiological intracellular levels of T_3 in this disease.

REFERENCES

1. Boado R, Brown TJ, Bromage NR, et al. Acta Endocrinol (Kbh) 89: 323, 1978.
2. Zaninovich AA, Brown TJ, Boado R, et al. Acta Endocrinol (Kbh) 86: 336, 1977.
3. Balsam A, Ingbar SH, and Sexton FC. J Clin Invest 62: 415, 1978.
4. Pittman CS, Lindsay RH, Senga O, et al. Life Sciences 28: 1677, 1981.
5. Kaplan MM and Utiger RD. Endocrinology 103: 156, 1978.
6. Jennings AS, Crutchfield FL, and Dratman MB. Endocrinology 114: 992, 1984.
7. Chopra IJ, Wiersinga W, and Harrison F. Life Sciences 28: 1765, 1981.
8. Gavin LA, McMahon FA, and Moeller M. Diabetes 30: 694, 1981.
9. Jennings AS. Abstracts of the 64th Annual Meeting of the Endocrine Society, Abstract 122, 1982, p 110.
10. Kjeld JM, Kuku SF, Diamant L, et al. Clin Chem Acta 61: 381, 1975.
11. Borges DR, Limaos EA, and Prado JL. Naunyn-Schimiedeberg's Arch Pharmacol 295: 33, 1976.
12. Jennings AS, Ferguson DC, and Utiger RD. J Clin Invest 64: 1614, 1979.
13. Kaplan MM and Utiger RD. J Clin Invest 61: 459, 1978.
14. Dunnett CW. In JW McArthur and T Colton (eds), Statistics in Endocrinology, The MIT Press, Cambridge, Mass., 1970, p 86.

METABOLISM OF rT_3 BY ISOLATED RAT HEPATOCYTES

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Outer ring deiodination (ORD) of T_4 in peripheral tissues produces T_3, the predominant bioactive form of thyroid hormone. In contrast, the inactive metabolite rT_3 is formed if T_4 undergoes inner ring deiodination (IRD). Three enzymes are known to catalyze these reactions (1). The type I deiodinase of liver is probably identical to the kidney enzyme and converts T_4 to either T_3 or rT_3, while it also catalyzes the further IRD of T_3 and ORD of rT_3 with, in both cases, $3,3'\text{-}T_2$ as the product (1). The type II deiodinase, which has been detected in the brain, pituitary, and brown adipose tissue, is a true ORDase that converts T_4 only to T_3 and rT_3 to $3,3'\text{-}T_2$ (1). The brain contains an additional, type III, deiodinase which specifically catalyzes the IRD of T_4 to rT_3 and of T_3 to $3,3'\text{-}T_2$ (1).

Considering the high rate of rT_3 ORD by the type I deiodinase (1), it is likely that little rT_3 produced from T_4 in the liver is released into the circulation. Most plasma rT_3 is, therefore, derived from type III deiodination of T_4 in tissues such as the brain, while it is cleared predominantly by the liver. *In vivo* studies employing the selective inhibitory effects of PTU on the type I deiodinase have demonstrated that this enzyme is the predominant site for the peripheral production of T_3 in normal rats (2). Opposite variation in plasma T_3 and rT_3 concentrations has been observed in a number of clinical situations, which changes are due to a decrease in both the production of plasma T_3 and the clearance of plasma rT_3 (3).

To investigate the potential importance of changes in type I deiodinase activity for the regulation of thyroid hormone metabolism, we initiated studies of the deiodination of iodothyronines by isolated rat hepatocytes. Reverse T_3 was considered as the substrate of choice for these experiments, since (while analogous with the conversion of T_4 to T_3) ORD of rT_3 is the most effective reaction catalyzed by the type I deiodinase (1). Initial results showed that rT_3 is rapidly metabolized by rat hepatocytes with radioiodide as the main product from outer ring ^{125}I-labeled substrate (4). Although the involvement of the type I deiodinase was demonstrated by the inhibitory effects of PTU, little production of $3,3'\text{-}T_2$ from unlabeled rT_3 could be detected by RIA (4). These results were unexpected, since they implied I^- formation from further metabolism of $3,3'\text{-}T_2$, while it is a poor substrate for the type I deiodinase. Subsequent studies have revealed that sulfation is the main, initial step in the hepatic metabolism of $3,3'\text{-}T_2$, and that $3,3'\text{-}T_2$ sulfate is a highly effective substrate for ORD by the

type I deiodinase (5,6). A similar facilitative effect of sulfation has been observed on the IRD of T_3 in hepatocytes (5,7).

The rapid metabolism of added 3,3'-T_2 in liver cells by successive sulfation and ORD may be the reason for our failure to detect significant production of 3,3'-T_2 from rT_3 in this system. If so, the yield of 3,3'-T_2 produced by this pathway should increase if its further sulfation is inhibited. This hypothesis was tested in the present study employing rat hepatocytes with diminished phenolsulfotransferase activity. The results show a dramatic increase in the proportion of rT_3 metabolized that is recovered as 3,3'-T_2.

MATERIALS AND METHODS

Rat hepatocytes were prepared by collagenase perfusion as described previously (8). Suspensions of 10^6 cells in 2 ml of culture medium (8) were seeded into uncoated 2 cm wells of plastic 6-well dishes. The dishes were kept for 4 hours at 37°C in a culture stove under atmospheric condition. Non-viable cells were removed by aspiration of the medium.

The thus-obtained monolayers of viable hepatocytes were incubated with 10 nM outer ring ^{125}I-labeled rT_3 or 3,3'-T_2 in 2 ml incubation medium (Dulbecco containing either 1 mM $MgSO_4$ or 1 mM $MgCl_2$ and supplemented with 2 mM glutamine, 1 mM vitamin C, and 0.1% BSA). Incubations were carried out for 60 min at 37°C under atmospheric conditions while the dishes were placed on a slightly angled, slowly rotating plate. Incubations were also conducted in the presence of the deiodinae inhibitor PTU (10 µM), or the phenolsulfotransferase inhibitor salicylamide (SAM; 100 µM). Some experiments were performed with unlabeled substrates to quantify the degradation of added rT_3 and 3,3'-T_2, as well as the production of 3,3'-T_2 from rT_3 by RIA.

Analysis of the radioactive reaction products of rT_3 and 3,3'-T_2 was done by adsorption chromatography on small Sephadex LH-20 columns. For this purpose, 500 µl aliquots of incubation medium were mixed with 500 µl N HCl and the mixtures were applied to 1 ml Sephadex LH-20 columns equilibrated with 0.1 N HCl. Columns were eluted successively with 1 ml fractions of 0.1 N HCl (2x), H_2O (6x), 10% ethanol in 0.1 N NaOH (6x), and 50% ethanol in 0.1 N NaOH (3x). Fractions were collected in separate tubes and counted for radioactivity. The identity of the products in the different fractions was checked by HPLC (9).

RESULTS AND DISCUSSION

Fig. 1 demonstrates the Sephadex LH-20 analysis of media obtained after incubation of labeled 3,3'-T_2 or rT_3 with hepatocytes. Radioiodide which is eluted from the columns with diluted HCl represents the main product of the metabolism of 3,3'-T_2 in control incubations without inhibitors. In accordance with previous observations (5,6), I^- formation from 3,3'-T_2 is greatly inhibited in the presence of PTU, and large amounts of radioactivity appear in the H_2O fractions, representing the accumulation of 3,3'-T_2 sulfate. If the phenolsulfotransferase inhibitor SAM is added to the incubations, I^- production is again strongly reduced with a corresponding increase in the amount of 3,3'-T_2 recovered in the fractions eluted with 10% ethanol in NaOH. Similar results were obtained if sulfaction was inhibited with dichloronitrophenol (DCNP) or by incubations with sulfate-deplete media (9). These results again clearly illustrate that 3,3'-T_2 is metabolized in hepatocytes by successive sulfation and ORD.

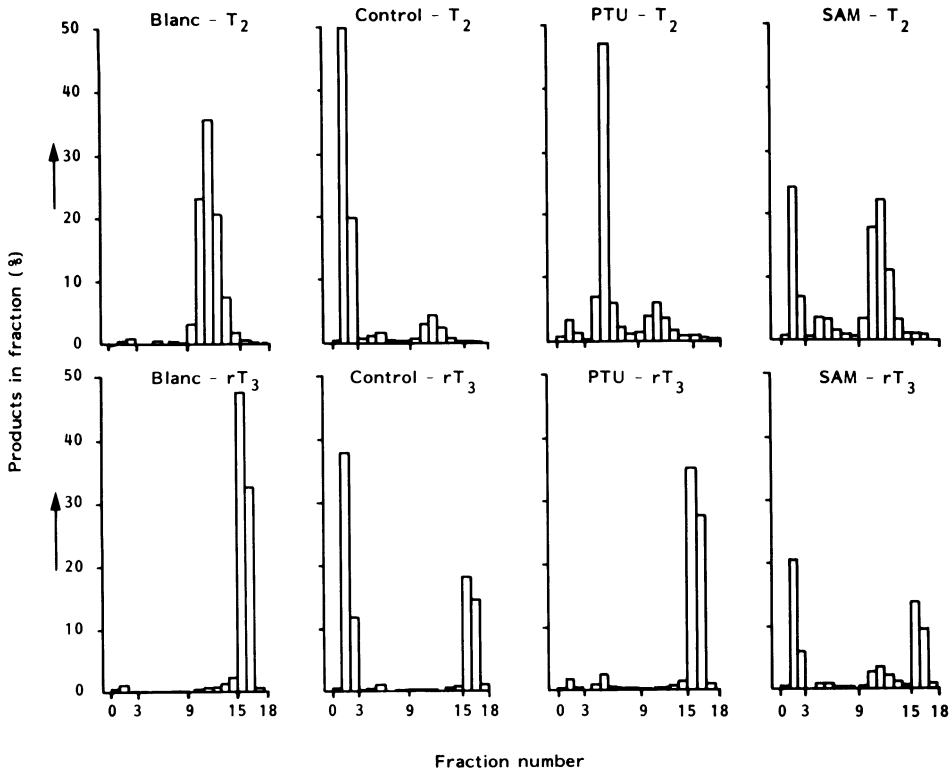

Fig. 1. Sephadex LH-20 chromatography of media obtained after incubation of 10 nM labeled 3,3'-T_2 or rT_3 for 60 min without (blanc) or with 10^6 hepatocytes in the absence (control) or presence of PTU or SAM. Fractions 1-3 were eluted with HCl, 4-9 with H_2O, 10-15 with ethanol/NaOH (10/90), and 16-18 with ethanol/NaOH (50/50).

Incubation of rT_3 with hepatocytes results predominantly in the formation of I^- together with small amounts of conjugate (Fig. 1, Table 1). Under control conditions, accumulation of 3,3'-T_2 from rT_3 is negligible, and unreacted rT_3 is eluted from the columns with 50% ethanol in NaOH. Addition of PTU leads to an almost complete inhibition of I^- production with an increased formation of conjugates, largely in the form of rT_3 glucuronide (9). In contrast to 3,3'-T_2, however, the increase in conjugates is negligible compared with the decrease in I^-, and degradation of rT_3 is inhibited by 90% in the presence of PTU. This indicates that ORD is the rate-limiting step in the metabolism of rT_3 in hepatocytes. Addition of SAM does not affect the disappearance of rT_3, but it induces a decrease in the release of I^- with an equivalent accumulation of 3,3'-T_2. The figures in Table 1 are not corrected for the fact that the specific radioactivity of the product 3,3'-T_2 is only half of that in the substrate rT_3. If this is done, it can be calculated that in the presence of SAM, roughly 60% of the medium rT_3 disappeared is recovered as medium 3,3'-T_2. Identical observations were made if incubations were carried out with unlabeled rT_3 and if the 3,3'-T_2 product was determined by RIA (9). Accumulation of 3,3'-T_2 from rT_3 was also observed in experiments with DCNP or with sulfate-depleted cells (9).

Table 1. Effects of PTU and SAM on the Metabolism of rT_3 by Rat Hepatocytes

Addition	% Product in medium*			
	I^-	Conjugates	$3,3'-T_2$	rT_3
---	47.0	1.5	0.7	43.7
10 μM PTU	1.8	5.3	-0.4	87.0
100 μM SAM	34.5	1.8	14.5	42.9

*Products were measured by Sephadex LH-20 chromatography after incubation of 10 nM labeled rT_3 for 60 min with hepatocytes. Formation of I^- was corrected by subtraction of I^- recovered from incubations without cells. Radioactivity in $3,3'-T_2$ fraction was corrected for slight contamination with rT_3. No corrections were made for changes in specific radioactivity. Results are the means of 5-10 experiments each performed in triplicate.

In conclusion, the above findings demonstrate that in rat hepatocytes, rT_3 is metabolized predominantly by ORD, but production of $3,3'-T_2$ is only observed if its subsequent sulfation is inhibited. This suggests that plasma $3,3'-T_2$ is mainly derived from extra-hepatic tissues, presumably by type III IRD of T_3.

REFERENCES

1. Leonard JL and Visser TJ. In G Hennemann (ed), Thyroid Hormone Metabolism, Marcel Dekker, New York, in press.
2. Silva JE, Gordon MB, Crantz FR, et al. J Clin Invest 73: 898, 1984.
3. Engler D and Burger AG. Endocrine Rev 5: 151, 1984.
4. Visser TJ, Otten MH, Mol JA, et al. Horm Metab Res (Suppl) 14: 35, 1984.
5. Otten MH, Mol JA, and Visser TJ. Science 221: 81, 1983.
6. Otten MH, Hennemann G, Docter R, et al. Endocrinology 115: 887, 1984.
7. Visser TJ, Mol JA, and Otten MH. Endocrinology 112: 1547, 1983.
8. Krenning EP, Docter R, Bernard HF, et al. FEBS Lett 91: 113, 1978.
9. Eelkman Rooda SJ, Van Loon MAC, and Visser TJ. Manuscript in preparation.

THYROID HORMONE METABOLISM IN KIDNEY EPITHELIAL CELLS IN CONTINUOUS CULTURE*

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Renal T_4 to T_3 conversion is catalyzed by membrane-bound enzyme, iodothyronine 5'deiodinase (5'D), that prefers rT_3 as a substrate, is inhibited by propylthiouracil (PTU), and shows ping-pong reaction kinetics with thiols as cosubstrate (1-3). Recent studies have shown that >90% of the kidney 5'D is associated with the tubular epithelium of the outer cortex (4). Since established renal epithelial cell lines that retain properties of either the proximal or the distal convoluted tubule are available, selected cell lines were examined for the presence of 5'D activity and for the presence of nuclear T_3 receptors.

METHODS

$LLC\text{-}PK_1$ (pig kidney) and MDCK (dog kidney) cells were obtained from the American Tissue Culture Collection, and OK (opossum kidney) cells were provided by Dr. R. L. Teplitz, Duarte, CA. Cultures were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics. $L\text{-}T_4$ and $L\text{-}T_3$ were purchased from Sigma, and rT_3 was purchased from Calbiochem. Radiolabeled iodothyronines were prepared by the method of Weeke and Orskov (5) and purified by descending paper chromatography (6). All other reagents were of the highest purity commercially available.

Confluent cell monolayers were washed with phosphate-buffered saline (PBS, pH 7.5), scraped from the flask, and collected by centrifugation (1000 g for 5 min). Pellets were resuspended in 250 µl of 20 mM HEPES buffer (pH 7.0) containing 1 mM EDTA and 10 mM dithiothreitol (DTT) and homogenized in a teflon-glass homogenizer. Cell homogenates were kept at 4°C until use.

5'D activity was determined in cell homogenates by measuring the release of $^{125}I^-$ from $[^{125}I]\text{-}rT_3$ (1) or by measuring the formulation of $[^{125}I]\text{-}T_3$ from $[^{125}I]\text{-}T_4$ (2). Products were analyzed by ion-exchange chromatography or descending paper chromatography, respectively (2). Equal amounts of I^- and $3,3'\text{-}T_2$ were formed with rT_3 as the substrate. One unit (U) of 5'D activity equals the formation of 1 pmol of product per minute.

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5'-Deiodination and T_3 degradation were also determined by incubating cell monolayers with 100 nM [^{125}I]-rT_3 (10,000 cpm/pmol) or 10 nM [^{125}I]-T_3 (30,000 cpm/pmol) in 1 ml of serum-free DMEM. Media samples were removed at 30, 60, and 120 min, and hormone metabolites were analyzed as described above.

Nuclear T_3 binding was determined as previously described (7) in isolated nuclei prepared by the method of Samuels and Tsai (8). Data were analyzed by the method of Scatchard (9).

RESULTS

Homogenates of LLC-PK_1 cells consistently catalyzed the 5'deiodination of rT_3 in vitro, while both OK and MDCK homogenates failed to deiodonate rT_3 (Table 1). At saturating rT_3 and DTT (10 µM and 20 mM, respectively) enzyme activity in LLC-PK_1 homogenates increased 1.7-fold and addition of 1 mM PTU blocked <95% of the 5'D activity (Table 1). T_4 to T_3 conversion was also catalyzed by LLC-PK_1 homogenates with 3.9 pmol of T_3 formed/hour/mg protein incubated with 100 µM [^{125}I]-T_4 and 5 mM DTT. No T_3 production was catalyzed by OK or MDCK homogenates. T_3 degradation was also absent in both OK or MDCK homogenates and was <5% of added T_3 in LLC-PK_1 homogenates.

Similarly, incubation of LLC-PK_1 cultures with 100 nM [^{125}I]-rT_3 resulted in the release of $^{125}I^-$ into the medium, 4.1 pmol/h/25 cm^2 flask (r=0.99), whereas no deiodination occurred in cultures of OK or MDCK cells. Addition of 100 µM PTU to the media completely blocked the 5'deiodination of rT_3 by LLC-PK_1 cells.

5'Deiodinating activity in LLC-PK_1 homogenates was linear with time and proportional to cell protein. Raising the [DTT] from 1.5 to 20 mM resulted in a stepwise increase in radioiodide release from rT_3 (Table 2). These data indicate that the LLC-PK_1 cells contain a 5'deiodinating enzyme with properties similar to those described for rat kidney (1-3).

Subcellular fractionation of the LLC-PK_1 homogenates revealed that 39% of the total 5'D activity was present in the plasma membrane containing

Table 1. 5'Deiodinase Activity in Established Renal Epithelial Cell Lines

Cell line	Suspected Origin	5'D activity ($pmol\ I^-$ released/min/mg protein) Assay 1	Assay 2
MDCK	Thick ascending limb	-0-	-0-
OK	Proximal convoluted tubule	-0-	-0-
LLC-PK_1	Proximal convoluted tubule	12 ± 1, n=4	20 ± 1, n=8

Assay 1: 1 µM rT_3, 1 mM DTT. Assay 2: 10 µM rT_3, 20 mM DTT. >95% of 5'D activity was inhibited by 1 mM PTU.

Table 2. Effects of Increasing [DTT] on 5'D Activity in LLC-PK_1 Homogenates

[DTT] mM	5'D activity (pmol I^- released/min/mg protein)
1.5	0.7
2.5	1.4
5	2.5
10	4.2
20	6.6

Data reported as means of closely agreeing (± 10%) triplicate determinations.
Reactant conditions:
0.1 mM phosphate buffer (pH 7.0)
1 mM EDTA
250 nM [^{125}I]-rT_3

fraction, with 10% present in the microsomal fraction (Table 3). Crude microsomes showed the highest enzyme enrichment with a 3-fold increase in specific enzyme activity over that in the homogenate.

Nuclear T_3 binding, determined in isolated nuclei, was observed only in nuclei from LLC-PK_1 cells which showed specific high affinity T_3 binding

Table 3. Subcellular Distribution of 5'Deiodinase in LLC-PK_1 Homogenates

Fraction	5'D activity (specific enzyme activity)	% total Enzyme
Homogenate	94	100
Large membranes, whole cells, nuclei	129	27
Apical plasma membranes	95	12
Mitochondria, membrane vesicles	184	11.4
Microsomes (basolateral PM, ER, membrane fragments)	279	9.4
Cytosol	16	--

Data reported as means of closely agreeing (± 10%) tripliate determinations. Assay conditions: 1 μM rT_3; 1 mM DTT.

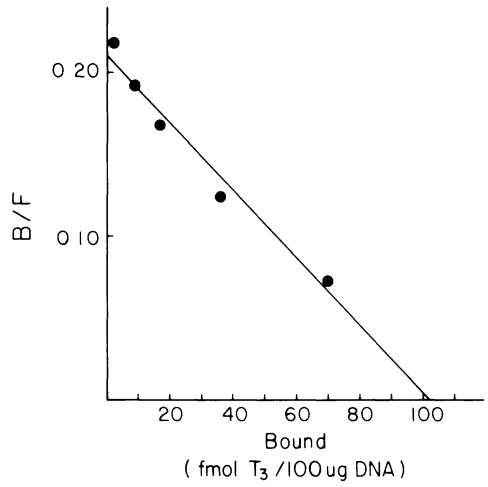

Fig. 1. Scatchard plot of nuclear T_3 binding to isolated LCC-PK_1 nuclei. Data reported as the means of triplicate determinations.

(Fig. 1) with a Kd of 0.5 nM and a MBC of 0.65 ng T_3 per mg DNA. Nuclei from either OK or MDCK cells failed to bind T_3.

DISCUSSION

The results of this study show that renal epithelial cells (LLC-PK_1) are capable of 5'deiodinating both T_4 and rT_3 by an enzyme that has the properties of the type I 5'deiodinase. These cells show properties associated with the proximal convoluted tubule such as Na^+-dependent glucose transport (10,11), and trehalase and maltase activities (12). Another cell also thought to be from the proximal tubule (OK) did not contain the 5'D. This raises the possibility that only a small portion of the proximal tubule may deiodinate thyroid hormone, since the properties of the OK cell (gluconeogenic and PTH responsive) (13) differ from those of the LLC-PK_1. However, it is also possible that selective loss of differentiated functions, such as T_4 metabolism, might account for the differences in 5'deiodinating capacity of the two proximal tubule cell lines. MDCK cells, derived from the distal tubule as judged by the presence of amiloride-sensitive Na^+ transport (14), and vasopressin stimulated transepithelial transport of salt and H_2O (15), also failed to metabolize thyroid hormone.

The properties of the 5'D in LLC-PK_1 cells are similar, if not identical, to those of the rat kidney enzyme. The amount of 5'D present in LLC-PK_1 is $\sim$50% of that in isolated cortical tubule preparations (4) and >95% of the activity is inhibited by PTU. Like the rat kidney enzyme, 5'D in LLC-PK_1 cells is associated predominately with the easily sedimenting membranes, presumably the plasma membrane (16). These data indicate that this cell line contains a type I 5'deiodinase offering the distinct advantage of a single cell type for the study of the biology of deiodination.

Finally, cultures of proximal and distal tubular epithelia were examined for their potential to respond to thyroid hormone by virtue of the presence of specific, high-affinity T_3 binding sites. Insofar as cell lines represent different segments of the nephron, only a portion of the proximal tubule (represented by the LLC-PK_1 cell) showed specific nuclear T_3 binding. Recently, the T_3 receptors were also identified in CV_1 cells from simian renal cortex (17). Interestingly, both the capacity to catalyze T_4 to T_3 conversion and to respond to thyroid hormone (presence of nuclear T_3 receptors) reside in the same renal epithelial cell. This suggests that, at least in the kidney, the ability to bioactivate thyroid hormone and to respond to thyroid hormone are linked.

REFERENCES

1. Leonard JL and Rosenberg IN. Endocrinology 107: 1376, 1980.
2. Leonard JL and Rosenberg IN. Endocrinology 103: 2137, 1978.
3. Leonard JL and Rosenberg IN. Biochim Biophys Acta 659: 205, 1981.
4. Leonard JL, Frank SJ, and Gogolin PJ. 60th Amer Thyroid Assoc Meeting, NY, T-43, 1984.
5. Weeke J and Orshov H. Scand J Clin Lab Invest 32: 357, 1973.
6. Bellabarba D, Petersen RE, and Sterling K. J Clin Endocrinol Metab 28: 305, 1968.
7. Kolodny JM, Leonard JL, Larsen PR, et al. Endocrinology 117: 1848, 1985.
8. Samuels HH and Tsai JS. Proc Natl Acad Sci USA 70: 3488, 1973.
9. Scatchard G. Ann NY Acad Sci 51: 660, 1979.
10. Perantoni A and Berman JJ. In Vitro 15: 446, 1979.
11. Sanders MJ and Misfeldt DS. Fed Proc 39: 736, 1980.
12. Handler JS, Perkins FM, and Johnson JP. Am J Physiol 238: F1, 1980.
13. Teitelbaum AP and Strewler GJ. Endocrinology 114: 980, 1984.
14. Rindler MJ, Taub M, and Saier Jr MH. J Biol Chem 254: 11431, 1979.
15. Misfeldt DS, Hammammoto ST, and Pitelka DR. Proc Natl Acad Sci USA 73: 1212, 1976.
16. Leonard JL and Rosenberg IN. Endocrinology 103: 274, 1978.
17. Savouret J-F, Eberhardt NL, Cathala G, et al. Endocrinology 116: 1259, 1985.

EXTRATHYROIDAL CONVERSION OF THYROXINE TO 3,5,3'-TRIIODOTHYRONINE IN RATS UNDERGOING ACUTE IMMOBILIZATION STRESS

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INTRODUCTION

Several experimental and clinical conditions sharing stress as a common factor (i.e., fasting, surgical procedures, severe non-thyroidal systemic illnesses) are known to induce marked alterations on the extrathyroidal metabolism of iodothyronines (1). These alterations, known as the "low T_3 syndrome," are characterized by low 3,5,3'-triiodothyronine (T_3), and high 3,3',5'-triiodothyronine (rT_3) serum concentrations, while thyroxine (T_4) values in serum may be decreased or unchanged (2).

The aim of the present investigation is to verify if acute stress, induced by restrainment, can interfere significantly with the peripheral metabolism of thyroid hormones, as assessed through the 5'deiodination (5'D) activity in liver and kidney homogenates.

MATERIAL AND METHODS

Young male rats of the Wistar strain were used throughout. They were housed in temperature-controlled quarters with light and dark cycles of 14 and 10 h, respectively. Food and water were available "ad libitum." Surgical bilateral adrenalectomy was carried out one week before the experiments, and thereafter each animal received daily injections of corticosterone (Sigma Chemical Co., St. Louis, EUA; 3 μg/100 g, i.m.). All experiments were done between 0800 and 1600 h. Rats were restrained continuously in tubes of variable diameters according to the rat's size, and were decapitated at 2, 4, 6, and 8 h thereafter. Blood samples were centrifuged and serum stored for iodothyronines radioimmunoassays (RIA) (3). Both kidneys and the liver were quickly dissected and homogenized with ice-cold PBS, 0.15 M, pH 7.4. 5'D activity was assessed using T_4 as the substrate, as described previously (4). Briefly, aliquots of tissue homogenates were incubated with non-radioactive T_4 for 1 h, 37°C, and the amount of T_3 generated was measured by RIA in an ethanol extract of tissue.

All results are mean values $\pm$ SEM for seven animals and the statistical significance was calculated by the unpaired Student's t test and analysis of variance (ANOVA).

Table 1. Serum Concentrations of Corticosterone (B), T_4, T_3, rT_3, and Conversion of T_4 to T_3 (5'D), in Intact Rats Subjected to Different Restrainment Periods

Determination		2 h		4 h		6 h		8 h	
		Control	Stress	Control	Stress	Control	Stress	Control	Stress
B	ng/ml	23 ± 3	225 ± 15	22 ± 4	340 ± 10	27 ± 5	455 ± 33	39 ± 5	574 ± 21
T_4	ng/ml	36 ± 3	36 ± 6	45 ± 10	39 ± 8	41 ± 7	35 ± 10	34 ± 8	32 ± 4
T_3	ng/dl	95 ± 10	95 ± 8	92 ± 15	80 ± 9	89 ± 9	60 ± 5*	92 ± 11	53 ± 7*
rT_3	ng/dl	12 ± 1	11 ± 2	13 ± 2	13 ± 1	11 ± 3	17 ± 2*	12 ± 3	21 ± 1*
5'D liver	ng T_3/tube	70 ± 10	75 ± 8	77 ± 11	59 ± 6*	83 ± 5	48 ± 7*	76 ± 13	43 ± 7*
5'D kidney	ng T_3/tube	118 ± 15	125 ± 13	122 ± 13	100 ± 11*	129 ± 10	89 ± 10*	125 ± 8	73 ± 5*

Values are means ± SD; *$p<0.05$ in relation to the control group; n=7 for all groups.

Table 2. Serum Concentrations of Corticosterone (B), T_4, T_3, rT_3, and Conversion of T_4 to T_3 (5'D), in Adrenalectomized Rats Subjected to Different Restrainment Periods

Determination		2 h		4 h		6 h		8 h	
		Control	Stress	Control	Stress	Control	Stress	Control	Stress
B	ng/ml	18 ± 5	22 ± 6	23 ± 3	20 ± 3	20 ± 3	20 ± 2	19 ± 2	18 ± 4
T_4	ng/ml	36 ± 7	32 ± 6	42 ± 8	40 ± 7	43 ± 5	33 ± 6	37 ± 4	41 ± 5
T_3	ng/dl	92 ± 8	85 ± 13	90 ± 10	93 ± 8	94 ± 13	96 ± 5	98 ± 6	91 ± 9
rT_3	ng/dl	10 ± 2	12 ± 2	11 ± 3	10 ± 3	9 ± 3	11 ± 3	12 ± 3	13 ± 2
5'D liver	ng T_3/tube	80 ± 9	89 ± 5	76 ± 7	92 ± 6	86 ± 13	95 ± 10	72 ± 8	91 ± 9
5'D kidney	ng T_3/tube	149 ± 13	143 ± 7	138 ± 10	155 ± 15	150 ± 14	160 ± 12	143 ± 8	158 ± 17

Values are means ± SD; n=7 for all groups.

RESULTS

In the intact rats following a four hour immobilization period, it was possible to detect decreased 5'D activity in both kidney and liver homogenates, as evidenced by decreased amounts of T_3 generated during the incubation period. Furthermore, animals sacrificed following 6-8 hour immobilization periods, in addition to the low 5'D activity, showed decreased T_3, increased rT_3, and unaltered T_4 serum concentrations (Table 1).

Following the same immobilization periods, adrenalectomized rats showed no modification of 5'D activity in both extrathyroidal sites studied, while iodothyronines serum concentrations remained unaltered (Table 2).

DISCUSSION

The mechanisms involved in the pathogenesis of the "low T_3 syndrome" are not completely understood. Previous reports have demonstrated that several conditions presenting variable degrees of stress are accompanied by decreased T_3 and increased rT_3 serum concentrations. These alterations are thought to be due to decreased extrathyroidal 5'D activity (1). Actually, decreased T_3 production rate was detected in cirrhotic patients (5) and in hepatic homogenates of fasting rats (6), although a recent report has suggested that, at least in the fasting rat, T_3 production rate is decreased primarily due to a decreased thyroidal secretion of T_4 (7).

It is well known that the administration of dexamethasone to healthy subjects can lead to the "low T_3 syndrome." It was further demonstrated in rats that treatment with corticosterone promoted a significant decrease in hepatic 5'D activity, suggesting that glucocorticoids may play a significant role in the stress-induced alterations in the extrathyroidal metabolism of iodothyronines.

The findings of the present investigation clearly demonstrate that the extrathyroidal metabolism of thyroid hormones is markedly altered following a six hour restrainment period. These modifications are characterized by decreased T_3, increased rT_3 serum concentrations, as well as by decreased 5'D activity in both liver and kidney homogenates.

When adrenalectomized rats, treated with physiological amounts of corticosterone, were restrained, no alterations could be detected in the iodothyronines serum concentrations or in the extrathyroidal 5'D activity, suggesting that increased adrenal secretion of corticosterone in response to restrainment is an important factor in the pathogenesis of the stress-induced alterations of the extrathyroidal metabolism of thyroid hormones.

In conclusion, from the results obtained in the present investigation, it is possible to assume that stress can markedly interfere with the peripheral metabolism of iodothyronines, and that these alterations are at least in part mediated by adrenal glands.

REFERENCES

1. Chopra IJ, Solomon DH, Chopra U, et al. Rec Prog Horm Res 34: 521, 1978.
2. Chopra IJ. Ann Intern Med 98: 946, 1983.
3. Russo EMK, Vieira JGH, Maciel RMB, et al. Arq Bras Endocrinol Metab 26: 23, 1982.
4. Chopra IJ. Endocrinology 101: 453, 1977.

5. Faber J, Thomsen HF, Lumholtz IB, et al. J Clin Endocrinol Metab 53: 978, 1981.
6. Harris ARC, Fang CS, Vagenakis AG, et al. Metab Clin Exp 27: 1680, 1978.
7. Kinlaw WB, Schwartz HL, and Oppenheimer JH. J Clin Invest 75: 1238, 1985.

EFFECT OF CADMIUM ON THYROID HORMONE METABOLISM

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Monodeiodination is a major pathway of T_4 metabolism in the rat as well as in man. The active thyroid hormone, T_3, is produced by outer ring deiodination of T_4. Thiol agents such as dithiothreitol (DTT), mercaptoethanol, and reduced gluthathione are potent stimulators of this reaction (1). The sulfhydryl-oxidizing agent, diamide, on the other hand, is a potent inhibitor. Heavy metals bind firmly to free sulfur groups and inhibit activities of a large number of enzymes having functional sulfhydryl groups (2). The inhibitory effect of mercury on the conversion of T_4 to T_3 *in vitro* has been reported (1,3). In the present paper we describe the effect of cadmium on the hepatic conversion of T_4 to T_3 in the rat. Furthermore, we have measured serum thyroid hormone concentrations in subjects living in a cadmium polluted area for a long time.

MATERIALS AND METHODS

Twenty-four female Wistar rats were used. In the first experiment, 12 rats were divided into two groups. There was no significant difference in the mean body weight between the two groups. Animals were maintained on Purina rat chow and water ad libitum. The $CdCl_2$ group was treated with 1 mg/kg BW/day $CdCl_2$ in 0.2 ml saline subcutaneously, five times a week for ten weeks. The control group was given 0.2 ml saline instead of $CdCl_2$. In the second experiment, the other two groups of rats studied consisted of normal controls and pair-fed rats. The pair-fed rats were given the same amount of food (12 g/day) consumed by the corresponding $CdCl_2$-treated rats for ten weeks. On the morning the treatment was completed, rats were weighed and then sacrificed by heart puncture. Blood was collected for the determination of serum T_4, T_3, and cadmium concentration.

T_4 5-'deiodinase activity in the liver homogenate was assayed as described previously (4,5). In brief, the liver homogenate (1 mg protein) was incubated with one μg T_4 for 60 min at 37°C in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.5 mM or 5 mM DTT. To study the effect of $CdCl_2$ on the conversion of T_4 to T_3 *in vitro*, 10^{-8} to 10^{-3} M $CdCl_2$ were added to the liver homogenates of the control rats with or without 0.5, 5, and 50 mM

Table 1. Effect of Treatment of Rats with $CdCl_2$ on Body Weight, Serum T_4, T_3, and T_4 to T_3 Monodeiodinating Activities in Liver Homogenates

	N	Body weight at the time of sacrifice (g)	Serum T_4 (µg/100 ml)	Serum T_3 (ng/100 ml)	Hepatic T_3 generation from T_4 (ng T_3/tube) 0.5 mM DTT	5 mM DTT
Control group	6	282 ± 14	3.0 ± 0.7	89 ± 6.0	14.0 ± 1.3	14.5 ± 3.2
$CdCl_2$ group	6	236 ± 7	2.6 ± 0.8	56 ± 7.9	1.1 ± 1.3	3.2 ± 1.0
		a)	ns	a)	a)	a)
					ns	
					a)	

Values given are mean ± SD; a) $p<0.001$; ns: not significant.

DTT. The amount of T_3 produced during incubation was determined by a specific RIA.

Sera of 258 subjects residing in the town of Izuhara, Tsushima, Nagasaki Pref., Japan, in an area of known cadmium pollution (cadmium group), were available for the present study. None had a history of thyroid disease, and none had any severe illness or were taking any medication known or suspected to influence thyroid hormone concentrations. Sera of 230 age- and sex-matched healthy subjects residing in cadmium nonpolluted areas (control group) were also collected. Daily cadmium ingestions in the cadmium and control groups were 200∿250 μg and 30∿50 μg, respectively. Mean urinary cadmium concentration in the cadmium group was 11.3 μg/g-creatinine, which was about 4.5 times as much as that in the control group (6). The difference of daily iodide intake between the two groups was not significant.

Serum T_4, T_3, and TSH concentrations were measured by RIAs. Free T_4 and free T_3 indexes (FT_4I and FT_3I) were calculated from serum T_4 and T_3 concentrations multiplied by T_3 uptake (percent).

Values reported are the mean ± SD and statistical analyses were carried out by means of Student's unpaired t test.

RESULTS

As shown in Table 1, $CdCl_2$ treatment caused a 16% reduction in the body weight and a 37% reduction in the serum T_3 concentration. Serum T_4 concentration in the $CdCl_2$-treated rats was slightly decreased, but was not significantly different from that in the control rats. This treatment was also associated with a 92% and a 78% reduction in the T_4 to T_3 conversion by rat liver homogenates with 0.5 mM and 5 mM DTT, respectively. Serum cadmium concentration in the cadmium-treated and control rats were 441 ± 132 μg/l and not detectable, respectively. In the pair-fed rats, the mean body weight was significantly reduced (10%), but serum T_4, T_3 concentrations and

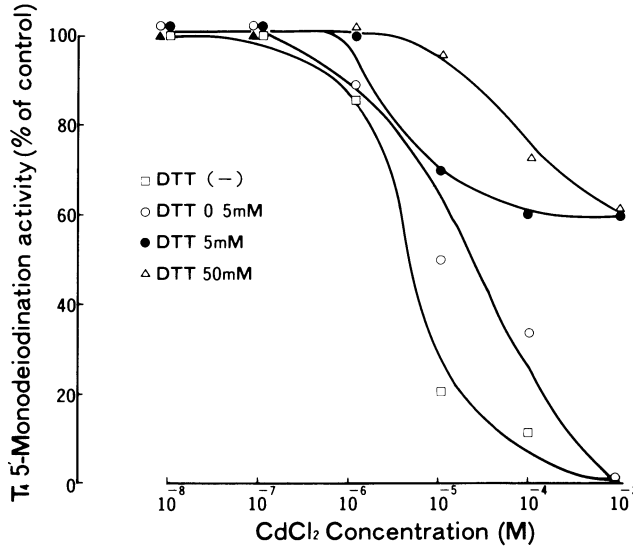

Fig. 1. Effect of $CdCl_2$ on T_3 generation from T_4 by rat liver homogenate.

Table 2. FT_4I and FT_3I in Subjects Living in the Cadmium-polluted and Control Areas

Age	FT_4I Control group	FT_4I Cadmium group	FT_3I Control group	FT_3I Cadmium group
30∿ 39	2.89 ± 0.44 (n=30)	2.88 ± 0.46 (n=16)	34.4 ± 6.1 (n=31)	38.6 ± 6.2 (n=16)
	n.s.		p<0.05	
40∿ 49	2.64 ± 0.43 (n=48)	2.92 ± 0.49 (n=6)	33.2 ± 6.8 (n=48)	38.4 ± 7.3 (n=4)
	n.s.		n.s.	
50∿ 59	2.71 ± 0.54 (n=79)	2.71 ± 0.58 (n=84)	35.4 ± 6.3 (n=79)	35.0 ± 6.0 (n=84)
	n.s.		n.s.	
60∿ 69	2.89 ± 0.55 (n=39)	2.70 ± 0.51 (n=37)	35.5 ± 6.0 (n=39)	31.6 ± 5.4 (n=37)
	n.s.		p<0.01	
70∿ 79	3.07 ± 0.50 (n=16)	2.68 ± 0.50 (n=36)	35.5 ± 3.9 (n=16)	31.2 ± 6.4 (n=36)
	p<0.05		p<0.01	

Mean ± SD

hepatic T_3 production from T_4 were not significantly different from those in the control rats.

In vitro addition of 10^{-6} to 10^{-3} M $CdCl_2$ to liver homogenates caused a dose-dependent reduction in T_3 generation from T_4 (Fig. 1). Without DTT, a 50% reduction in the T_4 to T_3 converting activity was caused by 4×10^{-6} M $CdCl_2$. Addition of DTT, 0.5, 5, or 50 mM, in the incubation mixture partially restored T_3 generation from T_4 in a dose-dependent manner, but concentration of 10^{-4} M or more $CdCl_2$ inhibited T_4 to T_3 conversion even in the presence of 50 mM DTT. 10^{-6} M to 10^{-3} M $HgCl_2$ and $PbCl_2$ also inhibited the T_4 to T_3 conversion by rat liver homogenates, but 10^{-6} M to 10^{-3} M NaCl, $CaCl_2$, and $MnCl_2$ had no effect.

Serum T_4 concentrations in the 60 to 69 and 70 to 79, and serum T_3 concentrations in the 50 to 59, 60 to 69, and 70 to 79 year age groups were both significantly lower in the subjects living in the cadmium-polluted areas than in the control areas. No significant difference in T_3 uptake was observed between the two groups in any age. Therefore, FT_4I in the 70 to 79 and FT_3I in the 60 to 69 and 70 to 79 year age groups were both significantly lower in the cadmium group than in the control group (Table 2). Serum TSH concentration in the cadmium group was not different from that in the control group in any age.

DISCUSSION

The present in vitro experiment indicated that $CdCl_2$ directly inhibits the T_4 to T_3 conversion in a dose-dependent manner. In the presence of DTT, the inhibitory effect of $CdCl_2$ on rat hepatic T_4 outer ring monodeiodination was diminished. These data suggest that $CdCl_2$ inhibition of T_4 outer ring deiodination may involve sulfhydryl groups of the deiodinase. It is likely that the decreased serum T_3 concentration in the $CdCl_2$-treated rats is due to both liver damage (7) and the direct inhibitory effect of $CdCl_2$ on T_4 outer ring deiodination.

Although there is a possibility that cadmium has some effects on thyroid hormone metabolism, detailed investigation of the effect of cadmium on serum thyroid hormone concentrations has not been reported yet. The present study indicates that FT_4I in the seventies and FT_3I in the sixties and seventies in the cadmium group are significantly lower than those obtained in the control group. The decrease in FT_3I in the cadmium group can be exexplained by the inhibitory effect of cadmium on the T_4 to T_3 conversion which was observed in the rat liver homogenate. The lower FT_4I in the seventies of the cadmium group, however, might be due to the decrease in T_4 secretion as seen in lead poisoning (8).

There is a report that the average cadmium concentration in the air of 28 cities shows a positive correlation with the death rate from arteriosclerotic heart disease (9). Bastenie et al. (10) reported strong clinical and pathological evidence supporting a relationship between atherosclerotic disease and hypothyroid state. Direct evidence in the relationship between increased frequency of atherosclerotic heart disease in subjects living in a cadmium-polluted area and the changes of thyroid hormone metabolism and secretion induced by cadmium awaits further investigation.

REFERENCES

1. Chopra IG, Solomon DH, Chopra U, et al. Recent Prof Horm Res 34: 521, 1978.
2. Vallee BL and Ulmer DD. Ann Rev Biochem 41: 91, 1972.

3. Visser TJ, Does-Tobe IVD, Docter R, et al. Biochem J 157: 479, 1976.
4. Yoshida K, Suzuki M, Sakurada T, et al. Acta Endocrinol (Copenh) 107: 495, 1984.
5. Murayama N, Yoshida K, Torikai S, et al. Horm Metabol Res 17: 197, 1985.
6. Nakano A, Saito H, Mitane Y, et al. Jpn J Hyg 39: 473, 1984.
7. Faeder EJ, Chaney SQ, King LC, et al. Toxicol Appl Pharmacol 39: 473, 1977.
8. Robins JM, Cullen MR, Connors BB, et al. Arch Intern Med 143: 220, 1983.
9. Carroll RE. J Amer Med Assoc 198: 177, 1966.
10. Bastenie PA, Neve P, Bonnyns M, et al. Lancet 1: 915, 1967.

PREFERENTIAL METABOLISM OF T_4 TO rT_3 (T_3 REVERSE) IN HYPERTHYROXINEMIA INDUCED BY L-THYROXINE (L-T_4) TREATMENT OF HYPOTHYROIDISM IN CHILDREN AND ADOLESCENTS

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INTRODUCTION

Preferential conversion to rT_3, an inert metabolite of T_4, has been proposed as a homeostatic mechanism for thyroid hormone inactivation in several physiopathological situations (1,2).

On the other hand, moderate elevation of T_4 and inhibition of TSH were frequently seen in our hypothyroid patients treated with "physiologic" doses of L-T_4 ($\bar{X}$ 6.7 µg/kg/1 between four months and one year of age, $\bar{X}$ 4.8 between one and nine years, and $\bar{X}$ 3.5 after 10 years).

In this work, we have studied the measurement of rT_3 to see if it could be used as a sensitive and simple parameter to evaluate L-T_4 treatment in hypothyroid children. A study of the dose as a function of age was also carried out in a large group of patients.

MATERIALS AND METHODS

T_4, T_3, rT_3, and/or TSH response to TRH were determined in the serum of: a) 19 normal children and adolescents, and b) 22 hypothyroid patients with normal TSH under treatment with L-T_4. TSH was also measured in a large group of 141 treated patients.

Hormones were determined by RIA; in the case of rT_3 using a kit provided by Serono Diagnostics. The specificity of the rT_3 assay was confirmed by addition of large doses of nonradioactive T_4 to the standard curve. TSH-releasing hormone (TRH) tests were carried out utilizing 200 µg of TRH (ELEA) intravenously.

RESULTS

Results are given as $\bar{X} \pm$ SD. Group a: T_4 (µg/dl) 9.18 $\pm$ 1.6; T_3 (ng/dl) 138 $\pm$ 34; rT_3 (ng/dl) 18.6 $\pm$ 5.3; TSH (µU/ml) 2.1 $\pm$ 1.1. Group b (p, group a vs b, t test): T_4, 13 $\pm$ 4 ($p<0.001$); T_3, 154 $\pm$ 35.7 (p=NS); rT_3, 30 $\pm$ 14 ($p<0.005$); TSH, 1.1 $\pm$ 0.5 ($p<0.001$). In group b, a statistically significant correlation was observed between rT_3 and T_4 ($p<0.001$) (Fig. 1), and between rT_3/T_3 ratio and T_4 ($p<0.05$) for values above the normal T_4 mean (Fig. 2). No correlation was found between T_4 and T_3.

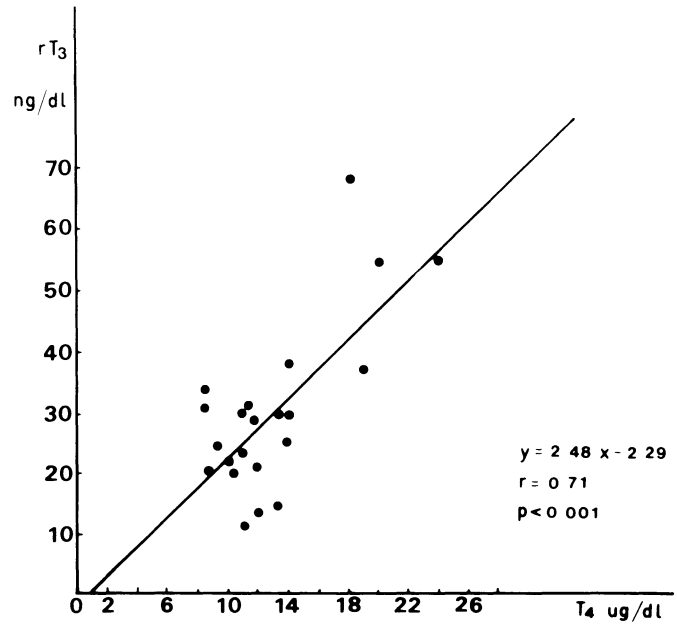

Fig. 1. Correlation between rT_3 and T_4 in treated primary hypothyroidism under treatment.

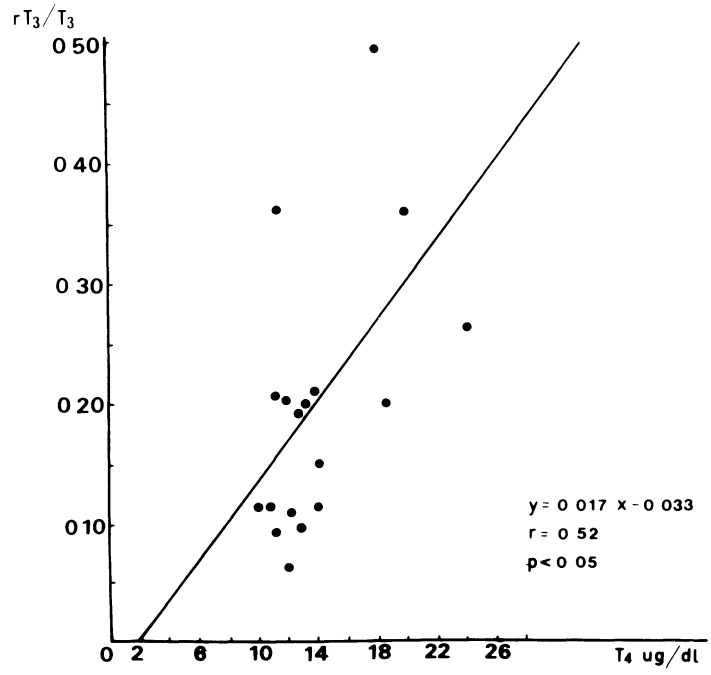

Fig. 2. Correlation between rT_3/T_3 ratio and T_4 for values above the normal T_4 mean in treated primary hypothyroidism.

Table 1. Patients Under L-T_4 Treatment Without Symptoms of Overdose

Name	Sex	Age years	Dose µg/kg	TSH-(TRH) basal µU/ml	rT_3 ng/dl	T_4 µg/dl	T_3 ng/dl
F.J.	M	2 4/12	4.30	0.30	21	12.0	185
D.G.	F	3 6/12	6.30	0.80	24	9.2	204
S.P.	F	6 0/12	4.70	0.50	29	11.8	146
R.L.	F	7 7/12	3.00	1.20	22	10.0	175
F.J.L.	M	8 7/12	6.20	0.40	11	11.0	141
L.A.	M	10 8/12	5.50	1.25	20	10.3	151
T.S.	F	14 9/12	3.30	1.00 (↓)	25	14.0	186
L.F.	M	14 6/12	3.40	1.20 (↓)	20	8.7	131
P.L.	F	15 2/12	2.92	0.90 (↓)	13	12.0	176
M.M.	F	16 8/12	2.50	1.25 (↓)	14	12.6	160
B.M.	F	16 8/12	2.65	1.25 (↓)	23	13.0	126
L.N.	F	16 8/12	4.30	2.30	33	8.5	109
I.M.	F	16 8/12	3.70	1.10	31	11.3	148
$\bar{X}$				1.03**	22*	11.1**	156*
SD				0.53	6.7	1.7	27.3

*p : NS vs normal subjects; **p<0.005 vs normal subjects;
(↓) blunted response.

Hypothyroid patients (group b) were divided according to the presence or not of symptoms of overdose.

As shown in Table 1, $\bar{X} \pm$ SD rT_3 in patients under L-T_4 treatment without symptoms of overdose was similar to that of normal subjects, while T_4 was significantly higher.

On the other hand, when symptoms of overdose were present, both rT_3 and T_4 were elevated compared to either normal subjects or patients without symptoms (Table 2).

In order to evaluate the dose of L-T_4, clinical evaluation and determinations of TSH and T_4, collected in a large group of 141 patients treated over a period of several years, was reexamined. Patients received between 2.5 and 10 µg/kg b.w.

Since TSH was frequently elevated (>5 µU/ml) in patients below six years of age, it was possible to calculate the minimal mean inhibitory dose from the regression line of TSH vs dose in six different age groups younger than six years (Fig. 3).

The following mean minimal inhibitory doses were found: 4-m- to 1-y-old patients, 11.1 µg/kg/1; 1-y- to 2-y-old, 6.8; 2-y- to 3-y-old, 5.7; 3-y- to 4-y-old, 5.3; 4-y- to 5-y-old, 5.4, and 5-y- to 6-y-old, 5.1.

Table 2. Patients Under L-T_4 Treatment With Symptoms of Overdose

Name	Sex	Age years	Dose µg/kg	TSH-(TRH) basal µU/ml	rT_3 ng/dl	T_4 µg/dl	T_3 ng/dl	Symptoms
I.M.	M	1 2/12	10.00	1.50	55	24.0	193	T. Irr.
G.P.	F	2 3/12	7.00	1.00	68	18.0	121	T. Irr.
I.R.	F	6 1/12	6.10	0.90	30	11.0	80	T.
P.C.	M	8 1/12	8.10	0.50	30	14.0	202	Irr.
R.B.	M	13 0/12	3.40	2.10	31	8.2	96	T.
G.M.	F	13 6/12	3.70	0.50	54	20.0	146	T. Irr.
V.A.	F	12 1/12	3.36	1.25 (↓)	37	19.0	184	H. T.
U.A.	F	13 8/12	3.70	1.25 (↓)	30	13.2	149	T.
M.M.	F	16 2/12		1.25 (↓)	38	14.0	171	T. Irr. H.
$\bar{X}$				1.14	41.44*	15.7**	149***	
SD				0.49	14.05	4.9	43	

*$p<0.001$ vs patients without symptoms and vs normal subjects; **$p<0.01$ vs patients without symptoms and $p<0.001$ vs normal subjects; ***NS vs patients without symptoms and vs normal subjects; (↓) blunted response; T: tachycardia; Irr: Nervousness, irritability; H: Headache.

In five different age groups older than six years, most TSH values were suppressed. In these groups, mean $\pm$ SD L-T_4 dose in subjects with inhibited TSH was calculated (Fig. 4). A TRH-TSH test under treatment was carried out in eight patients between 13 and 16 years of age receiving 2.5 - 3.7 µg/kg of L-T_4. All of them presented a blunted TRH curve.

CONCLUSION

rT_3 and T_4 are sensitive indicators of excessive L-T_4 treatment, but, while serum T_4 indirectly indicates the levels at which target cells are exposed to the hormone, rT_3 represents an intrinsic biological indicator of excessive stimulation acting through 5' or 5 deiodinases (3). The preferential metabolism of T_4 to rT_3 rather to T_3 at high T_4 levels was confirmed by the positive correlation between T_4 vs rT_3 and vs rT_3/T_3.

Furthermore, rT_3 showed better correlation with clinical findings than T_4, since patients without symptoms of overdose had T_4 higher than normals and rT_3 within the normal range.

Contrary to TSH response to TRH, which is equally blocked at small or large overdoses, rT_3 measurements permit quantitation of the excessive treatment since the homeostatic mechanism is active in this situation.

It was possible to establish the minimal mean TSH inhibitory dose in patients up to six years of age. These doses are slightly higher than those reported by Guyda for ages 1-5 years, but much higher before one year (4). In older patients, particularly in adolescents, usually recommended doses seem to be excessive, as shown by the blunted TSH response to TRH.

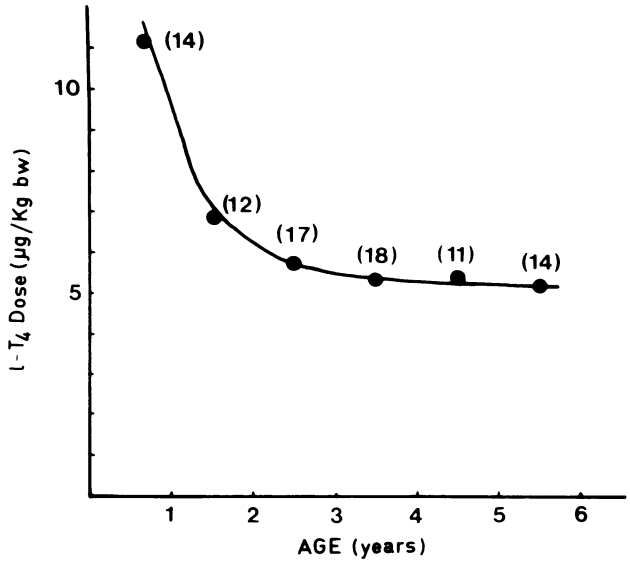

Fig. 3. Minimal mean inhibitory dose of L-T_4 under 6 years of age. TSH was increased above normal (0-5 µU/ml) in a way inversely proportional to dose. The regression line was calculated by the method of least squares and the dose corresponding to 5 µU/ml of TSH is plotted in the figure for every age group (number of subjects in parenthesis). Serum T_4 was within normal range in all patients.

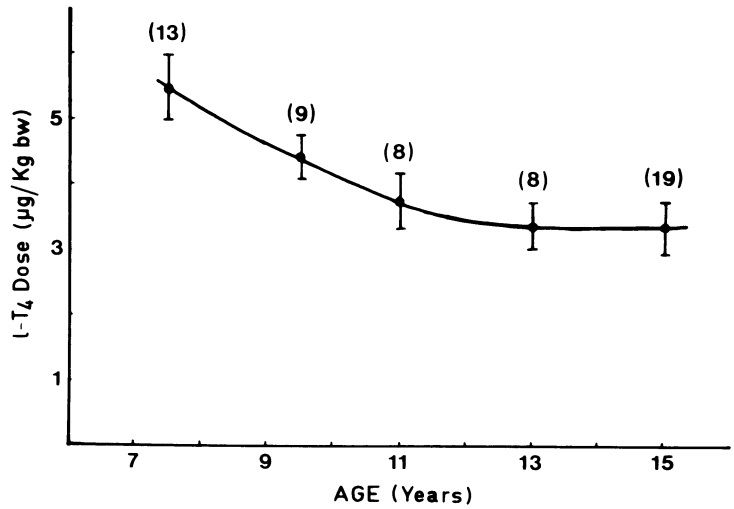

Fig. 4. Mean ± SD L-T_4 dose in 5 different age groups with inhibited TSH (number of patients in parenthesis). This information cannot differentiate physiologic from excessive L-T_4 inhibitory dose.

REFERENCES

1. Chopra IJ. J Clin Invest 54: 583, 1974.
2. Wartofsky L and Burman KD. Endocrine Rev 3: 164, 1982.
3. Visser TJ, Leonard JL, Kaplan MM, et al. Proc Natl Acad Sci USA 79: 5080, 1982.
4. Guyda HJ. In GN Burrow and JH Dussault (eds), Neonatal Thyroid Screening, Raven Press, New York, 1980, p 247.

NADPH WITH CYTOSOL STIMULATES DEIODINATION BY DETERGENT-SOLUBILIZED HEPATIC MICROSOMES: EVIDENCE FOR NADPH-DEPENDENT CYTOSOLIC NON-GLUTATHIONE REDUCTASE SYSTEM*

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INTRODUCTION

Based upon reconstitution experiments in which very low 5'-deiodinase (5'-DI) activity of isolated rat liver microsomes was restored to various degrees by the addition of cytosol, the existence of an endogenous cytosolic-stimulating cofactor has been previously demonstrated (1). From similar reconstitution experiments using starved rats, it has been proposed that NADPH and/or GSH were cofactors for 5'-DI, the augmenting action of NADPH, being attributed to the generation of GSH through glutathione reductase (2). However, supporting evidence indicating that glutathione, as well as NADPH are essential endogenous cofactors in mediating 5'-DI stimulation has been controversial (3,4). We previously observed an important role of NADPH in stimulating microsomal 5'-DI in the presence of cytosol (5). Furthermore, using a microsome preparation, we have recently demonstrated a new non-glutathione NADPH-dependent cytosolic reductase system, which operates in the presence of intermediate (fraction B) and high molecular weight (MW) components (fraction A), without very low M.W. components including glutathione (GSH) (5). On the other hand, our laboratory has recently achieved solubilization of 5'-DI by detergents (6) and partial purification of the enzyme by DEAE-Sephacel column chromatography. Accordingly, it was the purpose of the present investigation to examine the effect of NADH, NADPH, and GSH on the stimulation of 5'-DI in a reconstitution assay system utilizing a detergent solubilized 5'-DI preparation with cytosol or fractionated cytosolic components (i.e., fraction A and B).

MATERIALS AND METHODS

Cytosol and microsomes were prepared by standard differential centrifugation using young male Sprague-Dawley rats maintained on regular chow ad libitum as previously described (5-7). Solubilization of 5'-DI was achieved from hepatic microsomal preparation using 5 mM 3-[(3-chloramidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS), as previously described (6). Fraction A and fraction B were prepared as previously described (5). Fractionation with polyethylene glycol (PEG) was also performed similar to the technique used by others with cholate extracts of

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microsomes, being performed as recently described (6). Further chromatography of solubilized 5'-DI preparation on DEAE-Sephacel was performed by a method previously described for cytochrome P_{450} (8). A PEG fraction (5 ml containing 33.5 mg protein) of 5'-DI was applied to a column of DEAE-Sephacel (0.9 x 30 cm) which had been equilibrated with CHAPS and a 0.125 M phosphate buffer with 1 mM EDTA and glycerol with a continuous gradient of NaCl (0 - 0.3 M) in the same buffer. The DEAE-Sephacel column bound approximately 80% of added 5'-DI at pH 7.4, with a single major peak of 5'-DI activity being eluted with a continuous gradient of NaCl (0 - 0.4 M). Fractions which had 5'-DI activity were combined and stored at -70°C. The preparation contained 190 µg protein per ml.

5'-iodothyronine deiodinase assay was performed using outer ring (3' or 5') labeled ^{125}I reverse T_3 (^{125}I-rT_3) as substrate essentially as previously described (7). Briefly, in experiments on the reconstitution of soluble 5'-DI with cytosol, the reaction mixtures consisted of the deiodinase preparation (21 µg protein), cytosol (790 µg protein), ^{125}I-rT_3 (10^5 cpm, final concentration 0.21 nM), and either DTT, NADPH, or GSH, to which PB-EDTA buffer was added for a final assay volume of 300 µl. A similar system with slight modification was as follows: in reconstitution experiments of fraction A, fraction B, and NADPH, the reaction mixtures consisted of various combinations of soluble 5'-DI preparation, fraction A (142 µg protein), fraction B (23 µg protein), NADPH (final concentration 1 mM), ^{125}I-rT_3 (10^5 cpm), and PB-EDTA in a final volume of 350 µl. 5'-DI rate was expressed as % $^{125}I^-$ released per 12 or 15 minutes with correction made for counts at zero time for non-specific iodide counts in deiodinase-free preparations. To obtain the true $^{125}I^-$ release rates from our data, 1% = 3.0×10^{-3} pmoles rT_3 deiodinated.

RESULTS

The influence of various concentrations of NADPH, NADH, and GSH on the activity of detergent-solubilized 5'-DI in the presence of cytosol is summarized in Table 1. While NADPH alone showed no signifiant stimulatory effect on the activity of 5'-DI in the soluble fraction in the absence of cytosol (data not shown), the NADPH activity was greatly enhanced by the simultaneous addition of cytosol. On the other hand, the stimulation of 5'-DI by GSH was observed irrespective of cytosol.

Table 1. Effect of NADPH, NADH, or GSH on Activity of Solubilized (Unpurified) Rat Liver 5'-DI in the Presence of Cytosol

	Concentration (mM)				
	0.00	0.05	0.20	0.50	1.00
NADPH	1.3 (a)	2.8(b)	2.9 (c)	2.9 (d)	3.1 (e)
NADH	1.3 (f)	1.2 (g)	1.3 (h)	1.6 (i)	2.0 (j)
GSH	1.3 (k)	N.D.	N.D.	2.0 (l)	2.2 (m)

p = not significant for: a vs. f & k; f vs. g & h;
p = <0.01 for: f vs. j; b vs. m; k vs. l & m;
p = <0.001 for: a vs. b, c, d, & e; b vs. g & l;
N.D. = not done; Student's t test was used for statistics.

However, by comparison with the 5'-DI activity with 50 μM NAHPH, 500 μM NADH and 500 μM GSH had significantly less stimulatory effect ($p<0.001$). When pooled active DEAE-Sephacel column chromatographic fractions were substituted for solubilized 5'-DI preparation, the effect of 200 μM NADPH was greater ($p<0.001$) than 1000 μM GSH in the presence of cytosol.

Assays of 5'-DI activity were performed substituting fraction A and fraction B for cytosol. Similar results to our previous observations using washed microsomes were obtained (data not shown). In particular, 5'-DI activity was linear with the concentration of fraction B in the presence of fixed amounts of fraction A and NADPH (data not shown).

DISCUSSION

Using detergent-solubilized, as well as partially purified preparation of 5'-DI, we have obtained a marked stimulatory effect of NADPH in the presence of cytosol, which is similar to our previous observations using washed microsomes. Such observations have indicated that the effects of NADPH and cytosol in activating 5'-DI do not depend upon a maintenance of the integrity of the microsomal membrane. Furthermore, in the present study, using fraction A and B, we have again observed significant stimulation of soluble 5'-DI in the presence of NADPH which is similar to our previous observations using washed microsomes (5).

Previous studies have shown a marked stimulatory effect of NADPH on 5'-DI activity (9) while other investigators found little effect (2). Differences in the tissue level of endogenous NADPH, possibly due to changes in nutritional status of the animals, as well as uncontrolled metabolic breakdown or oxidation of NADPH during tissue processing, may account for the discrepancies between the results of various investigators, perhaps influenced by whether fresh or stored (9) hepatic tissues were utilized.

Although GSH can stimulate 5'-DI and has been postulated to be a major *in vivo* cofactor mediating these effects, other experimental work does not confirm this theory (4). Since it is known that 2 moles of GSH are produced from 1 mole of GSSG by reduction with 1 mole of NADPH through glutathione reductase, our present results do not support the possibility that the NADPH effect could be mediated by glutathione reductase alone. Furthermore, fractionation of the cytosolic components to exclude fractions containing GSH + GSSG has clearly demonstrated that stimulation by NADPH is not mediated exclusively through a glutathione reductase system. Thus, results of the present study suggest that a hitherto unreported reducing system involving the interaction between NADPH and a cytosolic component might be operating in the 5'-deiodination of rT_3. Our findings present the first demonstration of a 5'-DI activating system which contains neither GSH nor added exogenous thiol and provides another potent activating system that is not mediated by glutathione-glutathione reductase or NADPH alone. The physical and enzymatic characteristics of the cytosolic components of fractions A and B suggest the possibility of a system similar to the thioredoxin-thioredoxin reductase system which plays a role in ribonucleotide reductase systems and possibly other biological reductions (10). However, Goswami and Rosenberg recently reported that a rat cytosolic hepatic glutaredoxin-like protein could stimulate the action of GSH on 5'-DI but that thioredoxin did not (11). Thus, a compound similar to but slightly different from thioredoxin may account for the activity of our endogenous 5'-DI cofactor, and has been shown in this report to be active even in the presence of detergent-solubilized 5'-DI.

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REFERENCES

1. Visser TJ, van der Does-Tobe I, Docter R, et al. Biochem J 157: 479, 1976.
2. Balsam A and Ingbar SH. J Clin Invest 63: 1145, 1979.
3. Gavan LA, McMahon FA, and Moeller M. J Clin Invest 65: 943, 1980.
4. Sato K and Robbins J. In V Loos and L Wartofsky (eds), Peripheral Metabolism of Thyroxine, Thieme-Stratton, New York, 1984, p 30.
5. Sawada K, Hummel BCW, and Walfish PG. 67th Annual Meeting of the Endocrine Society, Baltimore, MD (Abstract #1110), 1985, p 278.
6. Hummel BCW and Walfish PG. Biochim Biophys Acta (in press).
7. Sawada K, Hummel BCW, and Walfish PG. Endocrinology 117: 1259, 1985.
8. West SB, Huang M-T, Miwa GT, et al. Arch Biochem Biophys 193: 42, 1979.
9. Sato T, Murayam S, and Nomura K. Endocrinol Japon 28: 451, 1981.
10. Holmgren A. In J Jeffery (ed), Dehydrogenases, Experientia, Supplementum, Vol. 36, Birkhausen, Verlag, Boston, pp 149-280.
11. Goswami A and Rosenberg IN. J Biol Chem 260: 6012, 1985.

THE IONIC MICROSCOPE ENABLES THE VISUALIZATION OF STABLE IODINE WITHIN FOLLICLES OF THE HUMAN THYROID GLAND

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The functional characterization of thyroid cells is an important factor in thyroid tumor typing (1). This characterization should be possible with the development of cytochemical (2) and in situ hybridization techniques (3). Analytical ion microscopy (4,5), which is based on secondary ion spectrometry, makes possible an imaging of the distribution of a given stable or labeled element on a tissue section (6,7). The purpose of this study was to visualize stable iodine within the human thyroid follicle.

PRINCIPLE OF ANALYTICAL ION MICROSCOPY

An ion beam (primary ions) is focused on a given area of the surface of a histological preparation. Submitted to this ion bombardment, the atoms of the most superficial atomic layers of the specimen are progressively sputtered; some of them are in ionized form. These secondary ions are characteristic of the atomic composition of the analyzed area of the sample. They are accelerated, collected by ion optics, and focused into a beam. This beam carries the image of every ion emitted from the specimen. This complex image is filtered with a mass spectrometer which enables the separation of ion species. Thus, it is possible, by adjusting magnetic and electrostatic fields, to observe successively the images of distribution of the different elements emitted from a given area of the sample. This analytical image is displayed on a fluorescent screen connected either to a TV monitor or a photographic plate. The minimum detectable concentration varies from 1 ppm to 1 ppb.

MATERIAL AND METHODS

Human pathological tissues with an iodine concentration below 100 µg per g of fresh tissue (colloid goiter, Graves' disease, cold nodules) were processed for electron microscopy. They were embedded in eposy resin and 3 µm thick sections were placed in a gold specimen holder. They were observed in a CAMECA IMS3F ionic microscope. The bombardment times of the section with a cesium primary beam ranged from 10 seconds to two minutes, according to the element selected. The size of the observation field was 200 µm.

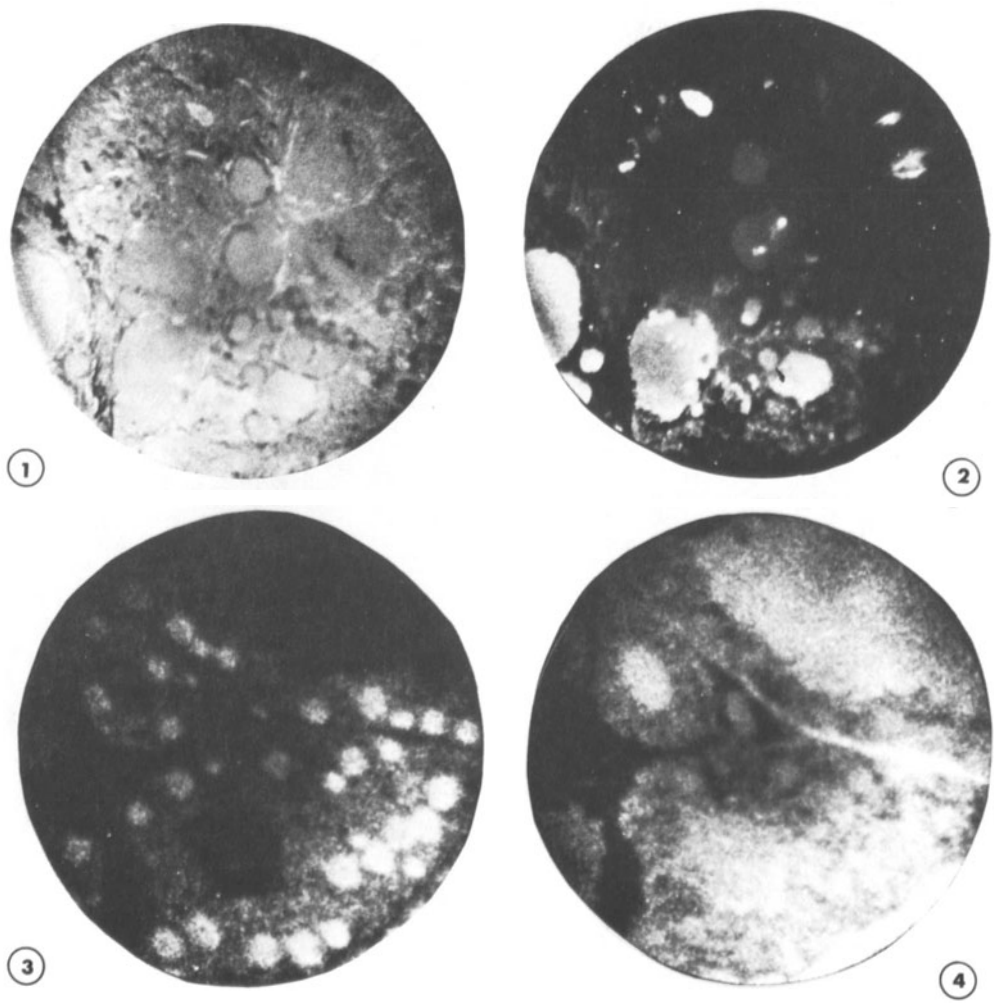
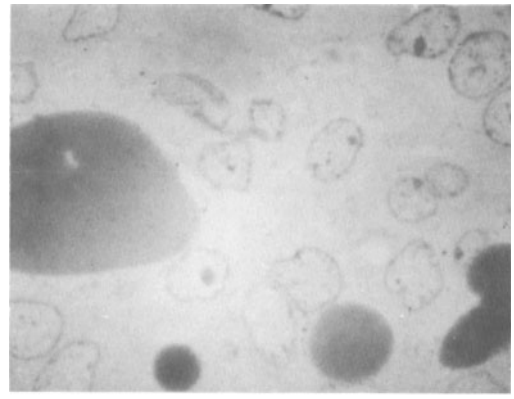


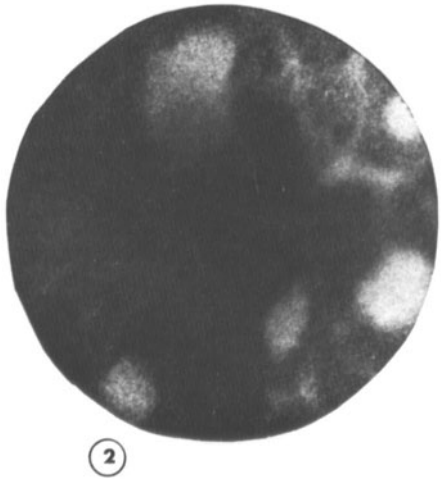
Plate 1. Thyroid cold nodule: comparative distributions of $^{32}S^-$ (Fig. 1) and $^{127}I^-$ (Fig. 2) in normal perinodular tissue; topographical distributions of $^{31}p^-$ (Fig. 3) and $^{32}S^-$ (Fig. 4) in nodular tissue.

RESULTS

The morphology of the secion can be recognized in $^{26}CN^-$ and $^{31}p^-$ images (Pl. 3, Fig. 1,3). The image of $^{26}CN^-$ showed the general topography of the tissue. $^{31}p^-$ localization was strongly associated with the nucleus of the epithelial cells surrounding the thyroid follicles.

In the normal perinodular tissue, $^{32}S^-$ of the thyroglobulin (Ig) was detected in all the follicles and its distribution density appears to be homogeneous (Pl. 1, Fig. 1). The corresponding distribution of the halogen was seen inside the follicles; in some, this element was plentiful, whereas in others it was absent (Pl. 1, Fig. 2). $^{32}S^-$ was still present in the "cold" nodular tissue (Pl. 1, Fig. 3,4), however, $^{127}I^-$ was not visualized. In a Hurthle cell tumor (Pl. 2, Fig. 1), $^{127}I^-$ was not visualized in tumor cells, but was present, as well as $^{32}S^-$, in follicular lumen (Pl. 2, Fig. 2,3).





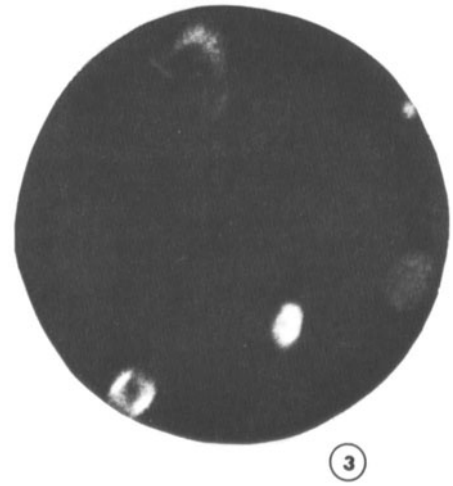


Plate 2. Hurthle cells thyroid tumor: histological aspect (Fig. 1) and comparative distributions of $^{32}S^-$ (Fig. 2) and $^{127}I^-$ in the same area (Fig. 3).

In colloid goiter, $^{127}I^-$ was always found within the follicular lumen associated with $^{32}S^-$ (Pl. 3, Fig. 2,4). However, some follicles rich in $^{32}S^-$ did not contain $^{127}I^-$.

In Graves' disease treated with MMI, a drug which leads to a poorly iodinated Tg, $^{127}I^-$ distribution was extremely heterogeneous (Pl. 4, Fig. 3,4). However, high amounts of $^{127}I^-$ were found in some small follicles which probably escaped the antithyroid drug (Pl. 4, Fig. 1,2).

DISCUSSION

The images of the distribution of stable iodine which we have observed with the ion microscope confirm the functional heterogeneity of the thyroid gland revealed by radioactive iodine and autoradiography techniques (8). In hypofixing thyroid nodules with iodine uptake defects (9), $^{127}I^-$ was not

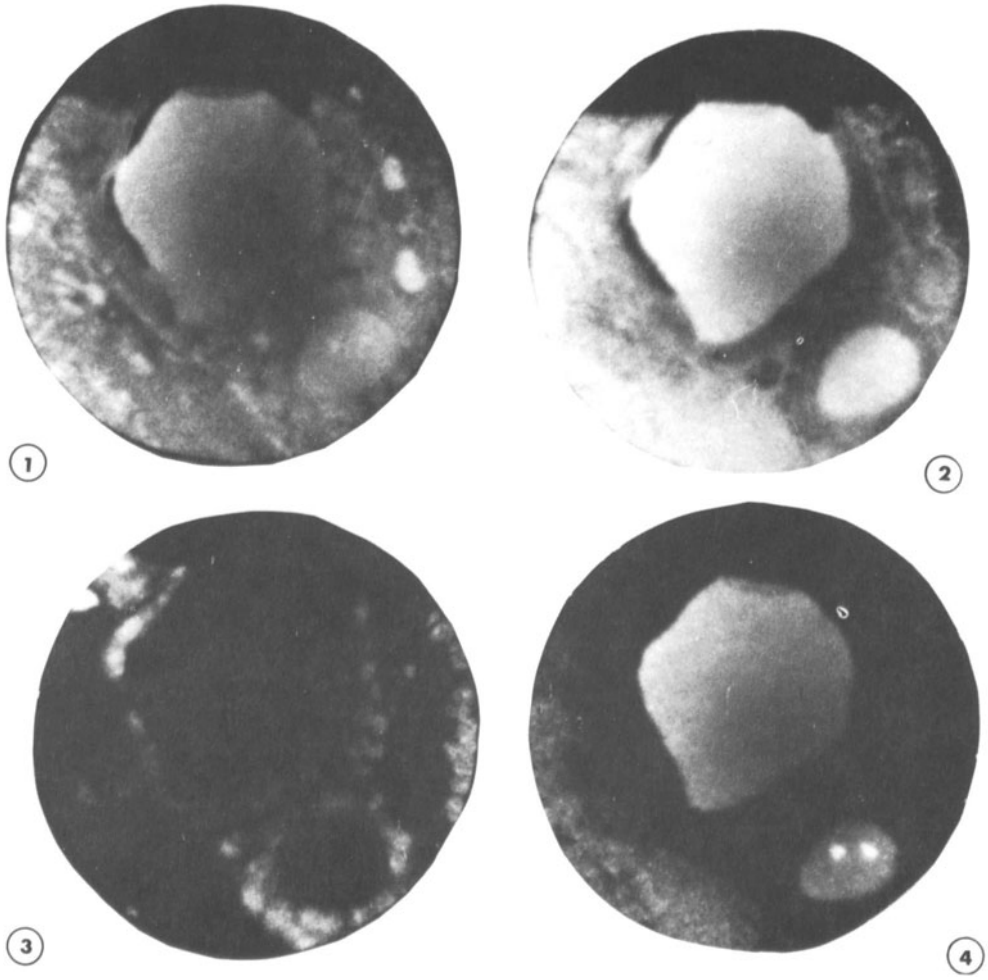


Plate 3. Colloid goiter: topographical distributions of $^{26}CN^-$ (Fig. 1), $^{32}S^-$ (Fig. 2), $^{31}P^-$ (Fig. 3), and $^{127}I^-$ (Fig. 4).

observed, whereas the $^{32}S^-$ of Tg was detected. Associated with detection of the iron contained in the thyroid peroxidase and with *in situ* hybridization methods for quantification of messenger RNA, the ionic microscope must give access to the quantitative and kinetic study of the different factors, among which iodine, taking part in the synthesis of thyroid hormones at the cellular level. This will be of interest in thyroid cancer typing, allowing the definition of new prognostic factors (10,11).

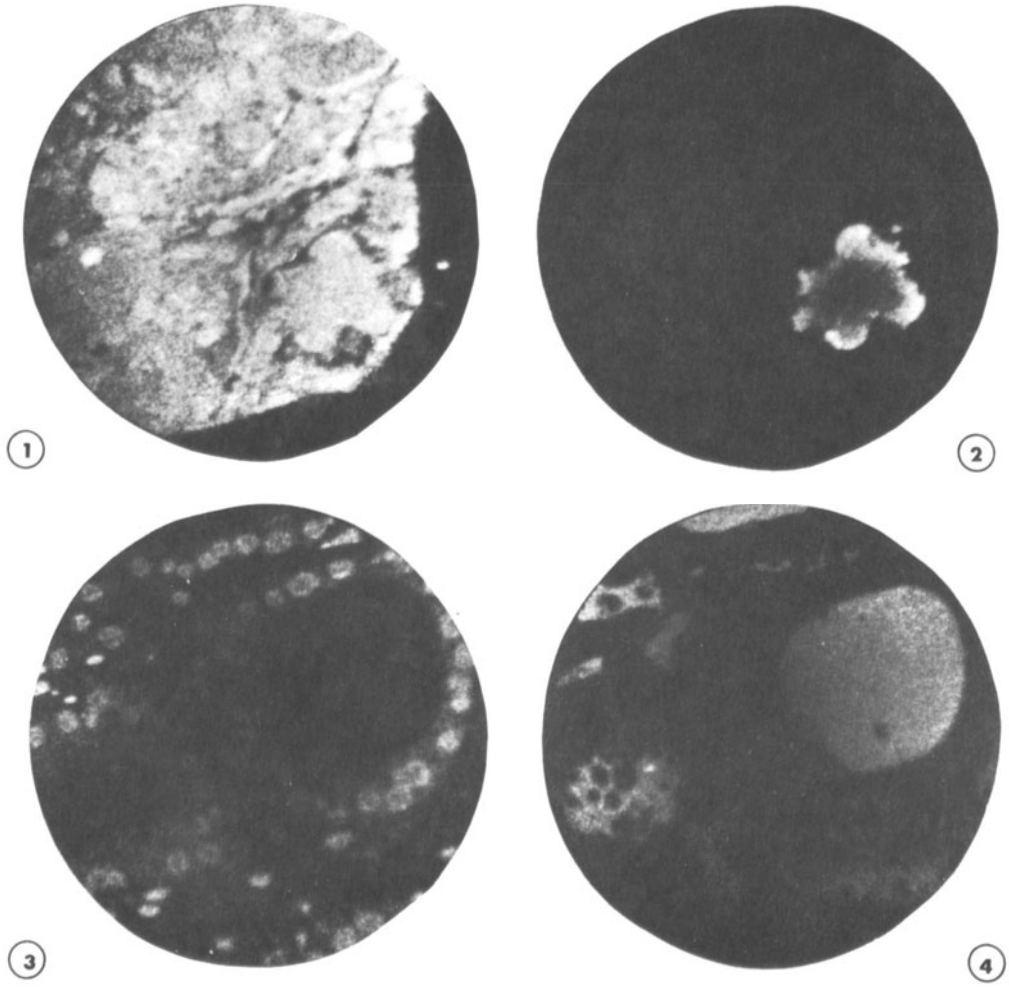


Plate 4. Graves' disease treated with MMI: comparative distributions of $^{32}S^-$ (Fig. 1), $^{127}I^-$ (Fig. 2,4), and $^{31}P^-$ (Fig. 3).

REFERENCES

1. Fragu P. In C Jaffiol (ed), Proceedings of the 1st European Meeting on Thyroid Cancer, Montpellier 1985, Except Med ICS, Amsterdam, 1985, in press.
2. Caillou B, Talbot M, Schlumberger M, et al. Ann Med Interne 135: 365, 1984.
3. Berge-Lefranc JL, Cartouzou G, Bignon C, et al. J Clin Endocr Metab 57: 470, 1983.
4. Castaing R and Slodzian G. J Microscopie 1: 395, 1982.
5. Galle P. J Nucl Med 23: 52, 1982.
6. Chandra S, Harris WC, and Morrison GH. J Histochem Cytochem 32: 1224, 1984.
7. Larras-Regard E and Siami K. CT Acad Sci, 1985 (in press).
8. Triantaphyllidis E and Verne J. Ann Endocr 24: 39, 1963.
9. Field JB, Larsen PR, Yamashita K, et al. J Clin Invest 52: 2404, 1973.
10. Tubiana M, Schlumberger M, Rougier P, et al. Cancer 55: 794, 1985.
11. Fragu P and Larras-Regard E. CR Acad Sci, 1985 (in press).

ENDOCYTOSIS OF ORGANIFIED IODINE IN THE ENDOSTYLE OF THE AMPHIOXUS*

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Parts of the protochordate endostyle are considered homologous to the vertebrate thyroid (1-3). In the adult amphioxus, the endostyle forms a groove in the floor of the pharynx lined by paired, longitudinal zones of epithelial cells. In the larval amphioxus, the endostyle is an unpaired structure in the right buccal wall (4). In the adult (5-7), as well as larval endostyle (8,9), iodination takes place in the lumen at the apical surface of a well-defined cell zone in the lateral portion of the endostyle. Iodination is catalyzed by a peroxidase, present in the apical plasma membrane (9). A thyroglobulin-like protein and iodothyronines have been found in the amphioxus (10,11). Thus, the mechanism of iodination shares many features with that in thyroid follicle cells (12).

Another important function of the thyroid follicle cell is the uptake of hormone-containing thyroglobulin from the follicle lumen by endocytosis and, after intracellular proteolysis, the release of thyroid hormones (13). We have made preliminary observations (7,8) suggesting a considerable endocytotic uptake of iodinated products also in the endostyle and have in this study examined this uptake in more detail.

Adult and larval amphioxus (Branchiostoma lanceolatum) were incubated for 10 min in sea water containg $^{125}I^-$ (8 mCi/40 ml). Adult animals were then chase incubated for 30, 60, 120, and 180 min and larvae for 40 and 80 min in sea water without label. At least two animals were examined at each time interval. The animals were fixed and embedded, and sections prepared for light and electron microscopic autoradiography as described elsewhere (7,9).

The endostyle of the adult amphioxus consists of different cell zones of narrow, tall, ciliated cells (Fig. 1). The thyroid-like properties are confined to the lateral portion (zones 5a, 5b, 6). We have recently, using electron microscopic autoradiography after incubation for 5 min in $^{125}I^-$, localized the primary site of iodination to the apical surface of zone 5a (7). This cell zone, not previously recognized, differs ultrastructurally from other zones, including zone 5b (zone 5 in previous studies) and zone 6 (Figs. 2,3).

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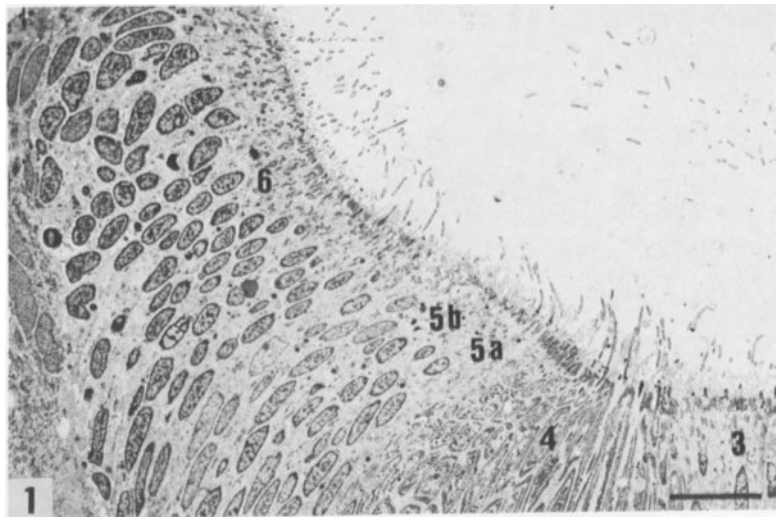

Fig. 1. Survey electron micrograph of the lateral (zones 3-6) portion of one half of the endostyle in an adult amphioxus. Bar - 10 μm.

After incubation for 10 min in $^{125}I^-$, autoradiographic grains were, in accordance with previous findings, concentrated at the apical surface of zone 5a. This accumulation of grains, often extending over the medial portion of zone 5b (Fig. 2), decreased progressively and had disappeared after 180 min. After 10 min, some grains were also associated with cilia and microvilli of zones 5b and 6. The concentration of grains in this location was considerably increased after 30 and 60 min, and at these time intervals, grains also appeared over the supranuclear cytoplasm of zones 5a, 5b, and 6 (Fig. 2). After 120 and 180 min, grains were distributed over all parts of the cytoplasm (Fig. 3), including the infranuclear region (Fig. 4). The overall picture was that of a gradual translocation of label

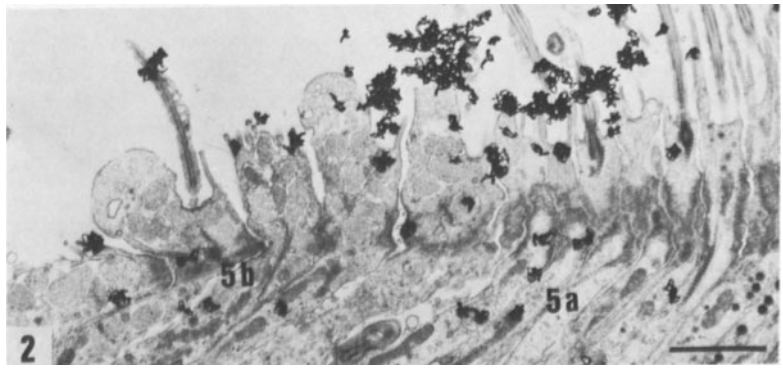

Fig. 2. Electron microscopic autoradiograph of an adult endostyle. Chase for 60 min. Grains are concentrated in the lumen at zone 5a and medial portion of zone 5b, characterized by large granules. Grains are also present over the supranuclear cytoplasm. Bar = 2 μm.

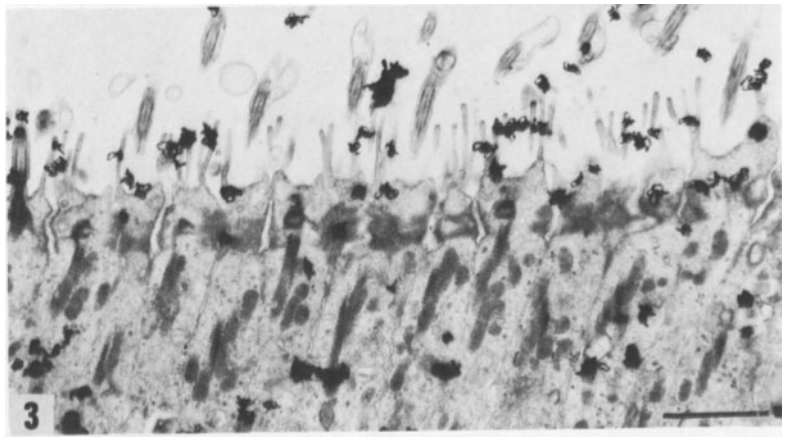

Fig. 3. Adult endostyle. Apical portion of zone 6. Chase for 180 min. Grains are present in the lumen, associated with cilia and microvilli, as well as over the apical cytoplasm. Bar = 2 µm.

from the surface of zone 5a laterally along the surface of zones 5b and 6, and then into the cytoplasm towards the base of the cells (Fig. 6).

The cytoplasmic grains were associated with vesicles, vacuoles, and lysosomes (Fig. 4), suggesting uptake by endocytosis and processing of iodinated products in the lysosomal apparatus. No significant uptake of label occurred in zones 1-4. Grains were present together with food particles in the gut lumen and were also located over vacuoles in the intestinal cells.

The same zones as in the adult (except zone 1) are present in the larval endostyle (4,9). After 10 min, the grains were almost exclusively located extracellularly (96%) at the apical surface of zones 5a, 5b, and 6. The highest concentration of grains was found at the surface of zone 5a. Grains were still mainly extracellular after chase incubation for 40 min. However, after 80 min, the majority of grains (58%) was located over the cytoplasm of zones 5a, 5b, and 5 (Fig. 5). Grains were frequently associated with vesicles and lysosomes.

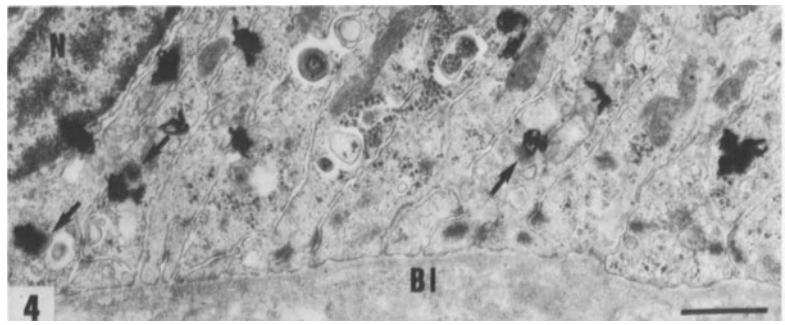

Fig. 4. Adult endostyle. Basal portion of zone 6. Chase for 180 min. Grains are associated with lysosome-like structures (arrow). N = nucleus; Bl = basal lumina. Bar = 1 µm.

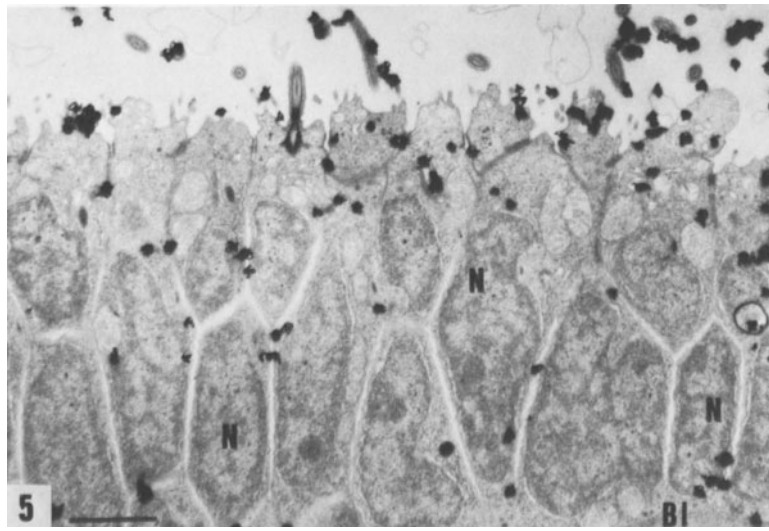

Fig. 5. Endostyle of larval amphioxus. Zone 6. Chase for 80 min. Grains are present in the lumen associated with cilia and microvilli, as well as over the cytoplasm. Nuclei, some of which are indicated (N), are essentially unlabeled. Bl = basal lamina. Bar = 2 μm.

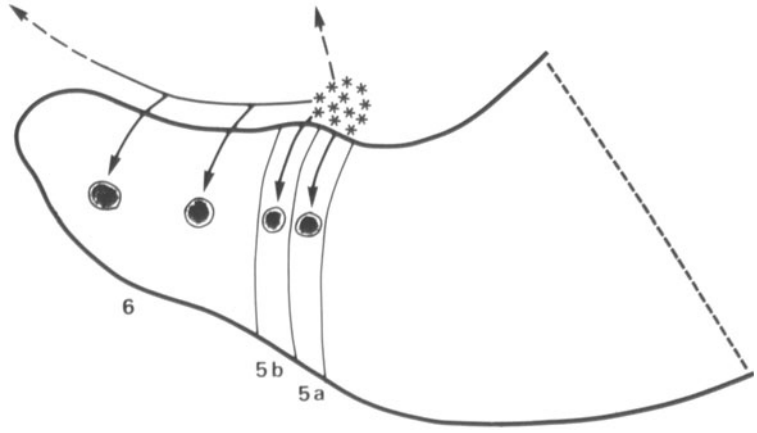

Fig. 6. Schematic presentation of the "protothyroid region" (zones 5a, 5b, 6) in one half of the endostyle in the adult amphioxus. The autoradiographic observations indicate that ^{125}I is organified in the lumen at the surface of zone 5a (*). Label is then translocated laterally and into the cells as indicated by the arrows. Within the cytoplasm, label is present in vesicles, vacuoles, and lysosomes. It is also possible that iodinated products are released directly into the lumen (--->), maybe to be digested in the gut.

Together our observations show that not only iodination, but also uptake by endocytosis of iodinated products from the lumen, occur in the lateral portion of the endostyle of the adult and larval amphioxus. Iodination is mainly confined to zone 5a, while uptake also occurs in zone 5b and in the large zone 6. These three cell zones might constitute a "protothyroid" region of the endostyle (Fig. 6).

A thyroglobulin-like protein (10), as well as iodothyronines (11), have been found in the amphioxus. Methimazole abolishes the autoradiographic reaction, indicating that the grains represent enzymatically-bound iodine (7, 9). We do not know to what extent the grains represent iodine bound to a protein or to what extent formation of iodothyronines by coupling of iodotyrosyles occurs in the endostyle. The coupling reaction appears to require a peroxidase (13). Histochemical (14) and cytochemical (9) observations show that peroxidase is present in the protothyroid region of the adult and larval endostyle, and that the enzyme activity is distributed within the cytoplasm, as well as to the apical plasma membrane in the same way as in thyroid follicle cells (9). Our present observations show that enzymatically-bound iodine remains attached to the peroxidase-containing plasma membrane for a time period which might be sufficient for coupling to take place. Hypothetically, the hormone-containing protein is then internalized by endocytosis and iodothyronines released after lysosomal digestion. However, observations in this and previous (5,6) studies clearly show that iodine, perhaps organified in the endostyle, is transferred into the gut and even into the intestinal epithelial cells. Provided this iodine includes iodine in iodothyronines, an alternative mechanism for thyroid hormone secretion might be digestion of the prohormone protein in the gut.

Two important functions of the thyroid follicle cell are extracellular iodination (12) and uptake by endocytosis of organified iodine (13). The present study shows that these functions are present also in the endostyle of the amphioxus.

REFERENCES

1. Barrington EJW. An Introduction to General and Comparative Endocrinology, Clarendon Press, 1963.
2. Olsson R. Acta Zool 44: 299, 1963.
3. Olsson R. Gen Comp Endocrinol Suppl 2: 485, 1969.
4. Olsson R. Zoomorphol 103: 1, 1983.
5. Thomas IM. J Mar Biol Ass UK 35: 203, 1956.
6. Barrington EJW. J Mar Biol Ass UK 37: 117, 1958.
7. Ericson LE, Fredriksson G, and Ofverholm T. Cell Tiss Res 241: 267, 1985.
8. Fredriksson G, Ericson LE, and Olsson R. Gen Comp Endocrinol 56: 117, 1984.
9. Fredriksson G, Ofverholm T, and Ericson LE. Cell Tiss Res 241: 257, 1985.
10. Monaco F, Dominici R, Andreoli M, et al. Comp Biochem Physiol B 70: 341, 1981.
11. Salvatore G. Gen Comp Endocrinol Suppl 2: 535, 1969.
12. Ekholm R. Mol Cell Endocrinol 24: 141, 1981.
13. Ericson LE. Mol Cell Endocrinol 22: 1, 1981.
14. Tsuneki K, Kobayashi H, and Ouji M. Gen Comp Endocrinol 50: 188, 1983.

HUMAN HEPATOMA (HEP G2) CELLS SECRETE A NOVEL T_4-BINDING PROTEIN (27 K PROTEIN)*

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INTRODUCTION

TBG, prealbumin, and albumin represent the major thyroid hormone transport proteins in the blood (1), but other proteins interact with thyroid hormones, although their physiological role is still unclear (2). We have recently reported purification of a novel T_4-binding protein from human serum, called 27 K protein for its apparent molecular weight in SDS-PAGE (3). In the present paper we show that 27 K protein is synthesized and secreted by human hepatoma (Hep G2) cells, and partially characterize it both in whole-cell and cell-free systems.

MATERIALS AND METHODS

All experimental procedures have been already described in a previous report showing TBG biosynthesis by Hep G2 cells (4).

Confluent Hep G2 cells, after preincubation overnight in serum-free methionine-free or serum-free low glucose (10 mg/dl) medium, were labeled for 4 hours with 100 μCi/ml [^{35}S]methionine (600 Ci/mmol, Amersham) or 200 μCi/ml [^{3}H]mannose (1-5 Ci/mmol, Amersham), respectively. In pulse-chase experiments, cells were pulse-labeled for 10 min with [^{35}S]methionine and then chased for 2 hours after addition of a 20,000-fold excess of unlabeled methionine.

Samples of media and cell lysates were immunoprecipitated by anti-27 K serum and protein A, and analyzed by 12.5% SDS-PAGE and subsequent fluorography. Alternatively, gels were stained, sliced into 1 mm sections, solubilized overnight at 55°C in 0.7 ml H_2O_2, and the radioactivity was measured in 10 ml of Ultrafluor in a beta counter.

RNA was extracted from Hep G2 cells by the LiCl-urea procedure and fractionated by discontinuous sucrose gradient centrifugation. Total RNA

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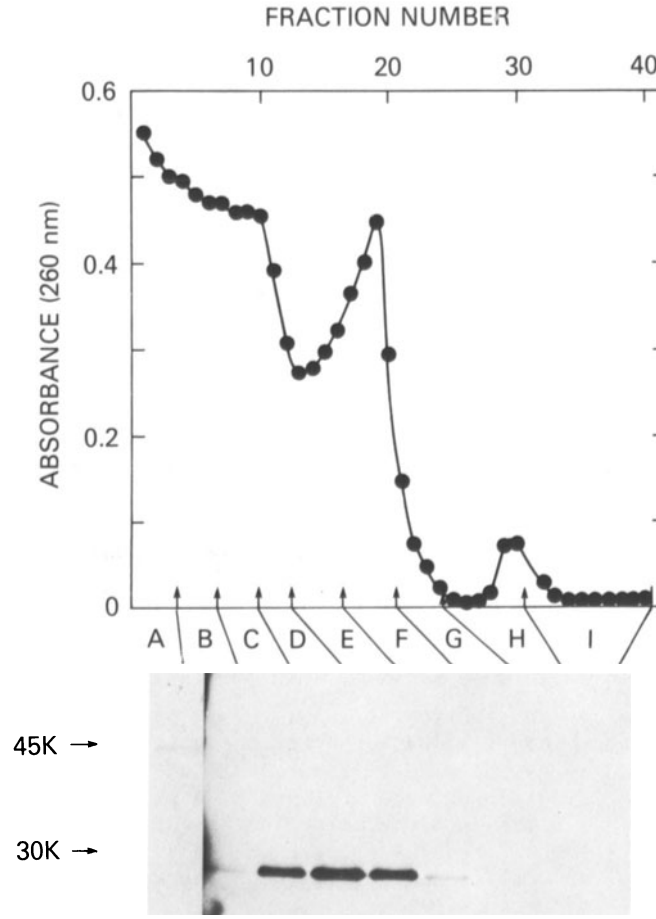

Fig. 1. Translation of Hep G2 RNA fractions by a rabbit reticulocyte lysate. Upper panel: RNA distribution after centrifugation at 35,000 rpm (18 h, 4°C) in a 0-30% discontinuous sucrose gradient (10 mM Na acetate, pH 5.2, 1 mM EDTA, 0.1% SDS). Lower panel: translation products using fractions taken from the bottom (A) to the top (I) of the tube. [^{35}S]methionine-labeled bands were immunoprecipitated by anti-27 K serum (also containing anti-TBG) and analyzed by SDS-PAGE and fluorography.

and sucrose gradient fractions were translated by a rabbit reticulocyte lysate using [^{35}S]methionine as a precursor. In other experiments, Hep G2 RNA was injected into Xenopus laevis oocytes, as previously described (4). Medium from oocytes was incubated with [^{125}I]T_4 in the presence or absence of a 1,000-fold excess of unlabeled T_4 and immunoprecipitated by anti-27 K serum.

RESULTS

Demonstration that 27 K protein is synthesized by Hep G2 cells was obtained by immunoprecipitation with anti-27 K serum both in whole-cell and

cell-free systems followed by SDS-PAGE. The molecular weight of 27 K protein was identical in both systems, suggesting that 27 K protein does not contain carbohydrates. In the same experiments, the molecular weight of TBG (precipitated by antibodies to TBG) shifted from 54 kDa in whole cells to 45 kDa in the rabbit reticulocyte lysate in agreement with the glycoprotein nature of TBG.

To examine whether TBG and 27K protein are related or different proteins, Hep G2 RNA was fractionated by sucrose gradient centrifugation, the fractions were translated by a rabbit reticulocyte lysate, and the translation products were immunoprecipitated. Figure 1 shows that TBG mRNA was found at the bottom of the tube, whereas 27 K protein mRNA was distributed in lighter fractions.

T_4-binding activity of 27 K protein was evaluated by $[^{125}I]T_4$ precipitation in the medium of oocytes injected with Hep G2 RNA. Figure 2 shows that $[^{125}I]T_4$ bound to 27 K protein was precipitated by anti-27 K serum that had been preabsorbed with pure TBG. This precipitation appears to be specific, since it was blocked by an excess of unlabeled T_4. No $[^{125}I]T_4$ was precipitated in the medium of oocytes injected with ovalbumin RNA or not injected with RNA.

The nonglycoprotein nature of 27 K protein was further supported by the finding that no $[^{3}H]$mannose was incorporated into the newly synthesized protein and no difference in the size of the immunoprecipitated protein was observed after treatment with the glycosylation inhibitor, tunicamycin (data not shown).

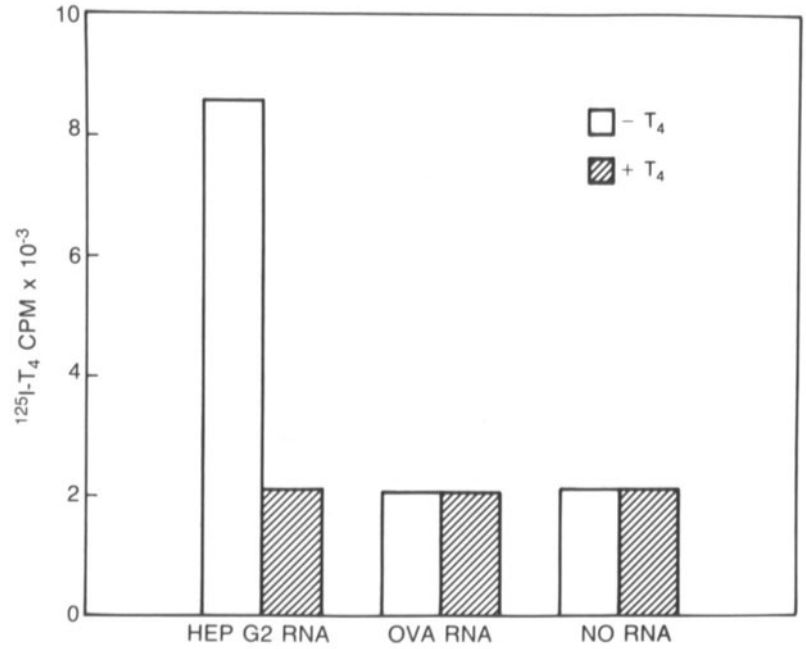

Fig. 2. $[^{125}I]T_4$ precipitation with anti-27 K serum (preabsorbed with pure TBG) in the medium from Xenopus laevis oocytes injected with Hep G2 RNA. Precipitation was in the absence (open bar) or presence (shaded bar) of a 1,000-fold excess of unlabeled T_4. $[^{125}I]T_4$ precipitation in the medium from oocytes injected with ovalbumin (OVA) RNA or not injected with RNA (No RNA) are also shown.

Table 1. Kinetics of TBG and 27 K Protein Secretion by Hep G2 Cells*

	Chase time (min)					
	10	20	30	40	60	120
^{35}S-labeled protein in medium (% of total intracellular + secreted)						
TBG	3	6	32	47	62	89
27 K protein	25	52	61	70	81	92

*Cells were pulse labeled with [^{35}S]methionine for 10 min and chased with excess methionine.

Table 1 illustrates the kinetics of secretion of TBG and 27 K protein from Hep G2 cells. 27 K protein was released earlier than TBG, 50% being secreted after only 19 min.

DISCUSSION

27 K protein is often copurified with TBG from human serum (3). One possible explanation for this finding might be that 27 K protein represents a fragment of TBG. This does not appear to be the case, since the RNA fractionation experiments indicate that two distinct mRNAs direct the synthesis of TBG and 27 K protein.

The latter protein appears to be nonglycosylated and is probably not synthesized as a larger molecular weight precursor, since whole-cell and cell-free system products had the same molecular weight. The nonglycoprotein nature of 27 K protein is confirmed by the lack of mannose incorporation and by the observation that tunicamycin caused no change in its molecular weight.

Finally, 27 K protein is not an inert contaminant of TBG preparations. As shown by the [^{125}I]T_4 precipitation experiments using the medium from Xenopus laevis oocytes that had been injected with Hep G2 RNA, the protein is able to bind thyroxine.

In conclusion, this study provides evidence that the liver synthesizes this novel T_4-binding protein, readily distinguishable from TBG. Its physiological role in thyroid hormone transport remains to be clarified, but Hep G2 cells represent a useful tool to further elucidate the mechanism regulating the synthesis and secretion of 27 K protein.

REFERENCES

1. Robbins J and Bartalena L. In G Hennemann (ed), Thyroid Hormone Metabolism, Marcel Dekker, 1985, in press.
2. Robbins J, Cheng S-Y, Gershengorn MC, et al. Recent Prog Horm Res 34: 477, 1978.
3. Grimaldi S, Bartalena L, and Robbins J. Ann Endocrinol (Paris) 44: Abstr. 113, 1983.
4. Bartalena L, Tata JR, and Robbins J. J Biol Chem 259: 13605, 1984.

PARTIAL PURIFICATION OF RAT SERUM THYROXINE-BINDING GLOBULIN

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INTRODUCTION

Rats have been frequently used experimentally in various aspects of thyroid research, but their serum-binding proteins for thyroid hormones (TH) remain unclear compared with those of human beings. Previous studies showed that in rats the major serum TH-binding protein was electrophoretically slow migrating prealbumin (R-TBPA) (1). Davis et al. (2) reported the presence of rat serum thyroxine-binding globulin (R-TBG) using polyacrylamide gel slab electrophoresis, but other studies showed little or no R-TBG (3,4). In special conditions such as on feeding rats a low protein, high carbohydrate diet (4) or on fasting (5), the rats were reported to gain clear R-TBG band. Thus, R-TBG has not yet been purified, and its binding characteristics have not been determined. We report that R-TBG was partially purified using Sephadex G-200 gel filtration from hypothyroid rat serum and its binding characteristics determined by charcoal-binding method.

MATERIALS AND METHODS

Hypothyroid rat serum was obtained by puncturing the abdominal aorta 10 days after thyroidectomy. ^{125}I-T_4 with an initial specific activity of 1200 μCi/μg was purchased from New England Nuclear Corp., Boston, MA.

Sephadex G-200 Gel Filtration

Descending gel filtrations on 2.6 x 100 cm Sephadex G-200 column were performed at 4°C. The column was previously equilibrated and eluted with 0.14 M NaCl-0.01 M phosphate buffer, pH 7.4. 1.5 ml of rat serum was diluted to 5.0 ml with the same buffer, incubated with 0.16 μCi of ^{125}I-T_4 for 30 min at 25°C, and then applied on the column. A constant flow rate of 15 ml/hour was maintained, and consecutive 5.0 ml of effluent fractions were collected. Radioactivity and asorbance at 280 nm were monitored in each fraction.

Charcoal-binding Method

Rat serum T_4-binding proteins partially purified by gel filtration were diluted adequately with buffer (0.05 M Tris-0.01 M EDTA, pH 7.4). ^{125}I-T_4 in 50 µl of 0.01 N NaOH and graded doses of T_4 or its analogues in 50 µl of 0.01 N NaOH were added to tubes containing 250 µl of the diluted rat serum T_4-binding proteins. After incubation overnight at 4°C, 100 µl of a suspension of dextran-coated charcoal (250 mg/dl Norit A and 250 mg/dl Dextran T 70 in the Tris buffer) was added. The tubes were agitated on a Vortex mixer and allowed to stand for 10 min at 4°C. They were then centrifuged at 3000 rpm for 10 min. Counting rates of both supernatant and charcoal pellet were determined.

RESULTS

Gel Filtration

When normal rat serum labeled with ^{125}I-T_4 was fractionated on Sephadex G-200 column, the peak of radioactivity was eluted shortly after the albumin peak (Peak II) (Fig. 1; upper panel). But when hypothyroid rat serum labeled with ^{125}I-T_4 was eluted on the same column, the main peak emerged several tubes before the albumin peak (Peak I) with the small Peak II (Fig. 1; lower panel). This Peak I was also demonstrated from normal

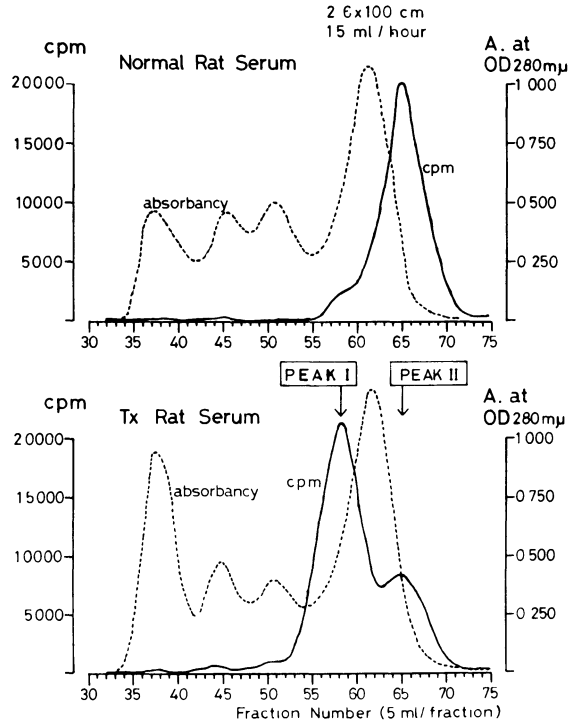

Fig. 1. Sephadex G-200 gel filtration of rat serum labeled with ^{125}I-T_4. Upper panel: normal rat serum. Lower panel: hypothyroid rat serum obtained 10 days after thyroidectomy.

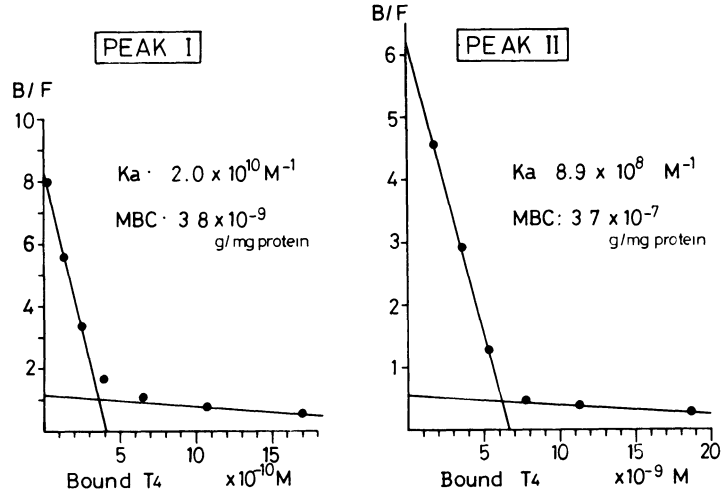

Fig. 2. Scatchard plot of T_4 bound to Peak I (left) and Peak II (right). Peak I and Peak II were obtained by Sephadex G-200 gel filtration of hypothyroid rat serum.

rat serum which was previously deprived of endogenous T_4 by charcoal adsorption. Charcoal adsorption of normal rat serum labeled with ^{125}I-T_4 (80 mg/ml Norit A) for 24 hours at 4°C removed about 94% of radioactivity. Estimation of molecular weight of these two peaks, using α-chymotripsinogen, ovalbumin, and lactic dehydrogenase as molecular markers, revealed that MW of the Peak I was 10 x 10^4 daltons and that of the Peak II was 5.6 x 10^4 daltons.

Binding Characteristics

A Scatchard plot analysis showed that both Peak I and Peak II had high affinity-small capacity binding sites for T_4 (Fig. 2). For Peak I, the affinity constant (Ka) and maximal binding capacity (MBC) values were 2.0 x 10^{10} M^{-1} and 3.8 x 10^{-9} g/mg protein, respectively. As for Peak II, the Ka and MBC were 8.9 x 10^8 M^{-1} and 3.7 x 10^{-7} g/mg protein, respectively. The relative affinities of T_4 analogues for these binders were determined by comparing the molar concentrations of L-T_4 with those of T_4 analogues required to depress by 50% B/Bo ratio of tracer ^{125}I-T_4. Relative affinities of various T_4 analogues for Peak I and Peak II are shown in Table 1, where the affinity of L-T_4 was assigned a value of 100. Thus, the Peak I had a rigid specificity to alanine side chain of T_4 molecule, but the Peak II showed no specificity to this side chain.

DISCUSSION

A previous study on normal rat serum TH-binding proteins by Sutherland et al. (2) using Sephadex G-200 gel filtration had demonstrated only one high affinity T_4-binding protein. They reported that this T_4-binding protein eluted shortly after the albumin peak, had a Ka of 3.5 x 10^8 M^{-1}, and was electrophoretically R-TBPA. Our Peak II is considered to be the same as the T_4-binding protein reported by them. Furthermore, our data indicates that relative affinities of the Peak II for various T_4 analogues showed no specificity to the alanine side chain of the T_4 molecule. This characteristic resembles that of human TBPA (6).

Table 1. Relative Affinities of T_4 Analogues for Peak I and Peak II

	Peak I	Peak II
L-T_4	100	100
D-T_4	34.9	9.2
L-T_3	11.1	2.1
D-T_3	1.8	1.0
rT_3	6.8	14.3
Tetrac	0.25	69
Triac	0.1	26.3

In the present study, we reported for the first time that another T_4-binding protein (Peak I) was separated from hypothyroid rat serum. This Peak I had a Ka of 2.0×10^{10} M^{-1} for T_4 and rigid specificity to alanine side chain of T_4 molecule. These binding characteristics of the Peak I resemble those of human TBG (7,8). Thus, Peak I is considered to be rat TBG, though we have no data on electrophoretic mobility of Peak I. From the fact that this Peak I was also demonstrated from normal rat serum pretreated with charcoal adsorption, R-TBG in normal rat serum is supposed to be masked due to saturation with endogenous T_4. The possibility that R-TBG might be increased by hypothyroidism cannot be denied.

REFERENCES

1. Sutherland RL and Brandon MR. Endocrinology 98: 91, 1976.
2. Davis PJ, Spaulding SW, and Gregerman RI. Endocrinology 87: 978, 1970.
3. Refetoff S, Robin NI, and Fang S. Endocrinology 86: 793, 1970.
4. Young RA, Braverman LE, and Rajatanavin R. Endocrinology 110: 1607, 1982.
5. Young RA, Rajatanavin R, Moring AF, et al. Endocrinology 116: 1248, 1985.
6. Andrea TA, Cavalieri RR, Goldfine ID, et al. Biochemistry 19: 55, 1980.
7. Korcek L and Tabachnick M. J Biol Chem 251: 3558, 1976.
8. Snyder SM, Cavalieri RR, Goldfine ID, et al. J Biol Chem 251: 6489, 1976.

THYROID FUNCTION TESTS IN SUBJECTS WITH A GENETIC ISOELECTRIC FOCUSING VARIANT TBG*

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ABSTRACT

A variant thyroxine-binding globulin (TBG) with slower migration on isoelectric focusing (TBG-S) has been found in some American Blacks. It is inherited as an X-chromosome-linked trait. Hemizygous males with TBG-S exhibit TBG bands shifted cathodally. Heterozygous females with TBG-CS, show the summation pattern of TBG bands corresponding to sera from subjects with only TBG-S and those with only the common type of TBG (TBG-C). Because the effect of TBG-S on thyroid function has not been studied in detail, we compared data of thyroid function tests in sex and age matched subjects belonging to four TBG genotypes (10 males each with TBG-S vs. TBG-C, and 10 females each with TBG-CS vs. TBG-CC). The mean serum T_4 level of the group of males with TBG-S was 6.79 $\pm$ 1.52 (SD) µg/dl, significantly lower than that of the group of males with TBG-C (8.49 $\pm$ 1.29 µg/dl, $p<0.02$). A significant concomitant reduction in the mean serum TBG concentration was found in the group of males with TBG-S as compared to that with TBG-C (1.41 $\pm$ 0.30 mg/dl vs. 1.72 $\pm$ 0.29 mg/dl, $p<0.05$). Mean values of serum free T_4 concentrations and other tests of thyroid function, including TSH levels, were not significantly different among the four groups matched by age and paired by sex. Although the reason for the reduced serum TBG concentration in subjects with TBG-S remains unclear the associated reduction of serum T_4 levels is not accompanied by hypothyroidism.

INTRODUCTION

T_4-binding globulin (TBG) is the principal T_4-binding protein in serum. It is an acidic glycoprotein which exhibits three major bands on isoelectric focusing (IEF) with pI range of 4.3 to 4.5. A cathodal shift of these bands was found in some American Blacks (1,2), Polynesians, Melanesians, and Micronesians (3). This variant TBG, termed TBG-S for its slower mobility on IEF, is inherited as an X-chromosomal-linked trait. Although previous papers mention that subjects possessing the variant TBG-S appear to be euthyroid, their tests of thyroid function have not been studied in detail. Since our preliminary study showed lower concentrations of serum total T_4 in males with the

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Table 1. TBG Types and Gene Frequencies in American Blacks

	Number of Subjects				
Genotype	C	S	CC	CS	SS
Males					
Observed	62	8	--	--	--
Expected*	61.6	8.4	--	--	--
Females					
Observed	--	--	34	9	1
Expected*	--	--	34.1	9.3	0.6

*Calculated from the Hardy-Weinberg equilibrium assuming X-linked alleles. Males were assigned a single allele of TBG-C or TBG-S.

TBG-S variant, we carried out a systematic evaluation of their thyroid function tests. Results were compared to those of age and sex matched subjects with the common type of TBG (TBG-C).

MATERIALS AND METHODS

Serum samples from 114 unrelated American Blacks (70 men and 44 women) living in the Chicago area were screened by IEF. According to the IEF patterns of their TBG and sex, the subjects were divided into five groups: 62 men with TBG-C, 8 men with TBG-S, 34 women with TBG-CC, 9 women with TBG-CS and one woman with TBG-SS as summarized in Table 1. The gene frequencies of TBG-C and TBG-S were 0.880 and 0.120, respectively.

Serum from two more men with TBG-S and two women with TBG-CS were added to the study. One subject with TBG-CS found to have positive thyroidal autoantibodies and a serum TSH level of 52.5 μU/ml was excluded from the study, bringing the total number of subjects in each of the two groups with the variant TBG (TBG-S and TBG-CS) to 10. These two groups were each age matched with 10 subjects randomly selected from the larger number of samples with TBG-C (men) and TBG-CC (women).

IEF was performed on prefocused, horizontal polyacrylamide slab gels. Gel concentration was 5% total acrylamide (0.125% bisacrylamide) with 8% glycerol and 6.7% ampholines of pH 4.2 - 4.9 (Pharmacia Fine Chemicals, Piscataway, NJ). 5 μl serum samples were applied following incubation for 1 h at room temperature with 5000 cpm [^{125}I]-T_4 (New England Nuclear, Boston, MA), sp. act. 1250 μCi/μg. At the end of the run, the gel was immediately dried and exposed to Kodak XAR-5 film with an intensifying screen for 12 h at -70°C.

T_4, T_3, reverse T_3 (rT_3), TSH and thyroglobulin (TG) were measured by specific radioimmunoassays (RIAs). TBG and denatured TBG (dn TBG) levels in serum were measured by specific RIAs as previously described. The T_4-binding capacity of TBG was measured by the single T_4 load ion exchange resin method (5). Free T_4 index (FT_4I) was derived from the ratio of serum

total T_4/TBG. Serum free T_4 was measured by equilibrium dialysis (FT_4D). Antibodies to thyroglobulin and thyroid microsomes were measured by a hemagglutination method.

Grouped data were expressed as the mean $\pm$ SD and were analyzed using the Student's t test. Statistical significance was defined when the p value was less than 0.05.

RESULTS

The three major TBG bands identified on IEF in male subjects with TBG-C or females with TBG-CC had pIs of 4.35, 4.42, and 4.50. Sera from males with TBG-S and the single female with TBG-SS exhibited a cathodal pI shift of all bands of approximately 0.06. Heterozygous females with TBG-CS showed a 6 banded pattern corresponding to the combined pIs of TBG-C and TBG-S.

Grouped data of thyroid function tests are summarized in Table 2. Mean serum T_4 concentration in the group of men with TBG-S was significantly lower than the age matched group of men with TBG-C. However, neither the mean FT_4D nor the mean TSH levels were significantly different among these two groups. Mean TBG concentration in sera with TBG-S was found to be significantly lower than that of TBG-C. Serum levels of dn TBG were less than 4 µg/dl in all samples except for 3, in which it was within the normal range. Other tests of thyroid function were similar between these two groups. Moreover, the two groups of women with TBG-CC and TBG-CS showed no significant differences in all determinations including serum T_4 and TBG concentrations. None of the subjects examined had circulating thyroid autoantibodies.

When a group of 10 sera with TBG-C were selected to match individual TBG concentrations of the 10 serum samples with TBG-S, the statistical difference between the mean T_4 levels of these two groups with different types of TBG disappeared (Fig. 1).

DISCUSSION

Family studies have shown that the TBG-S variant is inherited as an X-chromosome-linked trait (1). This finding is in agreement with the inheritance of other congenital TBG abnormalities such as TBG excess, TBG deficiency (6), or TBG-A in Australian Aborigines (7). Assuming that the frequency of the TBG-S allele is the same in both sexes, the frequency of hemizygous males should be the same as the gene frequency of 0.12 in the sample population of Black Americans we studied. The frequencies of the TBG-CS and TBG-SS genotypes in females are expected to be 2×0.12 and $(0.12)^2$, respectively. Indeed, observed numbers of the subjects with these genotypes were close to those expected from this calculation (Table 1). This, and the finding of TBG-CS in females, only confirms the X-chromosome-linked mode of inheritance of the TBG-S variant.

The finding of significant reduction of T_4 concentration in serum of subjects possessing the variant TBG-S could be attributed to many factors including altered affinity for T_4 or associated abnormalities in hormone secretion or metabolism. However, it is best explained on the basis of concomitant reduction in the concentration of circulating TBG. Indeed, the FT_4I and FT_4D were not significantly different between the groups with TBG-S and TBG-C. All individual TSH values were in the normal range, and there was no significant difference between the mean values for the two groups. Furthermore, the obliteration of the significant difference in the mean T_4 concentration of the group with TBG-S when matched by TBG concentration to a group of subjects with TBG-C proves that the observed reduction in serum

Table 2. Thyroid Function Tests in Four Groups of American Blacks Divided According to Their IEF Patterns of TBG

Subject	Age (yr)	T_4 (μg/dl)	T_3 (ng/dl)	rT_3 (ng/dl)	TSH (μU/ml)	TG (μg/ml)	FT_4D (ng/dl)	Ft_4I (T_4/TBG)	TBG Conc. (mg/dl)	TBG Cap (μg T_4/dl)	TBG Cap/Conc. (μg T_4/mg TBG)
TBG-S											
Mean	39	6.79	127	19.1	1.7	16.2	1.32	4.92	1.41	17.8	12.7
SD	15	1.52	24	3.8	1.4	18.5	0.30	1.01	0.30	3.6	1.1
TBG-C											
Mean	39	8.49	127	20.5	1.5	13.2	1.70	5.01	1.72	20.4	11.9
SD	17	1.29	13	4.2	0.7	5.1	0.25	0.70	0.29	3.3	0.7
P value	NS	<0.02	NS	NS	NS	NS	NS	NS	<0.05	NS	NS
TBG-CS											
Mean	33	9.25	149	--	1.7	16.0	--	4.48	2.12	25.2	12.1
SD	16	1.62	31		1.2	6.1		0.66	0.58	5.5	1.0
TBG-CC											
Mean	33	9.13	140	--	1.3	17.3	--	4.29	2.16	25.0	11.6
SD	17	1.75	25		0.5	8.4		0.56	0.46	5.9	1.2
P value	NS	NS	NS	--	NS	NS	--	NS	NS	NS	NS

Number of individual sera in each group was 10. Paired groups of males (TBG-S and TBG-C) and females (TBG-CS and TBG-CC) were age matched.

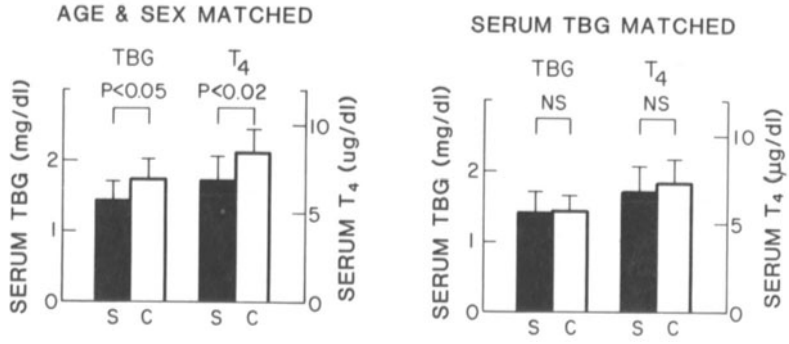

Fig. 1. Comparison of serum concentrations of T_4 and TBG between subjects with TBG-S and TBG-C. Ten subjects with TBG-S were matched by age or by TBG concentration to 10 subjects each with TBG-C. Data are given as mean ± SD for each group.

total T_4 is due to quantitative rather than functional differences between the two types of TBG. Although the data on TBG concentration is more reliable owing to the smaller interassay variation, because of the lack of statistical difference in T_4-binding capacities of TBG between the two groups, one might consider that a single molecule of TBG-S could have an additional binding site for T_4. However, the mean ratio of TBG capacity/TBG concentration was not significantly higher in subjects with TBG-S, as compared to that of TBG-C (12.71 ± 1.13 vs. 11.93 ± 0.74, respectively). As expected, the small reduction in T_4 and TBG concentrations associated with TBG-S in hemizygous males could not be detected in heterozygous females with TBG-CS.

The reason for decreased levels of TBG in serum of subjects with TBG-S is open to speculation. It could be that secretion of TBG-S is reduced. Our recent *in vitro* study using a human hepatoblastoma cell line demonstrated that glycosylation of TBG is essential for its secretion (8). The shift of the IEF pattern in TBG-S could be the consequence of an alteration in glycosylation due to a genetic difference in amino acid sequence. This may also affect the rate of TBG-S secretion. Nevertheless, an increased rate of degradation cannot be excluded without further studies on the metabolism of TBG-S *in vivo*.

Since a substantial number of American Blacks carries the TBG-S gene, Black men found to have low concentrations of serum T_4 require a careful evaluation to avoid a possible erroneous diagnosis of hypothyroidism.

REFERENCES

1. Daiger SP, Rummel DP, Wang L, et al. Am J Hum Genet 33: 640, 1981.
2. Grimaldi S, Bartalena L, Ramacciotti C, et al. J Clin Endocrinol Metab 57: 118, 1983.
3. Kamboh MI and Kirwood C. Am J Hum Genet 36: 646, 1984.
4. Refetoff S, Murata Y, Vassart G, et al. J Clin Endocrinol Metab 59: 269, 1984.
5. Refetoff S, Hagen SR, and Selenkow HA. J Nucl Med 13: 2, 1972.
6. Refetoff S, Robin NI, and Alper CA. J Clin Invest 51: 848, 1972.
7. Refetoff S and Murata Y. J Clin Endocrinol Metab 60: 356, 1985.
8. Murata Y and Refetoff S. Endocrinology suppl 115: T45, 1984.

EFFECT OF LONG-CHAIN FATTY ACIDS ON THE BINDING OF T_4 AND T_3 TO HUMAN THYROXINE-BINDING GLOBULIN (TBG)*

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Long-chain free fatty acids (FFA) such as oleic and linoleic acids have been shown to inhibit the binding of T_4 to binding proteins in serum (1-3). In a recent report, Chopra and coworkers (4) have presented evidence indicating tht circulating FFA, especially oleic acid, makes an important contribution to thyroid hormone binding inhibitor (THBI) activity found in sera of certain patients with nonthyroidal illnesses (NTI). Although oleic acid and linoleic acid are known to inhibit T_4 binding to albumin (5), the effect of FFA on T_4 binding to TBG, the major thyroid hormone-binding protein in serum, has not been studied directly. In the present study, we compared the effect of different FFA on the binding of T_4 to pure TBG.

MATERIALS AND METHODS

TBG was purified from pooled fresh frozen human serum as described previously (6). [^{125}I] labeled T_4 and [^{125}I]-T_3 both having SA $\sim$ 150 μCi/μg were obtained from NEN (Boston, MA, USA). Fatty acids (at least 99% pure) were obtained from U. S. Biochemical Corp. (Cleveland, OH). Aqueous solutions of the sodium salts of the fatty acids were made up as described by Goodman (7). Equilibrium dialysis (8) was used to study binding at pH 7.4 and 37°C (see legend to Fig. 1).

RESULTS

Scatchard plots showing the effect of different fatty acids on the binding of [^{125}I]-T_4 to TBG are given in Fig. 1A. In each binding experiment the overall strength of binding, as represented by the nK value, was determined by extrapolation of the Scatchard plot to the $\bar{\nu}/A$ axis (see legend to Fig. 1 for definitions). Table 1 shows the degree of binding inhibition as calculated from the percent change in nK value in the presence of fatty acid as compared to the control.

Fasting FFA levels in normal human plasma are of the order of 0.5 mM (10). Assuming that the concentration of TBG (M_r = 60,000) in normal serum

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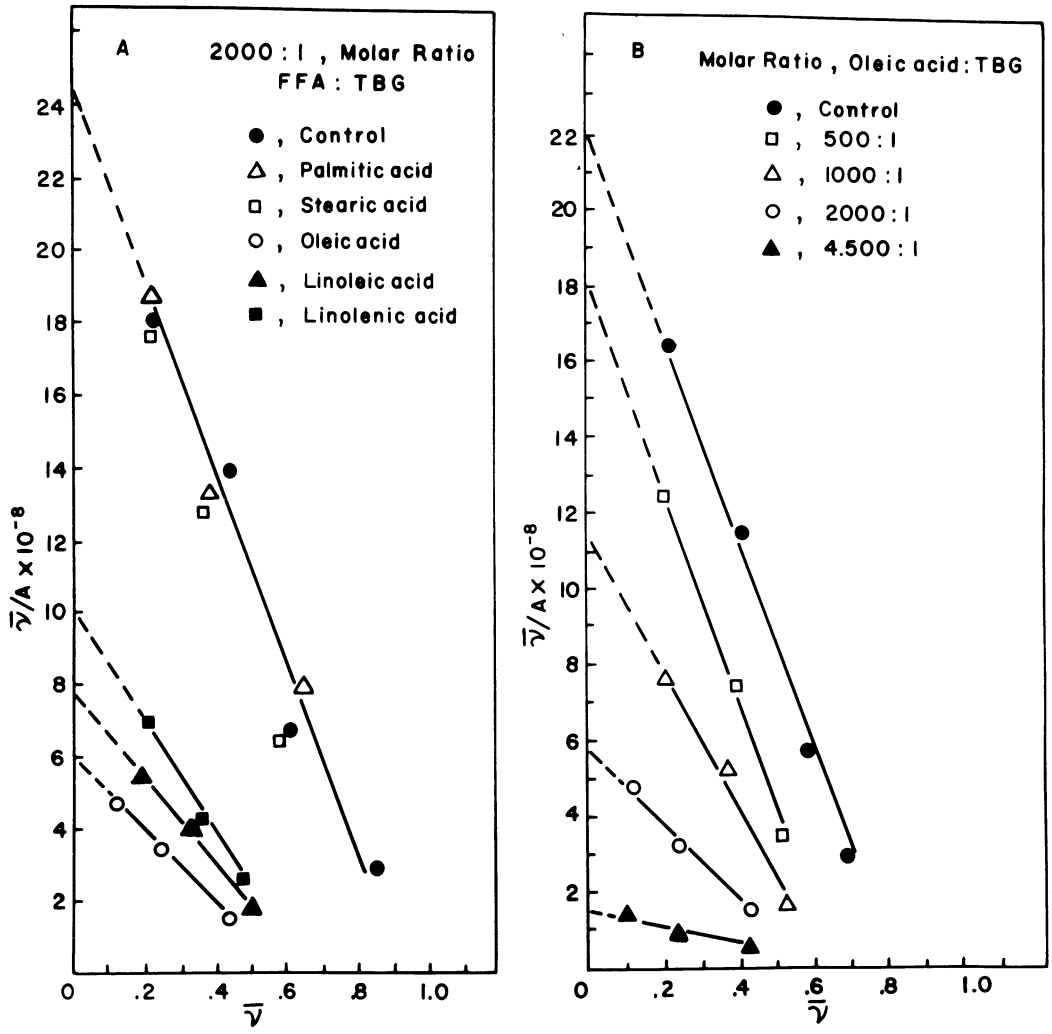

Fig. 1. A. Effect of long-chain fatty acids on the binding of T_4 to TBG at pH 7.4 and 37°C. The molar ratio of fatty acid to TBG was 2000:1. B. Effect of Oleic acid concentration on the binding of T_4 to TBG at pH 7.4 and 37°C. Binding was studied by equilibrium dialysis performed in 0.06 M potassium phosphate buffer containing 0.7 mM EDTA (8). The data were plotted according to the Scatchard equation: $\bar{\nu}/A = -k\bar{\nu} + nk$ (9) where $\bar{\nu}$ is the average number of moles of ligand bound per mol of protein, A is the molar concentration of free ligand, n is the apparent number of binding sites, and K is the apparent association constant for binding. Extrapolation to the $\bar{\nu}/A$ axis as $\bar{\nu}$ approaches zero gives the nK value for binding (the dashed lines). The fatty acids were in the form of their sodium salts. The data points are averages of duplicate determinations.

is about 2.5×10^{-7} M, then a molar ratio of FFA to TBG of 2000:1 as used in the binding experiments (Fig. 1A) represents a fatty acid concentration in serum of 0.5 mM, which is in the physiological range.

The effect of different concentrations of oleic acid on binding was also studied and the results are given in Fig. 1B. As can be seen in Fig.

Table 1. Inhibition of T_4 Binding to TBG by Long-chain Fatty Acids[a]

Fatty Acid	nK (x 10^{-8}) M^{-1}	T_4 bound (% of control)	% Inhibition
None, control	24.8 ± 2.2	100	-
Palmitic acid (16:0)	24.1 ± 1.4	100	0
Stearic acid (18:0)	22.7 ± 1.9	100	0
Oleic acid (18:1)	5.9 ± 0.7	24	76
Linoleic acid (18:2)	7.7 ± 0.6	31	69
Linolenic acid (18:3)	9.8 ± 0.9	39	61

[a]At pH 7.4 and 37°C in 0.06 M potassium phosphate buffer containing 0.7 mM EDTA. Binding was determined at a 2000:1 molar ratio of fatty acid to TBG. The nK value (mean ± SEM) was determined by extrapolation of the Scatchard plot as shown in Fig. 1.

1B, oleic acid is capable of inhibiting T_4 binding to TBG at the lowest concentrations studied, 500:1 and 1000:1 molar ratios of TBG, corresponding to 0.125 and 0.250 mM fatty acid, respectively, in serum. Based on the change in nK values at molar ratios of oleic acid to TBG of 500:1, 1000:1, and 4,500:1 (Fig. 1B), binding was inhibited 18%, 48%, and 93%, respectively. Oleic acid also inhibits T_3 binding to TBG. At molar ratios of oleic acid to TBG of 1000:1, 2000:1, and 4000:1, T_3 binding was inhibited 24%, 41%, and 76%, respectively (data not shown).

DISCUSSION

The relative inhibitory effectiveness of the FFA used in our study is in the same order as that reported by Chopra et al. (3) for inhibition of $[^{125}I]$-T_4 binding in normal serum. The data correlates very well with the observation that oleic acid is more important to THBl activity of NTI sera than other fatty acids (4). It is also of interest to note that fatty acids have been found in association with TBG; about 20% of the total is in the form of oleic acid (11).

There is a question as to whether thyroid hormone transport in normal individuals can be influenced by changes in FFA levels owing to binding of FFA to albumin. However, the possibility exists that transitory surges in FFA levels in extracellular fluid may affect T_4 transport into certain tissues, i.e., adipose tissue.

SUMMARY

Unsaturated long-chain fatty acids (oleic, linoleic, and linolenic) are potent inhibitors of T_4 binding to TBG, whereas saturated fatty acids (palmitic and stearic) have little effect on binding.

REFERENCES

1. Hollander CS, Scott RL, Burgess JA, et al. J Clin Endocrinol Metab 27: 1219, 1967.
2. Tabachnick M, Hao YL, and Korcek L. J Clin Endocrinol Metab 36: 392, 1973.
3. Chopra IJ, Huang TS, Hurd RE, et al. J Clin Endocrinol Metab 58: 619, 1984.
4. Chopra IJ, Teco GNC, Mead JF, et al. J Clin Endocrinol Metab 60: 980, 1985.
5. Tabachnick M. Arch Biochem Biophys 106: 415, 1964.
6. Korcek L and Tabachnick M. J Biol Chem 251: 3558, 1976.
7. Goodman DW. J Am Chem Soc 80: 3887, 1958.
8. Siegel JS, Korcek L, and Tabachnick M. Endocrinology 113: 2173, 1983.
9. Scatchard G. Ann NY Acad Sci 51: 660, 1959.
10. Dole VP. J Clin Invest 35: 150, 1955.
11. Korcek L and Tabachnick M. Biochim Biophys Acta 371: 323, 1974.

AN UNUSUAL TYPE OF FAMILIAL DYSALBUMINEMIC HYPERTHYROXINEMIA IN A JAPANESE FAMILY*

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INTRODUCTION

Familial dysalbuminemic hyperthyroxinemia (FDH) is a rare entity characterized by elevation of serum thyroxine (T_4) due to an increase in the binding of T_4 to serum dysalbumin. Serum T_4 levels in affected subjects have been reported to be 16-40 μg/dl, while serum free T_4 is in normal range. Values for serum T_3 and reverse T_3 are normal in most cases. In this communication, we present patients with extremely elevated serum T_4 (111-136 μg/dl) in a Japanese family.

MATERIALS AND METHODS

The propositus was a 1.5-year-old boy who was referred to our hospital because of a slight growth retardation. He had no signs or symptoms suggestive of thyroid disease, but serum total T_4 was found to be unusually elevated (136 μg/dl). Serum T_3 and reverse T_3 were also higher than normal (Table 1). T_3-resin uptake (29.9%) was within normal range, but TBG was slightly decreased. Basal serum TSH (1.9 μU/ml) and response to TRH were normal. Thyroidal ^{123}I-uptake (24 h) was 33.1%, which was suppressed to 15.1% after T_3 administration (25 μg/day) for 7 days. Serum free T_4 was significantly elevated when measured by Amerlex kit, but normal by micro-membrane capsule method. Antibodies to thyroglobulin and microsomes were negative.

Serum total T_4, T_3, and reverse T_3 were determined by radioimmunoassay kits. Serum free T_4 was measured by a kit using micro-membrane capsule (Liquisol), a kit using ^{125}I-T_4-analog (Amerlex) and by equilibrium dialysis method. Equilibrium dialysis was performed using Tris/HCl buffer as described by Stockigt et al. (1) and magnesium chloride precipitation. T_4-binding protein was analyzed by reverse flow paper electrophoresis after trace-labeling with T_4. Serum albumin was prepared from serum using DEAE-Affigel-blue column. Competitive binding of T_4 to serum was studied as follows: Patient's serum or albumin was incubated with ^{125}I-T_4 in the

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Table 1. Thyroid Function Tests in Affected Family Members

	Grandfather	Father	Patient	Sibling	Normal
T_4 (μg/dl)	111	112	136	126	5.1 - 11.4
T_3 (ng/ml)	216	227	292	388	90 - 170
rT_3 (ng/dl)	273	238	405	316	14 - 41
RT_3U (%)	-	34.8	29.9	-	23 - 34
TSH (μU/ml)	2.1	1.2	1.8	2.7	1 - 4
TBG (μg/ml)	10.6	10.5	15.3	16.9	22 - 30
Tg (ng/ml)	59	16.3	68.1	154	5 - 35
free T_4 (ng/dl)					
Amerlex	5	5	5	5	1.03 - 2.42
Liquisol	1.06	1.27	1.44	0.9	0.8 - 2.4
Dialysis	-	-	3.42	-	1.0 - 3.5

presence or absence of various concentrations of unlabeled T_4, T_3, and reverse T_3 at 4°C for 18 h. Protein-bound ^{125}I-T_4 was separated from unbound by adding charcoal-coated dextran as described by White et al. (2). Competitive equilibrium dialysis was carried out using patient's albumin and ^{125}I-T_4 with or without various concentrations of unlabeled T_4. The procedure was identical to that employed for determination of serum free T_4.

RESULTS AND DISCUSSION

Although serum total T_4 was markedly elevated in this 1.5-year-old boy, he was considered to be euthyroid judged by physical findings, normal response of TSH to TRH, and by normal suppression of thyroidal ^{123}I-uptake after T_3 suppression. Marked hyperthyroxinemia along with elevated serum rT_3 and was also found in the other three family members (Table 1), suggesting that the abnormality was inherited by autosomal dominant transmission. None of these affected family members showed signs and symptoms suggestive of hyperthyroidism. Hyperthyroxinemia was not due to the presence of antibodies to T_4. An increase of serum TBG or TBPA was ruled out by paper electrophoresis experiments. Serum TBG levels determined by radioimmunoassay were rather decreased. These observations strongly suggest that hyperthyroxinemia may be due to FDH, characterized by an increase of T_4-binding albumin. Serum free T_4 levels in the affected subjects were significantly elevated when determined by Amerlex kit using ^{125}I-T_4-analog, but the values obtained by micro-membrane capsule method (Liquisol) were normal. Furthermore, serum free T_4 in propositus was normal when assayed by equilibrium dialysis. It has been reported that serum free T_4 determined by Amerlex kit was higher than normal in patients with FDH because of binding of ^{125}I-T_4-analog to serum dysalbumin (3). To confirm the diagnosis of FDH, we performed reverse flow paper electrophoresis of patient's serum after ^{125}I-T_4 labeling. As expected, most of ^{125}I-T_4 migrated with albumin. However, when unlabeled T_4 was added to normal serum to a concentration equivalent to that in patient's serum, ^{125}I-T_4 also co-migrated with albumin. Thus, the results obtained from paper electrophoresis were compatible with FDH, but not conclusive.

When subjected to equilibrium dialysis, free T_4 fraction was 0.0234% for normal control serum and 0.0023% for patient's serum. Addition of unlabeled T_4 (25 ng) resulted in an increase of free T_4 fraction to 0.0644% for normal serum and to only 0.0211% for patient's serum, indicating higher T_4-binding capacity in patient's serum. Remarkably, there was a much greater increase of serum free T_4 fraction in patient's serum compared to normal (0.0639% vs. 0.2681%), when dithiothreitol (DTT) was added in the dialysis buffer. These observations are compatible with an earlier report on FDH (4). Disulfide bond(s) may be involved in maintaining T_4-binding activity of dysalbumin.

We next studied ^{125}I-T_4 binding to serum or albumin using charcoal-coated dextran for BF separation. It has been reported that the T_4-uptake test using charcoal is very specific to FDH (5). The patient's serum (1:100 diluted) was incubated with ^{125}I-T_4 in the presence of 10^{-6} M of unlabeled T_4. We found that as much as 89.6% of added ^{125}I-T_4 bound to serum proteins, while the value was only 8.5% for normal serum. We also performed similar experiments using serum albumin prepared from patient's serum. As shown in Fig. 1, albumin from patient's serum bound 44.5% of added ^{125}I-T_4. The binding was dose-dependently inhibited by unlabeled T_4. Analysis of binding data revealed T_4-binding sites with affinity of 3.6×10^{-6} M^{-1} and binding capacity of 1.3 mg/g albumin. Affinity of 1.06×10^{6} M^{-1} has been obtained when determined by equilibrium dialysis assays. These values are similar to those reported for FDH by others (4,6).

Taken together, these observations strongly suggest that hyperthyroxinemia found in four members in a Japanese family is due to the presence of T_4-binding dysalbumin with high binding affinity and capacity. The present cases are unique in several respects. First, serum T_4 level is the highest among the patients so far described. Second, the present cases showed a marked increase in serum reverse T_3. This could be accounted for by binding of reverse T_3 to T_4-binding dysalbumin. Supporting this, ^{125}I-T_4 binding to albumin was inhibited by reverse T_3 in a dose-dependent manner, although the potency of reverse T_3 to inhibit ^{125}I-T_4 binding was only 1/20 that of T_4. Recently, Lalloz et al. (7) have reported a patient having abnormal albumin with increased affinity for reverse T_3. In contrast to reverse T_3, the ability of T_3 to bind to T_4-binding albumin was much lower as shown in Fig. 1. This is consistent with marginal elevation of serum T_3 in in these patients. Yabu et al. (8) recently proposed that the abnormality of serum albumin in patients with FDH might be increase of albumins with particularly high affinity for T_4 that are present in normal serum at a low concentration (8). It is not clear, however, whether T_4-binding albumin(s) found in sera of the cases under discussion are present in normal serum.

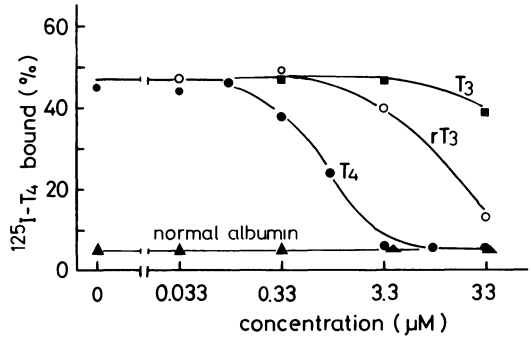

Fig. 1. Binding of thyroid hormones by the albumin of the FDH patient.

REFERENCES

1. Stockigt JR, Topliss DN, Barlow JW, et al. J Clin Endocrinol Metab 53: 353, 1981.
2. White EL, Barlow JW, Burke, et al. In JR Stockigt and S Nagataki (eds), Thyroid Research VIII, Australian Academy of Science, Canberra, 1980, p 512.
3. Stockigt JR, DeFaris M, Csicsmann JM, et al. Clin Endocrinol (Oxf) 15: 313, 1981.
4. Barlow JW, Csicsmann JR, White EL, et al. J Clin Endocrinol Metab 55: 244, 1982.
5. Stockigt JR, Stevens V, Dyer S, et al. Abstract of the International Congress of Endocrinology #2426, 1984.
6. Lalloz MRA, Byfield PGH, and Himsworth RL. Clin Endocrinol (Oxf) 18: 11, 1983.
7. Lalloz MRA, Byfield PGH, and Himsworth RL. Clin Endocrinol (Oxf) 22: 521, 1985.
8. Yabu Y, Amir SM, Ruiz M, et al. J Clin Endocrinol Metab 60: 451, 1985.

PERIPHERAL METABOLISM OF THYROID HORMONES IN CONGENITAL THYROID HORMONE TRANSPORT ABNORMALITIES: FAMILIAL DYSALBUMINEMIC HYPERTHYROXINEMIA AND TBG INCREASE

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INTRODUCTION

Familial dysalbuminemic hyperthyroxinemia (FDH) and inherited increase of thyroxine-binding globulin (TBG) in plasma are two very uncommon thyronine serum-binding protein abnormalities that cause hyperthyroxinemia without hyperthyroidism. In FDH, subjects have an abnormal albumin molecule which shows increased affinity only for thyroxine (T_4). As a result, serum total T_4 (TT_4) value is increased, while the serum total triiodothyronine (TT_3) concentration is normal or slightly elevated. Nevertheless, the increase in the free T_4 index frequently leads to an erroneous diagnosis of thyrotoxicosis. On the contrary, in the inherited high TBG syndrome, proportionate increments in serum TT_4 and TT_3 concentrations are seen because TBG has a high affinity for T_4, as well as T_3, whereas the free T_4 index remains normal.

The effect of alterations in the affinity or concentrations of plasma-binding proteins on the in vivo distribution and metabolism of thyroid hormones in man is not yet well known; few and incomplete kinetic data are available (1,2). The recent finding of the bioavailability of the albumin-bound fraction of the thyroid hormones (3) is not in agreement with Mendel's results which deny a primary role for albumin in T_4 transport into the cells (2). The main purpose of the present study was to test the hypothesis that the availability of T_4 bound to albumin is greater than that of T_4 carried by TBG; therefore, we have carried out complete kinetic studies of T_4 and T_3 metabolism in five subjects affected by FDH and in two subjects with inherited increase of TBG serum levels.

MATERIAL AND METHODS

A total of 22 turnover studies were performed: 14 euthyroid normal subjects served as the control group, five members of three families were affected by FDH and two kindred had congenital elevation of TBG. Their ages ranged from 37 to 73 years. Serum TT_4, TT_3, free T_3, rT_3, TSH, and TBG levels were measured with commercial RIA kits. The free T_4 fraction was measured by equilibrium dialysis. TRH tests were done by IV bolus injection of 200 µg of TRH. Distribution of ^{125}I-T_4 among serum proteins

Table 1. Serum Concentrations and Affinity Constants of T_4 Carrier Proteins (37°C, pH 7.4, ionic strength 0.15 m) in FDH

	PA (mg/dl)	TBG (µg/ml)	K_{TBG} (1/mol)	HSA (g/dl)	K_{HSA} (1/mol)	aHSA %	K_{aHSA} (1/mol)
Mean (n=5)	35.2	23.2	3.9×10^9	4.5	3.5×10^5	20.4	4.9×10^6
SD	5.3	2.6	0.8	0.2	2.1	2.9	1.1

PA = prealbumin (radial immunodiffusion, Behring); TBG = thyroxine-binding globulin (Immophase, Corning); K_{TBG} = affinity constants of T_4 to TBG; HSA = serum albumin concentration (radial immunodiffusion, Behring); K_{HSA} = affinity constants of T_4 to HSA; aHSA = abnormal serum albumin (percent HSA); K_{aHSA} = affinity constants of T_4 to aHSA.

was determined by poliacrylamide gel electrophoresis in Tris glycine buffer, pH 8.9

T_4 and T_3 turnover studies were carried out as previously described and the T_4 to T_3 conversion rate (CR) was computed by the convolution method (4). The plasma disappearance curves of IV bolus injected ^{125}I-T_4 and ^{131}I-T_3 were analyzed by the noncompartmental approach; standard formulas were employed to compute total plasma clearance rate (MCR), total plasma equivalent distribution volumes (TDV), the production rates (PR), and the extrathyroidal body pool (Q_t). Con A-Sepharose was used to separate TBG from prealbumin (PA) and albumin, and to determine the affinity constants (Ka) of TBG for T_4 according to the technique described (5) (pH 7.4, .15 M ionic strength, 37°C). Cibacron blue F3 GA-Sepharose and Sephadex partition (pH 7.4, .15 M ionic strength, 37°C) were used to isolate the albumin and to determine the T_4 affinity to the abnormal binding site in FDH subjects (6,7). Data were analyzed according to the Scatchard method.

RESULTS

All subjects studied were euthyroid by clinical and laboratory criteria. Table 1 gives mean values of the plasma levels and of the measured Ka of serum-binding proteins for T_4 in the five subjects with FDH. The T_4 and T_3 serum concentrations and the main kinetic parameters for all subjects studied are reported in Table 2. As previously reported (8), the two subjects with congenital elevation of serum TBG showed markedly reduced MCR and TDV of both T_4 and T_3, whereas the respective PR were found to be in the normal range, due to the increased serum levels of both hormones. On the other hand, a slight but significant reduction of the average T_4 MCR was observed in the FDH subjects, who also showed a considerable increase in both T_4 and T_3 PR (respectively +57% and +38%), and in the Q_t respectively +87% and +38%) (Fig. 1). A highly significant inverse correlation ($p<0.01$) was observed between T_4 MCR and TT_4 serum concentration in this condition (Fig. 2).

DISCUSSION

Since the MCR is defined as the ratio between disposal rate and the plasma concentration of any hormone, it can be considered as an index of the

Table 2. "In vivo" T_4 and T_3 Kinetic Parameters in FDH and in Increased TBG

Subjects	TT_4 µg/dl	MCR $l/d/m^2$	PR $\mu g/m^2$	TDV l/m^2	Q_t $\mu g/m^2$	TT_3 ng/dl	MCR $l/d/m^2$	PR $\mu g/m^2$	TDV l/m^2	Q_t $\mu g/m^2$	CR %	SR %
FDH												
1	18.8	0.39	72.9	5.0	940	157	12.4	19.5	18.6	29.2	21.6	32.4
2	15.6	0.55	86.1	4.9	769	159	18.9	30.0	19.4	30.8	30.2	27.5
3	15.8	0.58	91.6	4.9	784	135	16.3	22.0	26.6	35.9	18.4	36.0
4	15.8	0.57	90.2	5.5	864	173	12.2	21.1	13.8	23.9	20.3	27.4
5	16.4	0.52	84.8	4.5	735	148	9.5	16.5	10.5	15.5	15.5	33.0
Mean	16.5	0.51	85.1	5.0	818	154	13.9	21.8	17.8	27.1	21.2	31.3
SD	1.3	0.07	7.0	0.4	82	14	3.7	5.0	6.0	7.7	5.5	4.0
TBG												
1	14.2	0.36	51.8	3.9	556	204	6.9	14.1	10.5	21.4	22.1	33.0
2	16.6	0.29	47.7	3.8	637	272	4.8	13.0	8.6	23.4	22.8	30.0
Mean	15.4	0.32	49.7	3.85	596	238	5.8	13.5	9.5	22.4	22.4	31.5
SD	1.7	0.05	2.9	0.07	58	48	1.0	0.8	1.3	1.4	0.5	2.1
Normals (14)												
Mean	8.0	0.68	54.1	5.4	433	122	13.0	15.8	16.1	19.6	26.3	25.4
SD	1.1	0.13	10.0	0.8	74	12	2.3	2.3	3.4	4.0	6.3	13.0

See text for symbols.

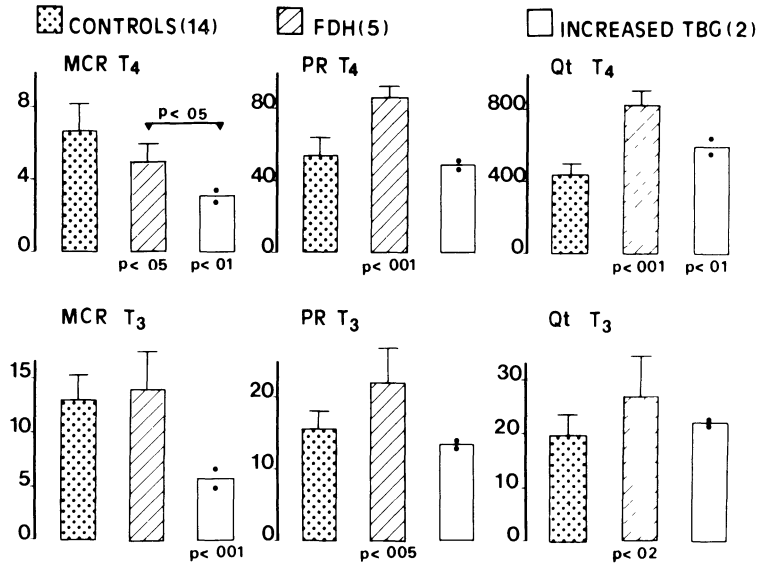

Fig. 1. Comparison of MCR, PR, and Q_t values for normals, FDH, and increased TBG subjects. The bars represent mean values and SD of MCR (1/day/m^2 bs), PR (μg/day/m^2 bs), and Q_t (μg/m^2 bs) for T_4 and T_3 in the studied groups. Statistical significance of differences between normals and FDH or increased TBG subjects is indicated (t test).

availability of the hormone for disposal and, as far as T_4 is concerned, for its conversion into T_3. Therefore, the availability of T_4 is markedly lowered in subjects with congenital TBG elevation (by 53% reduction in comparison with normal subjects); the higher T_4 plasma levels allow, however, a normal disposal rate of T_4 and, therefore, a normal peripheral T_3 PR. On the other hand, the observed reduction of the T_4 MCR in FDH subjects (by only 25% as compared to the control group) was relatively lower than that found in the increased TBG state, indicating that T_4 carried by the abnormal albumin is more available to peripheral tissues than that T_4 carried by TBG. This result is in agreement with the finding that the affinity of the abnormal albumin-T_4 binding site is ten-fold increased in respect to the normal T_4-albumin binding site but is still about 1000-fold lower than that of the TBG binding site (Table 1). It is worth noting that for identical values of serum T_4 concentration the subjects with congenital elevation of TBG show much lower T_4 MCR than the FDH subjects (Table 2). The inverse correlation observed between T_4 MCR and T_4 serum concentration suggests the existence in FDH of a variability in the affinity and/or capacity of the abnormal albumin binding sites. At variance with the results previously reported (1,2), the T_4 PR in all the FDH subjects studied was significantly increased in comparison with the control group. In fact, the increment in T_4 serum concentration was not associated with a proportionate decrement of T_4 MCR as observed in patients with increased TBG.

As far as distribution volumes are concerned, we found a significant reduction of TDV in TBG subjects, while this parameter was in the normal range in FDH subjects. Our TDV values in FDH are similar to those reported by Hennemann (1) and Mendel (2) who, however, report TDV values in the control subjects higher than those reported by other authors (9) and by us. As most of T_3 (about 70%) arises from peripheral T_4-monodeiodination, the

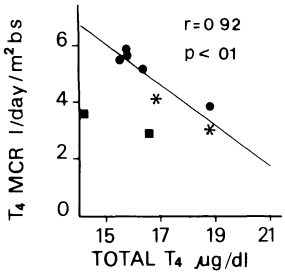

Fig. 2. Correlation between TT_4 serum levels and T_4 MCR in FDH.

- ● FDH present study.
- * FDH from Hennemann et al.
- ■ Increased TBG (not included in the correlation).

observation in FDH of a remarkable increase of T_3 PR and Q_t associated with normal values of T_3 MCR, T_4 to T_3 conversion fraction, and T_3 thyroidal secretion (SR) is compatible with an almost normal T_4 availability to the peripheral tissues. The underlying mechanism could be a hormone-carrier protein interaction in which increased bioavailability of albumin-bound hormone results from an intermediate T_4-albumin complex that dissociates faster than the T_4-TBG complex in response to a reduction of serum-free hormone levels due to tissue extraction (10).

REFERENCES

1. Hennemann G, Docter R, Krenning EP, et al. Lancet i: 639, 1979.
2. Mendel CM and Cavalieri RR. J Clin Endocrinol Metab 59: 499, 1984.
3. Pardridge WM. Endocr Rev 1: 103, 1981.
4. Bianchi R, Mariani G, Molea N, et al. J Clin Endocrinol Metab 56: 1152, 1983.
5. Dunn JF, Nisula BC, Rodbard D, et al. J Clin Endocrinol Metab 53: 58, 1981.
6. Lalloz MRA, Byfield PGH, and Himsworth RL. Clin Endocrinol (Oxf) 18: 11, 1983.
7. Barlow JW, Csicsmann JM, White EL, et al. J Clin Endocrinol Metab 55: 244, 1982.
8. Nicoloff JT, Low JC, Dussault JH, et al. J Clin Invest 51: 473, 1972.
9. Ramsden DB and Hoffenberg R. In DG Cramp (ed), Quantitative Approaches to Metabolism, John Wiley & Sons Ltd., 1982, p 301.
10. Giraudi G. J Clin Immunol 7: 268, 1984.

FREE T_4 INDEX RESULTS ARE NON-SPECIFIC IN PATIENTS WITH HYPOTHYROXINEMIA

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INTRODUCTION

Hypoproteinemia is well-recognized as a cause of hypothyroxinemia. Both hypoproteinemia and hypothyroxinemias are common among patients hospitalized for nonthyroidal illnesses, yet the role of T_4-binding proteins in the hypothyroxinemias of nonthyroidal illness is unclear. T_4-binding proteins can be measured indirectly by studies of either T_3 binding or T_4 binding to serum proteins, or they can be measured directly by protein-specific assays. Discrepancies between T_3 binding and T_4 binding are common in nonthyroidal illness, but it is unknown which, if either, represents T_4-binding protein levels. In this study we measured the three T_4-binding proteins by protein assays and compared the levels to the T_3 binding activity and the T_4 binding activity. We selected 150 hypothyroxinemic patients hospitalized for nonthyroidal illnesses solely on the basis of their serum T_4 concentrations. Albumin was measured colormetrically; prealbumin (PA) and TBG were measured by immunoassay. T_3 binding was measured by *in vitro* T_3 uptake or by equilibrium dialysis, and T_4 binding was measured by equilibrium dialysis. In 134 of the 150 hypothyroxinemic patients one or more of the T_4-binding proteins was low. The contribution of these hypoproteinemias to the hypothyroxinemias was assessed by calculating the overall T_4-binding protein level of each serum from the concentrations of each of the three individual T_4-binding proteins measured by protein-specific assays and comparing this overall T_4-binding protein level to the protein-bound T_4 concentration (i.e., Total T_4). The interrelationships between overall T_4-binding protein levels, tracer T_3 binding and tracer T_4 binding were examined. The biochemical mechanisms of hypothyroxinemia were determined from the free T_4 concentration, protein-corrected T_4 level, and serum TSH without consideration of free T_4 index values. The ability of the free T_4 index to identify different types of hypothyroxinemias was then evaluated.

METHODS

Albumin was measured by bromcresol blue colorimetry (Boehringer-Mannheim), and PA was measured by enzyme immunoassay (Abbott). TBG was measured by ^{125}I TBG RIA (Nichols Institute), T_4 and T_3 were measured by solid phase RIA (Clinical Assays), TSH was measured by RIA (Becton Dickinson) or by IRMA (Hybritech), *in vitro* T_3 uptake was measured using a barbital buffer with talc as the separating agent (Nuclear Medical Laboratories), and the % free T_4 and % free T_3 were measured by equilibrium dialysis.

Calculations

Free T_4 = Total T_4 x % Free T_4: Free T_3 = Total T_3 x % Free T_3

Free T_4 Index = Total T_4 x T_3 Uptake Ratio

Overall T_4-binding protein level as % of normal =

$$\frac{\text{TBG}}{\text{Normal TBG}} \times 70\% + \frac{\text{PA}}{\text{Normal PA}} \times 18\% + \frac{\text{ALB}}{\text{Normal Alb}} \times 12\%$$

$$\text{Overall protein-corrected } T_4 = \frac{\text{Total } T_4}{\text{Overall } T_4\text{-binding Protein Level}}$$

Normal Ranges

The expected values in normal subjects were: Total T_4 6.1-10.6 µg/dl; Total T_3 73-153 ng/dl; % Free T_4 0.019-0.037%; Free T_4 1.6-3.3 ng/dl; Albumin 3.8-4.9 gm/dl; PA 18-45 mg/dl; TBG 1.5-3.5 mg/dl; T_3 Uptake Ratio 0.85-1.15; Free T_4 Index 6.3-1.05; Overall T_4-binding Protein 74-134% of normal and Overall Protein-corrected T_4 6.1-10.4 µg/dl.

Subjects

One hundred and fifty hypothyroxinemic subjects hospitalized for nonthyroidal illness were selected for this study solely on the basis of their serum T_4 concentrations at the time of admission to the hospital. Fifty patients were selected in each of three T_4 ranges. Group I had serum T_4 levels $\leq$ mean -4 SD of normal ($\leq$3.7 µg/dl), Group II had T_4 levels between the mean -3 SD and the mean -4 SD of normal (3.8-4.9 µg/dl), and Group III had T_4 levels between the mean -2 and mean -3 SD of normal (5.0-6.0 µg/dl). Six individuals with congenital TBG deficiency were also studied. All six were healthy euthyroid males with normal serum TSH concentrations. All had normal albumin and PA concentrations, and all had TBG concentrations of 0.1 mg/dl or less. In two of the six subjects no measurable TBG was present. One hundred healthy controls were selected from among hospital personnel. Fifty were males and fifty were females. Each control subject underwent a general physical examination, medical history, screening chemistry panel, complete blood count, and serum TBG measurement. All subjects were medication-free at the time their blood sample was obtained.

To obtain sera with the same albumin, PA, and free hormone concentrations but varying TBG concentrations, serum from one healthy male with congenital TBG deficiency was dialyzed under sterile conditions against serum from another healthy male who had congenital TBG excess. These two sera were then mixed in varying proportions to given serum samples with varying TBG concentrations. Both subjects had normal albumin (4.2 and 4.4 gm/dl) and PA (25 and 23 mg/dl) concentrations, as well as normal free T_4 (1.9 and 1.6 ng/dl) and normal TSH levels (1.9 and 1.3 µU/ml by IRMA).

RESULTS

In the 150 patients with both nonthyroidal illness and hypothyroxinemia, serum albumin levels ranged from 1.8 gm/dl - 4.5 gm/dl and averaged 3.0 $\pm$ 0.6 (mean $\pm$ SD). PA levels ranged from 2 mg/dl - 46 mg/ dl and

averaged 17 ± 11, while TBG concentrations ranged from 0.5 mg/dl - 3.7 mg/dl and averaged 1.8 ± 0.5.

To assess the reliability of the calculation of the overall T_4-binding protein levels, these were calculated in the six subjects with congenital TBG deficiency. The results ranged from 25% - 33% with a mean of 29%. Serum T_4 levels ranged from 2.4 - 2.9 with a mean of 2.5 µg/dl. This represents 29% - 35% of the mean of normals with an average of 30% of normal. Overall protein-corrected T_4 values ranged from 7.5 - 10.4 with a mean of 8.7 µg/dl (normal 6.1 - 10.4). In these calculations, the TBG concentration was taken as 0.09 mg/dl for the two individuals who had undetectable TBG concentrations, since our assay is incapable of reliably distinguishing values of 0.09 mg/dl from zero.

Among the 150 hyothyroxinemic patients, the overall T_4-binding protein levels ranged from 34 - 109% with a mean of 68 ± SD 16% (normal 104 ± 15), the T_3 uptake ratio was 0.74 - 1.75 with a mean of 1.14 ± SD 0.18 (normal 1.00 ± 0.075), and the % free T_4 was .024 - .244% with a mean of .048 ± SD .025% (normal 0.028 ± 0.0045). As T_4-binding protein levels fell, T_3 uptake values rose, but their increase was proportionately less than the fall in T_4-binding proteins. As T_4-binding protein levels fell, the % free T_4 rose, but the % free T_4 rose proportionately more than the fall in T_4-binding proteins. The % free T_4 rose to a mean of 171% of normal, and the T_3 uptake rose to a mean of 114% of normal, while the overall T_4-binding protein level fell 32%. From inspection of the data it appeared that the discordance between T_3 uptake and % free T_4 was greater when T_4-binding protein levels were lower, and less when T_4-binding protein levels were higher. The difference between T_3 binding activity and T_4 binding activity (each expressed as a % of normal) was inversely correlated with the overall T_4-binding protein level ($y = 1.95 X + 193$; $r = 0.40$; $p<0.001$). Since TBG is the major protein contributing to the overall T_4-binding protein level, the relationship of TBG concentration to the difference between T_3 binding activity and T_4 binding activity was also studied. Again, a significant inverse relationship existed ($r = 0.31$, $p<.001$).

The relationships described above were found in the serum of the sick. We next explored these relationships in the serum of the well using two healthy males, one with congenital TBG excess and the other with congenital TBG deficiency. Their sera were mixed as described above to give serum samples with normal albumin, PA, free T_3, and free T_4 concentrations but with differing TBG concentrations. In this experiment, the dialyzable fraction of T_3 replaced the T_3 uptake as the measure of T_3 binding activity. The same negative correlation between TBG concentration and the difference between T_3 binding activity and T_4 binding activity was seen. At TBG concentrations of 10 mg/dl, the difference between % free T_4 and % free T_3 (expressed as the % of normals) was -2. At a TBG concentration of 20 mg/dl (in the normal range) the difference was 3%, while at TBG concentrations of <0.1 mg/dl, the difference rose to 79%. There was no appreciable change in the difference between T_3 binding activity and T_4 binding activity in the range of TBG concentrations between 30 and 100 mg/dl, but there was a consistent increase in the difference as TBG levels fell below 20 mg/dl.

Since the rise in T_3 uptake was not proportional to the fall in overall T_4-binding protein levels, we next looked at the classification of hypothyroxinemic patients using overall T_4-binding protein levels rather than the T_3 uptake ratios to correct T_4 levels for changes in T_4 hormone-binding concentrations. To do this, we calculated the overall protein-corrected T_4 as total T_4 ÷ overall T_4-binding protein level. Hypothyroxinemia due to hypoproteinemia was defined as serum with a normal overall protein-corrected T_4 and a normal free T_4 concentration. Hypothyroidism was defined as serum with a low overall protein-corrected T_4 and a low free T_4 concentration.

Hypothyroidism was further divided into primary hypothyroidism (when TSH was elevated) and apparent TSH deficiency (when TSH was not elevated). Defects in the T_4 binding activity of serum proteins could then be identified as the combination of a low overall protein-corrected T_4 with a normal free T_4 concentration. Any other patterns of results represented complex, multifactoral hypothyroxinemias (these included patients with a low overall protein-corrected T_4 but a high free T_4 concentration or patients with a high overall protein-corrected T_4 with a normal free T_4 concentration). Using these criteria, 73 of the 150 patients had hypothyroxinemia due to hypoproteinemia, 44 had hypothyroidism, 28 had defects in T_4 binding activity of their serum proteins, and 5 had complex hypothyroxinemia. Twenty-one of the 44 with hypothyroidism had primary hypothyroidism, and 23 of the 44 had apparent TSH deficiency.

The free T_4 index did not distinguish between the four biochemical mechanisms of hypothyroxinemia. It was below the normal range in all 21 patients with primary hypothyroidism, all 23 patients with apparent TSH deficiency, and all 28 patients with defects of T_4 binding activity. It was also below range in 51 of the 73 patients with hypoproteinemia. In this study the free T_4 index was of diagnostic value only when it was in the normal range, thereby indicating hypothyroxinemia due to hypoproteinemia, but this occurred in only 22 of the 150 patients studied.

DISCUSSION

Low concentrations of T_4-binding proteins were frequent among the 150 hypothyroxinemic patients studied. Only 16 had normal concentrations of all three thyroid hormone-binding proteins. There was a discordance between T_3 binding activity and T_4 binding activity. The T_3 uptake rose proportionately less than the % free T_4 as T_4-binding protein levels fell. Neither the increase in T_3 uptake nor the increase in the % free T_4 was proportional to the decrease in overall T_4-binding protein levels. This data indicates that T_3 binding activity and T_4 binding activity are partially separable characteristics of serum proteins. Low T_4-binding proteins are the most common cause of hypothyroxinemia in patients with non-thyroidal illnesses. These patients cannot be reliably distinguished by their free T_4 index values. Measures of T_3 binding activity provide little information about the biochemical mechanisms of hypothyroxinemia and low free T_4 index results are nonspecific in hypothyroxinemia.

MEASUREMENT OF "FREE THYROXINE" IN DRIED BLOOD SAMPLES ON FILTER PAPER BY RADIO- AND ENZYME-IMMUNOASSAY FOR MONITORING THYROID FUNCTION

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INTRODUCTION

Mass screening for congenital hypothyroidism in newborns has been developed because the irreversible mental retardation caused by this disorder can be prevented by early treatment. These programs include measurement of thyrotropin or total thyroxine (T_4), or both, in dried blood samples. Measurement of total T_4 is useful for detecting primary, secondary, and tertiary hypothyroidism, but gives false-positive results for subjects with abnormal concentrations of thyroxine-binding globulin (TBG), who do not need to be treated. Hence, we developed an enzyme immunoassay (EIA) and a radioimmunoassay (RIA) for measuring free T_4 (FT_4) in dried blood samples on filter paper.

MATERIALS

T_4-galactosidase conjugate: The conjugate was prepared by the method of Ito et al. (1). T_4 was mixed with 4-(maleimidomethyl)cyclohexane-1-carboxylic acid succinimide ester, and this mixture was added to D-galactosidase solution and applied to a column of Biogel A-5m.

^{125}I-T_4-analogue: ^{125}I-labeled T_4 analogue was from Amerlex Free T_4 RIA kit.

Free T_4 standard in dried blood samples: Heparinized pooled blood from normal subjects was centrifuged and the packed cells were washed with phosphate buffered isotonic saline (PBS). The washed packed cells were then mixed with an equal volume of the serum-based standard FT_4.

PROCEDURES

EIA for FT_4: Two 3-mm,-diameter blood discs, each of which contained about 2.7 µl of blood, were punched out from standard or test blood spots on filter paper. The discs were soaked in 100 µl of anti-T_4 IgG solution in a test tube for 30 min at room temperature. Fifty µl of T_4-galactosidase conjugate solution were added to the tube and the mixture was incubated for 5 h at room temperature. After adding 10 µl of normal rabbit IgG and 50 µl

of anti-rabbit IgG goat IgG, the mixture was incubated for 20 h at room temperature. Two ml of washing solution were then added to the mixture and centrifuged. The resulting precipitates were washed twice with 2 ml of washing solution and suspended in 1 ml of a 1 g/l solution of o-nitrophenyl-β-D-galactopyranoside containing 50 ml of methanol per liter. After incubating the suspensions for 2 h at 37°C, we mixed them with 1 ml of 30 g/l sodium carbonate solution and measured the absorbance at 405 nm.

RIA for FT_4: Blood spots 9 mm in diameter, equivalent to about 25 µl of blood, were soaked in 300 µl of PBS and vortex-mixed for 20 min at room temperature. To elute 100 µl of the mixture, 150 µl of $^{125}I^-$ T_4 analogue solution and 150 µl of solid-phase anti-T_4 antibody suspension were added, shaken gently, incubated at 37°C for 1 h, and centrifuged. The supernatant fluid was discarded and the radioactivity of the precipitate was counted.

Bindings of ^{125}I-T_4, ^{125}I-T_4 analogue, and T_4-galactosidase conjugate to TBG and albumin: Twenty µl of TBG (0.6 µg) were mixed with 200 µl of T_4-galactosidase conjugate solution (diluted 150-fold), 200 µl of ^{125}I-T_4, or 500 µl of ^{125}I-T_4 analogue, and incubated for 30 min at room temperature. After adding 200 µl of anti-TBG antiserum, the bound and free forms of these T_4 tracers were separated by adding the second antibody. The enzyme activity or the radioactivity in the precipitate was measured. After adding various amounts of albumin to the T_4-galactosidase conjugate, ^{125}I-T_4, or ^{125}I-T_4 analogue, and mixing this with anti-T_4 antiserum, the second antibody was added to precipitate the bound forms, and the enzyme activity or radioactivits of the precipitate was determined.

RESULT

Binding of T_4-galactosidase conjugate to TBG and albumin: Binding of ^{125}I-T_4 to TBG was calculated to be 90% of the total; whereas, bindings of the T_4-galactosidase conjugate and ^{125}I-T_4 analogue to TBG was negligible.

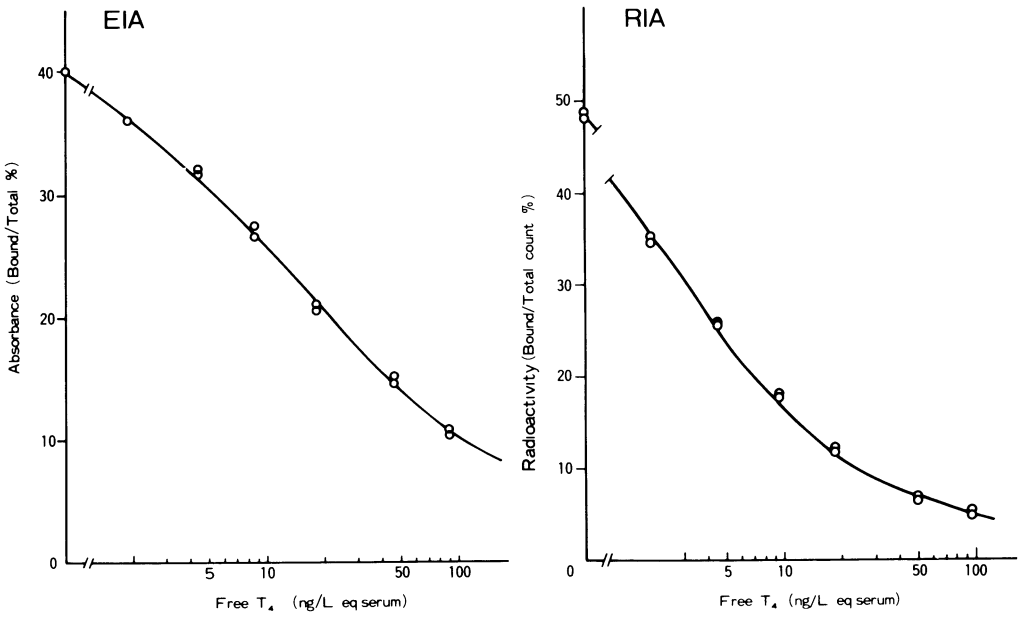

Fig. 1. Calibration curves for FT_4 in dried blood samples.

The bindings of ^{125}I-T_4 and ^{125}I-T_4 analogue with anti-T_4 antiserum decreased with the concentration of albumin; whereas, binding of the T_4-galactosidase complex with anti-T_4 was unaffected by concentrations of albumin up to 100 g/l.

Calibration curve: Fig. 1 shows typical calibration curves. The minimum detectable concentration of FT_4 determined by EIA was 1.9 ng/l of equivalent serum (5.1 fg per assay tube) from the point where the 95% confidence limit of the response at zero dose intersected the calibration curve, in six replicate determinations of FT_4 standard blood spots, and the measurable range of FT_4 was 1.9 to 93 ng/l. The minimum detectable concentration of FT_4 by RIA was 0.8 ng/l (4 fg per assay tube), and the measurable range of FT_4 was 1.8 to 93 ng/l.

Reproducibility: In both methods, when the blood spots were left to dry at room temperature, the changes in FT_4 values did not exceed 15% on subsequent storage at either -20°C, room temperature, or 37°C. Coefficients of variation (CVs) within and between assays of EIA for FT_4 were 6.0 to 9.0 (mean 7.6%) and 5.6 to 7.1 (mean 6.4%), respectively, for dried blood samples stored at -20°C. CVs within and between assays of RIA were 3.8 to 7.0 (mean 5.3%) and 5.9 to 6.4 (mean 6.2%), respectively.

Comparison of EIA and RIA: The FT_4 concentrations in 47 samples of dried blood, as determined by the EIA (x), correlated well with those as determined by RIA (y): ($y=1.1x$; $r=0.99$; $p<0.001$).

FT_4 concentrations in various subjects: FT_4 concentrations determined by EIA using dried samples of normal blood were 6.5 to 14.9 (mean 10.7) ng/l for adults, and 9.8 to 20.7 (mean 15.2) ng/l for neonates (Fig. 2). Those determined by RIA were 8.5 to 20.3 (mean 14.3) ng/l for adults, and 9.7 to 22.9 (mean 16.3) ng/l for neonates. Using both methods, the FT_4 concentration in blood spots was low in cases of primary and secondary hypothyroidism, high in cases of hyperthyroidism, and within normal range in case of low TBG. In cases of pregnancy (25th to 36th weeks of pregnancy), FT_4 concentrations determined by EIA were all within normal range, but two of those determined by RIA were slightly lower than the normal range.

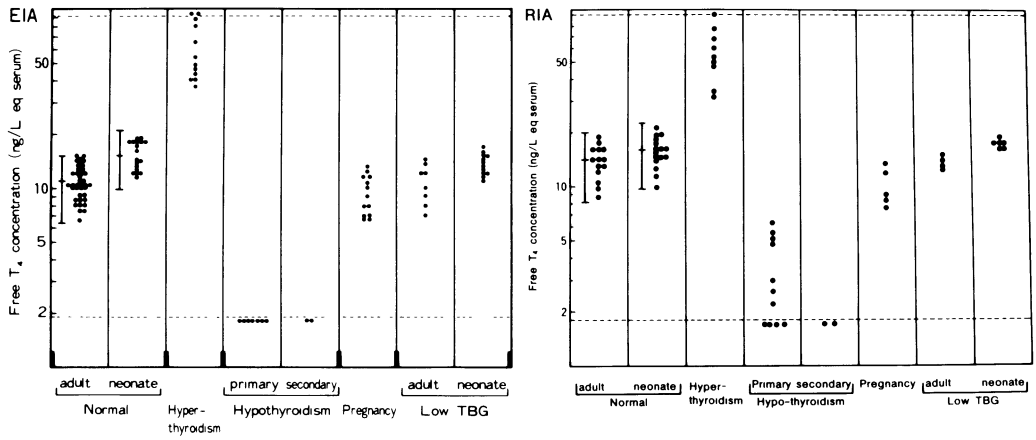

Fig. 2. Concentrations of FT_4 in dried blood spots from various subjects determined by EIA (left) or by RIA (right).

DISCUSSION

It was demonstrated that the present EIA and RIA for FT_4 in dried blood samples could detect clearly hyper- and hypothyroidism even in subjects with alteration of TBG concentration. However, the slightly lower values of FT_4 were observed in pregnancy by the present RIA. It may be due to changes in albumin concentration, because the T_4 analogue used in RIA does not bind to TBG, but binds to albumin. On the other hand, in the present EIA, the binding of T_4-galactosidase conjugate to anti-T_4 antibody is unaffected by the concentration of serum albumin. Furthermore, it can be done in a routine laboratory since isotope is not employed. Since a small amount of blood samples can be easily taken on filter paper and the properly dried samples may be sent by mail even at room temperature, the present methods were found to be useful for monitoring thyroid disorders in newborns.

SUMMARY

We described the developments of EIA and RIA for determining FT_4 in dried blood samples on filter paper. The measurable ranges of FT_4 were 1.9 to 93 ng/l in EIA and 1.8 to 93 ng/l in RIA. The precisions were proven by sufficient reproducibilities of within and between assays, and a good correlation between FT_4 levels determined by EIA and RIA. FT_4 in dried blood samples was stable for at least four weeks when kept dry. The present methods clearly differentiated patients with hyper- and hypothyroidism from normal subjects and those with abnormal concentrations of TBG.

REFERENCES

1. Ito M, Miyai K, Doi K, et al. Clin Chem 30: 1682, 1984.
2. Amino N, Nishi K, Nakatani K, et al. Clin Chem 29: 321, 1983.

EFFECT OF DILUTION ON FT_3 and FT_4 CONCENTRATION IN SERUM CONTAINING SUBSTANCES THAT INTERACT WITH THYROID HORMONE-BINDING PROTEINS

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INTRODUCTION

Almost all available methods for FT_3 and FT_4 determination include a dilution step of serum. Even in the direct determination using dialysis, a method that has been regarded as a reference technique, dilution is unavoidable. The effect of dilution will be more pronounced on an interacting but weakly bound substance than on the binding of thyroid hormones to the transport proteins. This means that, in serum that contains such compounds, the free concentration of thyroid hormones will be underestimated, if dilution is employed.

We, therefore, undertook to develop a method capable of separating free and protein-bound thyroid hormone without dilution of plasma and at circumstances as near *in vivo* conditions as possible. This method enabled us to determine the true concentration of free hormone also in the presence of an interacting substance.

The method is cumbersome and more expensive than existing methods, and is designed mainly for research and reference purpose.

MATERIALS AND METHODS

The method is based on ultrafiltration by centrifugation of undiluted serum through an especially prepared cellulose membrane (Visking seamless dialysis tube, 0.25 inch, Union Carbide Corporation, Chicago, USA). The T_3 or T_4 contents in the ultrafiltrate were determined with radioimmunoassays.

Preparation of the Visking Tube

The tube was initially boiled in distilled water for 10 min, thereafter rinsed in 0.1 M NaOH, then in 0.1 M HCL, and finally in 0.1 M phosphate buffer, pH 7.4. After cutting the Visking tube into suitable pieces, it could be stored at 5°C for one week in modified Krebs-Ringer bicarbonate buffer (KRB), pH 7.4.

Ultrafiltration

Serum was adjusted to pH 7.4 at 37°C with CO_2 and 2 ml of serum was introduced into a loop of Visking tube and placed in a glass tube gassed with 5% CO_2 just before plugging. The glass tube was incubated for 60 min at 37°C. After incubation, the Visking tube was transferred to a PYREX® glass tube (104 x 16 mm). The test tubes were centrifuged at 37.7 ± 0.3°C (mean ± SD, N = 9) at 260 G for 120 min.

Each 2 ml centrifuged in this way yields approximately 200 µl of ultrafiltrate, meaning that FT_4 and FT_3 RIA had to be performed on separate ultrafiltrates.

Radioimmunoassays

Radioimmunoassay for FT_3 and FT_4 was performed as previously described for serum dialysates (1) with modifications.

Albumin in ultrafiltrates was determined with a sensitive radioimmunoassay, described by Christensen and Orskov for urine analysis, with modification in employed tracer quantity only (2).

Serum

Serum for the various controls, inter- and intraassay CV determination and for use in the fenclofenac experiments was pooled from more than five healthy blood donors.

Sera from myxoedematous and thyreotoxic patients were obtained from inpatients. Their metabolic state was estimated by standard biochemical and clinical tests.

Blood was drawn from pregnant women within one month prior to expected delivery. All had normal pregnancy and absence of thyroid illness.

Serum for determination of normal range and serum from women on oral contraceptive therapy were obtained from healthy medical or physiotherapy students.

RESULTS

The manufacturer recommends that the Visking tube should be boiled and rinsed in distilled water before use. If this treatment is applied solely, the Visking tube will liberate substances which give crossreactions and spurious values in both T_3 and T_4 radioimmunoassays. Adsorption of hormone to the tube and a substantial binding of both thyroid hormones to ordinary plastic- and glassware made it also mandatory to find other ways of pretreatment of the Visking tube before use. Data to illustrate these three problems is given in Table 1.

The liberation of crossreacting substances could be abolished by flushing the Visking tube with large volumes of NaOH followed by HCL, and finally phosphate buffer, pH 7.4, to retain physiological pH. Apparently, substances bound by ion-dependent force were washed away by this procedure. This procedure did not change the ability of the Visking tube to retain proteins.

The prepared dialysis tube bound ^{125}I-tracer hormones to the same extent as unprepared dialysis tube, when ultracentrifugation was performed

Table 1. Changes in Measured Levels of T_3 and T_4 Dissolved in KRB After After Two Hours of Centrifugation Through a Visking Tube

	1) Added hormone concentration	2) Retentate	3) Ultrafiltrate	4) Glass tubes
T_3 (pmol/l)	0.0	9.8	12.4	0.1
	6.1	21.5	19.4	4.8
	12.3	26.7	25.8	9.5
	24.6	24.0	20.6	20.0
	49.2	42.4	36.9	40.9
T_4 (pmol/l)	0.0	0.6	0.6	0.6
	20.6	10.3	7.7	14.2
	41.2	23.2	21.7	37.3
	82.4	43.8	46.3	66.9
	164.7	95.2	94.0	127.4

Visking tube prepared solely by boiling and flushing with distilled water. Concentration is given for added standard (column 1), in the retentate (column 2), and in the ultrafiltrate (column 3). Furthermore, the measured concentrations are shown after two hours of incubation in ordinary glass test tubes (column 4) (mean, N = 3).

in KRB (without serum). But this binding is no problem when the protein-bound concentration of hormone is high inside the Visking tube. In normal serum diluted 1:100, the binding of hormone was less than 2%. The total hormone concentrations in 1:100 diluted normal serum are far less than total hormone concentrations in undiluted serum from any pathophysiological condition. The binding process is in equilibrium within one hour.

Another problem was binding of thyroid hormones to ordinary glassware. The only glass tube quality that could be used was Pyrex, that bound less than 1% during an incubation time of 2 hours.

Leakage of microquantities of binding proteins during the centrifugation will obviously give rise to spurious high FT_3 and especially FT_4 values.

Fig. 1 shows the T_4 or T_3 contents versus the albumin concentration in the ultrafiltrate. To illustrate the problem of leaking Visking tubes more clearly, 4 ml instead of the usual 2 ml were introduced into the Visking tubes and run by approximately double the G-force (470 G) than ordinary samples. On the basis of such experiments, the cutoff value for albumin in ultrafiltrate for FT_4 determinations was set at 0.03 µmol/l and, for FT_3, at 0.3 µmol/l. Every ultrafiltrate was tested and approximately 10% of FT_4 and 2.5% of FT_3 determinations were falsely too high, because the Visking tube had been unable to retain proteins, but there was great difference between different batch numbers of Visking tube.

The intraassay CV for the whole procedure was: FT_4: 11.0% (mean 31 pmol/l, N = 12); FT_3: 8.7% (mean 6.4, N = 12). The intraassay CV for two

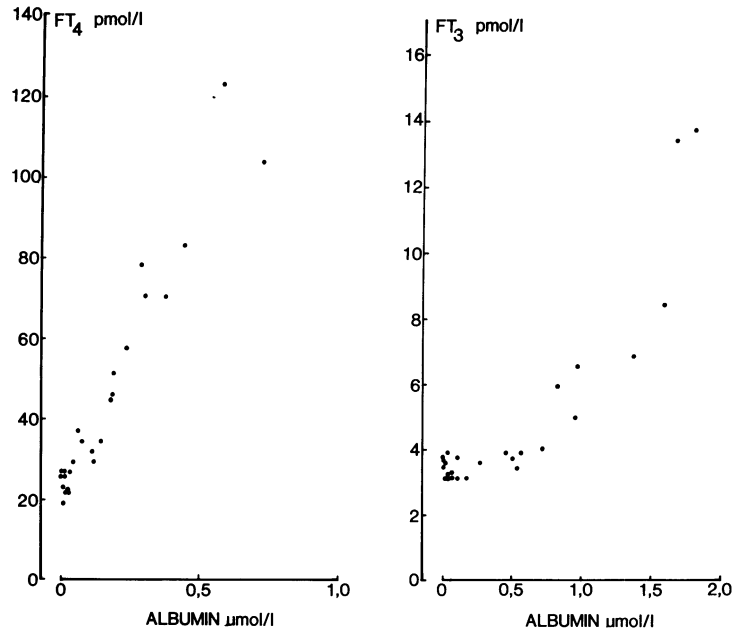

Fig. 1. Simultaneous determination of albumin and FT_4 or FT_3 in ultrafiltrates from the same normal serum pool. 4 ml of serum was placed in each Visking tube and run at 470 G for 2 hours.

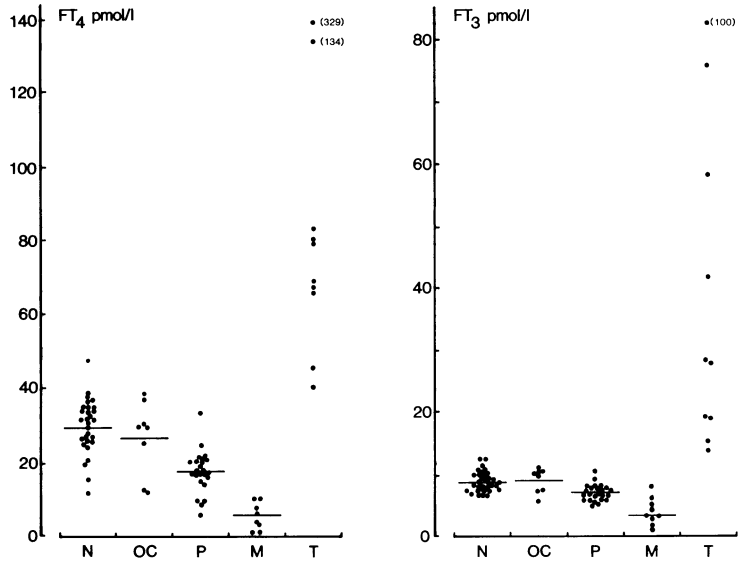

Fig. 2. FT_4 and FT_3 determination by ultrafiltration and RIA in various groups of subjects. N denotes normal persons, OC normal women on oral contraceptive medication, P normal pregnant women within one month prior to estimated delivery, M myxedematous patients, and T thyreotoxic patients.

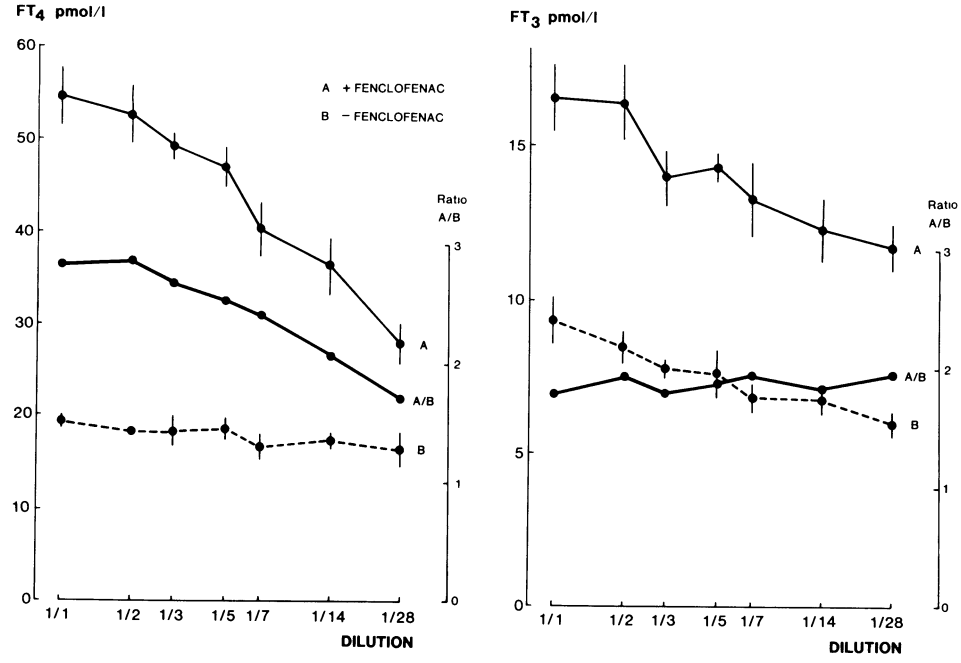

Fig. 3. A normal serum pool was spiked with fenclofenac in high therapeutic concentration (200 µg/ml). Values of measured FT_4 and FT_3 by ultrafiltration versus dilution of serum with modified KRB. Solid lines indicate serum with fenclofenac (A); dotted lines indicate unspiked serum (B). (Mean ± SE, N = 6). Heavy solid lines indicate ratio of spiked to unspiked serum for FT_4 and FT_3, respectively (A/B).

control sera (FT_4: 19 and 60 pmol/l, FT_3 9.8 and 24.6 pmol/l) was between 8.8 and 11.4%.

Fig. 2 gives the mean and range for various groups of subjects.

As an example of application of the ultrafiltration method to serum containing an interacting substance, the effect was studied by adding 200 µg/ml fenclofenac to normal serum. Fenlofenac is a potent anti-inflammatory drug with a high degree of binding to serum proteins. Data are shown in Fig. 3.

Fenclofenac has a more pronounced effect on T_4 binding to proteins than on T_3 binding. The relative amount of displaced T_4 was approximately 120% higher in undiluted than in diluted (1:28) serum, when fenclofenac was present in both.

DISCUSSION

Our aim with the present study was to develop a method capable of coping with the drawbacks of the current standard method for estimating free thyroid hormones in serum. Furthermore, the method should be usable as a reference for evaluation of new methods. These requirements implied that the separation of free and bound hormones should be without dilution, that it should take place in a milieu as near in vivo as possible, and that the binding between an interacting substance and thyroid hormone-binding proteins should

be as undisturbed as possible. The method described in this study fulfills these requirements. In the last years, many new and rapid methods have been introduced. These methods are devised for daily routine purposes, but insufficient in a number of cases (3), and for many research purposes.

During treatment with the anti-inflammatory drug fenclofenac, total hormone concentrations are drastically reduced and free hormone levels as assayed by equilibrium dialysis fall to low normal levels, after an acute minor rise at the start of the treatment (4). The 120% increase of FT_4 in serum spiked with a therapeutic concentration is in accordance with these data. At the start of medication, the FT_4 level is probably elevated more than the 20% found by Taylor et al., resulting in a reset of TSH secretion and a new level of thyroid hormones in the blood determined through the negative feedback mechanism. The patients had probbly normal FT_4 but due to different dilution effects on fenclofenac and thyroid hormone binding to serum proteins, this could not be demonstrated by equilibrium dialysis.

CONCLUSION

By using the present methods, drawbacks of the equilibrium dialysis method can be avoided, and a new standard for research purpose, evaluating exact thyroid hormone status and for evaluation of the increasing number of commercial analog methods is established.

REFERENCES

1. Weeke J and Orskov H. In KGMM Alberti (ed), Recent Advances in Clinical Biochemistry, Churchill Livingstone, Edinburgh, 1978, p 111.
2. Christensen CK and Orskov C. Diabetic Nephropathy 3+4: 92, 1984.
3. Stockigt JR, Stevens V, White EL, et al. Clin Chem 29: 1408, 1983.
4. Taylor R, Huttong C, and Weeke J. Clin Endocrinol (Oxf) 19: 683, 1983.

EVIDENCE FOR DUAL PERIPHERAL 5'DEIODINASES (5'D) REGULATING CIRCULATING T_3 LEVELS IN MAN*

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The majority of circulating T_3 in euthyroid man appears to be derived from conversion of T_4 to T_3 via 5'deiodinase (5'D) systems located in peripheral tissues. Recently, we have reported that a progressive reduction in the efficiency of T_4 to T_3 conversion occurs when circulating T_4 levels rise from subnormal to supraphysiological concentrations (1,2). As shown in Fig. 1 (left panel), the greatest change in conversion efficiency of T_4 to T_3 is seen over the span of T_4 values ranging between 0 and 5 µg/dl in eu-TBG-emic subjects. These data were obtained in 254 patients who had been placed on varying doses of chronic T_4 therapy. Some of the T_3 values in patients with identical serum T_4 levels have been averaged in order to obtain a better overview of the mean pattern of the T_3 response in the study population. In separate T_4 tracer kinetic studies, we have found that a 50% conversion efficiency of T_4 to T_3 occurs at low serum T_4 values which progressively drops to less than 15% at T_4 levels in excess of 20 µg/dl (3). It is of interest that no apparent alteration in the efficiency of T_4 to rT_3 was detected over this same range of serum T_4 values (2).

We initially chose to describe this variable T_4 to T_3 conversion as an "autoregulation" phenomenon in that there is a tendency for serum T_3 values to remain within the normal physiologic range despite widely varying T_4 levels. It is noteworthy that several studies have previously described this blunting in the rise of the serum T_3 response to exogenously administered T_4 when going from physiologic to supraphysiological T_4 concentrations (4-6). It has also been speculated that this failure of T_3 levels to proportionately rise may be responsible for the lack of development of thyrotoxic symptoms in patients who habitually receive excessive doses of exogenous T_4 (7).

ENZYME KINETIC ANALYSIS

The initial goal of the present study was to attempt to linearize the serum T_3 response curve at varying T_4 concentrations in order to be able to more accurately predict the serum T_3 level at any given serum T_4 value. Reasoning that the serum T_3 response to elevation of the circulating T_4

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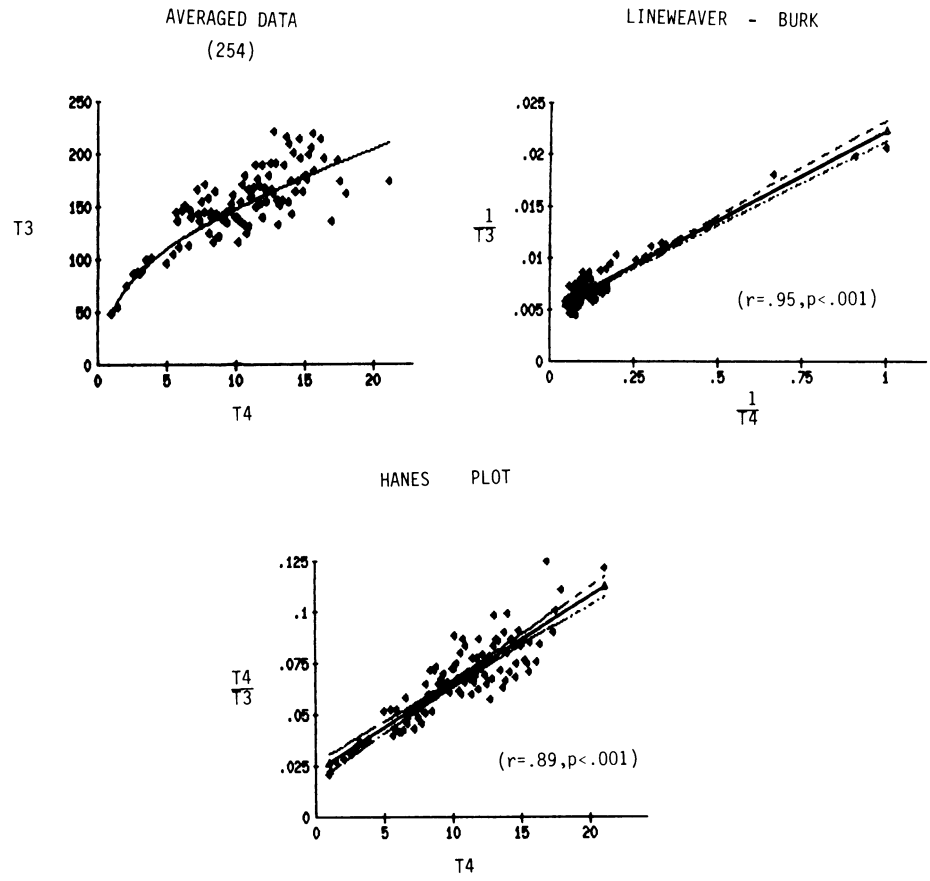

Fig. 1. Left panel displays comparison of serum T_3 (ng/dl) on the ordinate and serum T_4 (µg/dl) on the abscissa in 254 subjects on T_4 therapy. Serum T_3 values were averaged where serum T_4 was the same. Right and bottom panels display the same data on Lineweaver-Burk and Hanes plots, respectively.

level should be, in part, a reflection of the 5'D system(s) responsible conversion process, we applied standard double reciprocal plot analysis, (lineweaver-Burk and Hanes plots), to the data, as shown in the middle and right-hand panels of Fig. 1. In this analysis, we assumed that S (substrate) = serum T_4 concentration and V (velocity) = serum T_3 concentration. These assumptions are valid if serum T_4 production is constant and the clearances of T_3 and T_4 remain unaffected over the T_4 dosage range employed or are influenced in the same direction and by the same degree. It has previously been shown that T_3 and T_4 clearances are affected in the same direction by alterations in thyroid status (8), although T_3 clearance is altered to a greater degree than T_4. Efforts were made to minimize this disparity in clearances resulting from changes in metabolic status by withdrawing exogenous T_3 therapy a few days prior to study. Inspection of the Lineweaver-Burk plot (Fig. 1, middle panel) and the Hanes plot (Fig. 1, right panel) reveals an excellent correlation with both forms of analysis. The Hanes plot and the Lineweaver-Burk plot are derived from the same Michaelis-Menten equation. The principal advantage of the Hanes plot is that data is more evenly spread over the T_4 dose range, thereby reducinc errors at low substrate concentrations (9).

The close correlation seen between these two graphical methods for analyzing enzyme kinetics is suggestive that the serum T_3 to serum T_4 response curve is regulated by a process, possibly enzymatic in nature, that follows Michaelis-Menten kinetics. That is, that under steady-state conditions, the conversion of circulating T_4 to T_3 is subject to a rate-limiting first-order kinetic process (9). It is recognized that this rate-limiting step could represent a transport phenomenon, as well as an enzymatic process.

ENZYME INHIBITION STUDIES

To address the question as to whether the curvilinear response relating circulating T_4 and T_3 levels is enzymatic or not, studies were performed on an individual subject who was given a large oral load of T_4, (3.0 mg). After allowing two days for the oral T_4 load to equilibrate, serum T_4 and T_3 values were measured on a daily basis for the subsequent two week period, during which time, circulating T_4 levels returned to the normal range. This protocol was then repeated in the presence of oral PTU, (200 mg/q 6 h). The Lineweaver-Burk analysis of the T_4 and T_3 relationship following the T_4 load, with and without PTU, demonstrated nonparallelism between the PTU and control studies, as shown in the right panel of Fig. 2. Parallelism would have indicated that PTU produced an uncompetitive inhibitory effect, as has been shown for 5'D systems in rat liver homogenate systems (10). The lack of parallelism in the Lineweaver-Burk analysis was thought to be either 1) due to the variability of the data base, ($r = 0.64$, $p<0.002$), resulting from the short half-life of PTU in the circulation, or 2) might reflect the involvement of more than one enzyme system in the generation of circulating T_3 from T_4. This latter view is consistent with the observation that two major 5'D systems appear to exist in peripheral tissues (10). The Type I enzyme possesses a low affinity and a high K_m, (Michaelis constant or the substrate concentration at which one half maximal velocity of the reaction occurs), for T_4 and is inhibited by PTU, while the Type II system has a high affinity and low K_m for T_4. A further suggestion that two enzyme systems are responsible for T_4 to T_3 conversion, was that the serum T_3/T_4 ratio values seen on the Hanes plot (right panels of Fig. 1 and 3 and the left panel of Fig. 2) were consistently below the projected line for values <4 µg/dl.

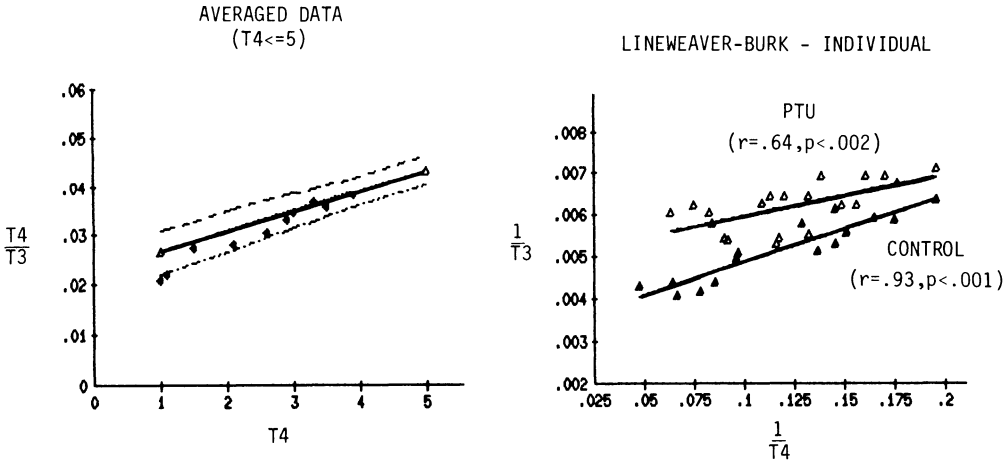

Fig. 2. Left panel shows an enlargement of the Hanes plot at low concentrations in the population study. Right panel shows the Lineweaver-Burk plot on an individual subject before and after propylthiouracil (PTU) therapy.

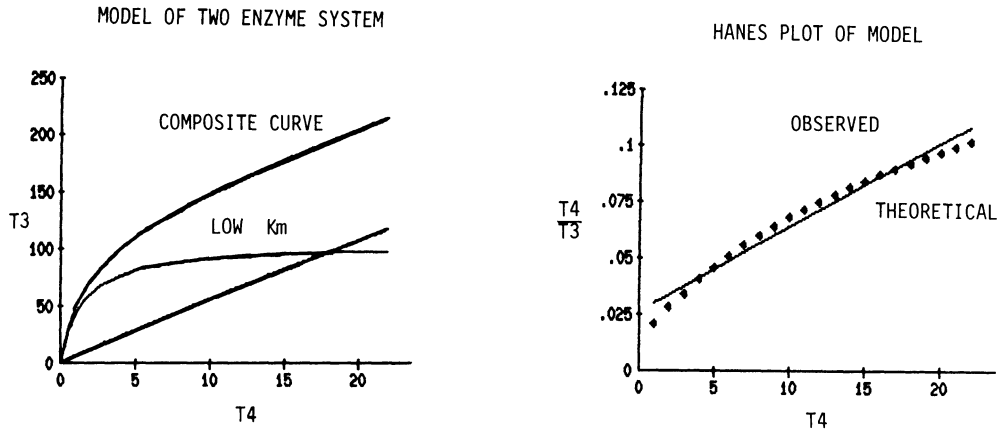

Fig. 3. Left panel shows a theoretical model of low and high K_m enzyme systems which may be responsible for generating circulating T_3. The right panel shows a comparison of the theoretical (solid line) and mean observed (dotted line) values on the Hanes plot of the large population data.

Working on the assumption that two enzyme systems are likely to be involved in the generation of circulating T_3 from T_4, we were able to construct a hypothetical model from our large population data. The results of this analysis are graphically shown in the left panel in Fig. 3. This model indicates that the K_m values were 240 and 1.5 μg/dl for the Type I and Type II enzyme systems, respectively. It is also noteworthy that the apparent K_m (right-hand panel of Fig. 2) changed from 4.3 to 1.2 μg/dl following PTU therapy. This decline in the apparent K_m is consistent with PTU's known selective inhibition of the Type I enzyme system and possibly indicates that the K_m of a high affinity system is in the range of 1-2 μg/dl of T_4. Other preliminary data (not shown) indiates that acute fasting may selectively inhibit the Type II enzyme system as shown in the apparent increase in K_m from 5.2 to 28 μg/dl.

By employing Michaelis-Menten kinetic analysis, it can be concluded that serum T_3/T_4 values can be accurately predicted in human subjects on T_4 therapy. This correlation is especially strong in individual subjects, (Fig. 2, right control, r = 0.93). Analysis of data at low T_4 concentrations shows that this is the product of two first-order rate limiting steps which may or may not be enzymatic in character. Although obvious caution must be exercised in interpreting such data with respect to whole body processes, it is hoped that by employing selective inhibitors of the enzymatic conversion of T_4 to T_3, the mechanisms regulating circulating T_3 will be elucidated in the future.

REFERENCES

1. Nicoloff JT, Lum SMC, Spencer CA, et al. Hormone and Metab Res Suppl #14, 74, 1984.
2. Lum SMC, Nicoloff JT, Spencer CA, et al. J Clin Invest 73: 570, 1984.
3. Morris RR, Lum SM, Kaptein EK, et al. Clin Res 31: 274, 1983.
4. Braverman LE, Vargenakis A, Downs P, et al. J Clin Invest 52: 1010, 1973.
5. Shimizu TC, Pittman JB, Chambers JB, et al. In Thyroid Research, Excerpta Med Int Congr Ser: 263, 1975.
6. Inada M, Kasagi K, Kurata S, et al. J Clin Invest 55: 1337, 1975.

7. Rendell M and Salmon D. Clin Endocr 22: 693, 1985.
8. Nicoloff JT and Dowling JT. J Clin Invest 47: 2000, 1968.
9. Cornish-Bowden A. Fundamentals of Enzyme Kinetics, Butterworths, London, 1979.
10. Larsen RR, Silva JE, and Kaplan ML. Endocr Rev V 2(1): 87, 1981.

EVIDENCE FOR PERIPHERAL AUTOREGULATION OF THYROXINE CONVERSION*

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INTRODUCTION

The reciprocal changes of triiodothyronine (T_3) serum concentrations and (SC) and thyroxine (T_4) SC observed in patients with varying degrees of primary hypothyroidism are well known. T_3 SC are often still within normal limits, when T_4 SC have already reached low-normal or subnormal levels. The patients do not have clinical evidence of hypothyroidism at that time (1). Considering the other extreme of the spectrum, most athyroid patients have a negative TRH test only if T_4 SC are raised to levels beyond the normal range by large doses of T_4. This is associated with T_3 SC in the upper normal range. These patients do not appear to be clinically hyperthyroid (2). These findings suggest that a non-thyroidal mechanism accounts for the reciprocal changes in T_4 SC and T_3 SC. The purpose of this clinical study was to clarify the role of these non-thyroidal mechanisms in regulating T_3.

METHODS

The study population consisted of 22 athyroid patients, who had undergone thyroidectomy and radioiodine therapy for thyroid cancer at least one year before. All patients were found to be free of metastases at regular follow-up examinations. Substitution therapy with T_4 was discontinued at least four weeks, and T_3 was withdrawn at least eight days before the initial TRH test was carried out. Substitution therapy with T_4 was then restarted. The dose was 0.7 µg/kg body weight per day in the first week, increasing by increments of 0.7 µg/kg body weight per day every week until the TRH test became blunted ($\Delta TSH \leq 3$ mU/l). The TRH test was carried out at the seventh day of each level of substitution therapy. Blood samples were obtained at the time of the TRH test. The specimens were frozen at -20°C and SC of TSH, T_4, T_3, and reverse T_3 (rT_3) were determined by radioimmunoassay. (T_4 and T_3 kits from Bayer-Diagnostic, sensitivity 0.5 µg/dl for T_4 and 20 ng/dl for T_3, respectively; rT_3 kit from Serono Diagnostics Inc., sensitivity 2 ng/dl; TSH kit from Henning Berlin.) In order to detect any alterations in thyroid hormone binding, the T_4-uptake was measured and

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the free thyroxine equivalent (FTE) was determined. The ratios of T_3 SC and T_4 SC (T_3/T_4) and of rT_3 SC and T_4 SC (rT_3/T_4) were calculated from gram concentration units. Conversion into molar concentration ratios can be performed by multiplying these values with the factor 1.194. Regression analysis was done by the method of least squares. Looking for mathematical funtions of curvilinear relations, several mathematical functions were applied to the data and their correlation coefficients were considered a measure of the goodness of fit.

RESULTS

The thyroxine doses per day required to render the TRH test negative varied between 2.1 and 5 µg T_4/kg b.w. The protocol resulted in a total of 141 studies. Each patient had 4 to 8 studies until the TRH test became negative. During the study period, T_4 SC ranged from 0.5 µg/dl to 18.9 µg/dl (normal range 4 to 12 µg/dl), T_3 SC from 20 µg/dl to 294 µg/dl (80 to 180 ng/dl), and rT_3 from 2 µg/dl to 59 µg/dl (16 to 40 ng/dl). Basal TSH SC varied from >50 mU/l to <0.5 mU/l. FTE values correlated highly with T_4 SC ($r = 0.92$, $p<<0.001$). Fig. 1 illustrates the relationship between T_3 SC and T_4 SC. The data can be fitted to an exponential function ($y = 29.6\ x^{0.68}$, $r = 0.88$, $p<<0.001$). When calculating this regression for each subject, the correlation coefficient varied between 0.84 and 0.99. The relation between T_3/T_4 ratio and T_4 SC was found to be an exponential one too ($y\ x^{-0.32}$, $r = 0.74$, $p<<0.001$, Fig. 2). The latter equation corresponds closely to a first derivative of the former one. As illustrated in Fig. 3, the association between rT_3 SC and T_4 SC was less stringent ($r = 0.79$, $p<<0.001$). No correlation could be established between rT_3/T_4 and T_4 SC ($r = 0.11$, $p>0.2$, Fig. 4).

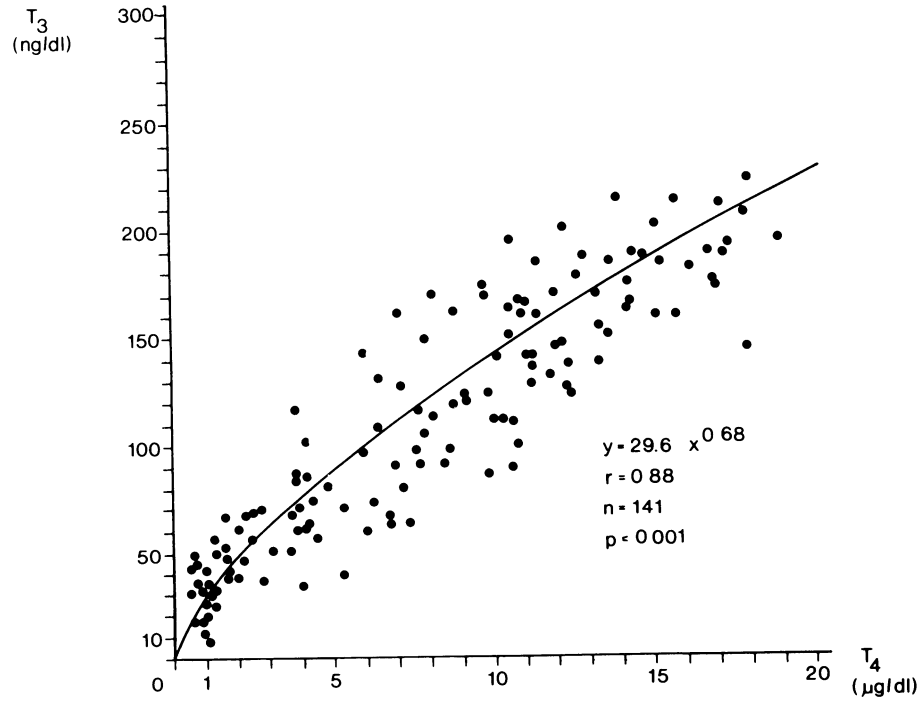

Fig. 1. Association between the serum concentrations of T_3 and T_4 in 22 athyroid patients, treated with varying doses of T_4.

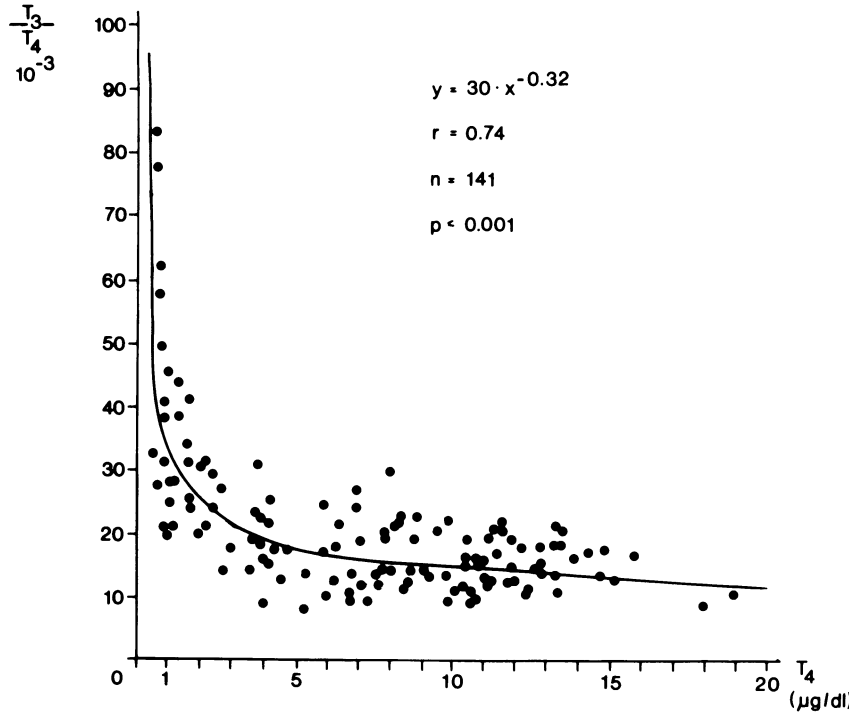

Fig. 2. Replotting of data presented in Fig. 1.

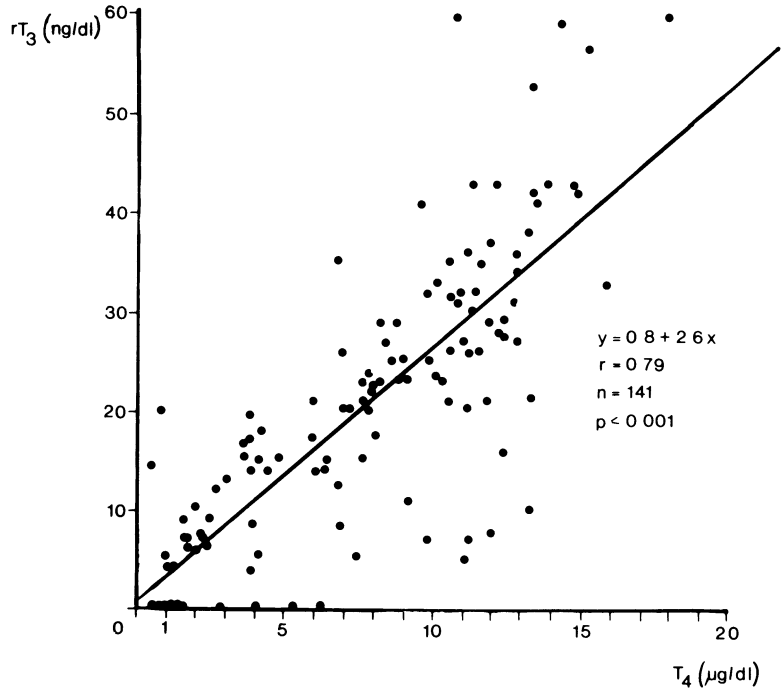

Fig. 3. Association between the serum concentrations of rT_3 and T_4.

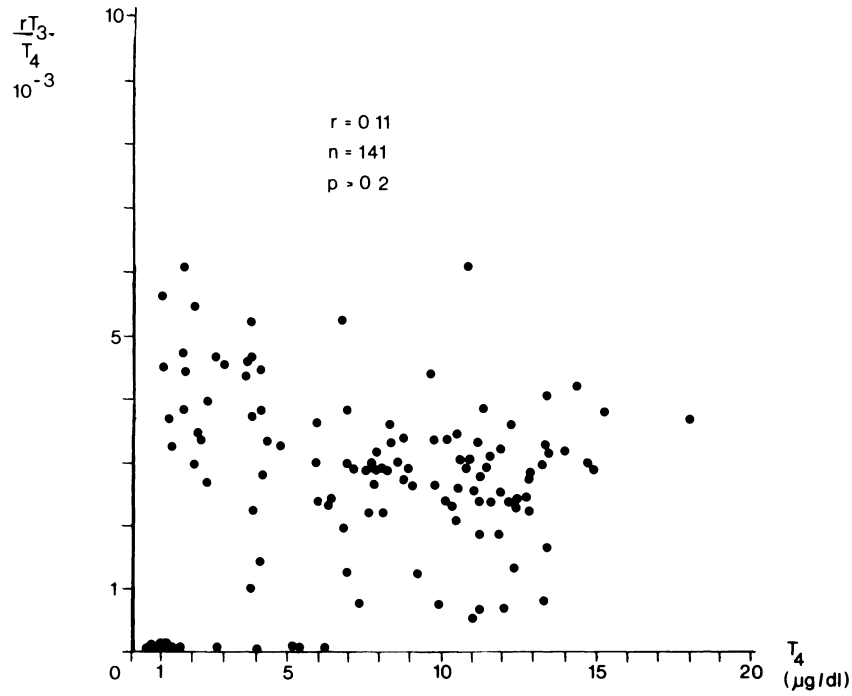

Fig. 4. Replotting of data presented in Fig. 3.

DISCUSSION

This study describes a non-thyroidal system which regulates T_3 SC in T_4 states varying from deficiency to excess. Since the study population consisted of athyroid patients, T_3 and rT_3 are produced by peripheral conversion of the administered T_4 only. The association between T_3 SC and T_4 SC is slightly superior to the association between rT_3 SC and T_4 SC. As illustrated in Fig. 3, the relation between T_4 SC and rT_3 SC is almost proportional. This does not apply to the relation between T_4 SC and T_3 SC. As evidenced by Fig. 1, increments and decrements in T_4 SC are not accompanied by alterations in T_3 SC to the same extent over a wide T_4 SC range. In the very low T_4 range, the increase in T_3 SC is pronounced, while it is flattened at the other end of the spectrum of T_4 values. These data suggest a mechanism besides the pituitary-thyroid axis for the maintenance of normal T_3 SC. This mechanism appears to be operative at both ends of the T_4 SC spectrum. As to rT_3 SC, the data provide no evidence for the existence of an analogous mechanism. The different character of the relationships between T_4 and its conversion products is further elucidated by calculating the ratios of the SC of T_3 and T_4, and rT_3 and T_4, and by relating these values to T_4 SC. As to T_3, this ratio becomes markedly increased when related to T_4 SC in the subnormal T_4 range. This ratio decreases exponentially with rising T_4 SC and has a slightly decreasing tendency in the normal and beyond the normal range. Obviously, the rT_3/T_4 ratio lacks this systematic relationship.

If these ratios reflect peripheral T_4 conversion activity, T_4 to T_3 conversion activity is markedly enhanced in T_4 deficiency states and slightly suppressed in T_4 excess. Therefore, it may be hypothesized, that the fore-mentioned non-thyroidal mechanism is brought about by the peripheral 5'-deiodination of T_4. Conversely, T_4 to rT_3 conversion activity appears to be unaffected by the available T_4.

It is conceivable that other factors are involved in maintaining T_3 SC normal. The altered metabolic states, per se, associated with T_4 deficiency and excess may cause the reciprocal changes in T_3 SC and T_4 SC. The data reported in the literature on this point are controversial. Inada et al. (3) found the metbolic clearance rate (MCR) of T_4 and T_3 to be reduced to about the same extent in hypothyroidism. This finding is at variance with a previous paper from Nicoloff and colleagues (4), who found the MCR of T_3 to be slightly decreased in comparison to T_4. This divergence in serum T_3 and T_4 clearance rates, however, is too slight to account for the steep increase in T_3/T_4 ratio. The MCR of T_3 and T_4 are changed in the same direction and to the same extent by treatment with T_4 in hypothyroidism (3). Moreover, the administration of large doses of T_4 in euthyroid persons resulting in T_4 SC beyond the normal range predominantly increases the MCR of T_4, while the MCR of T_3 is raised to a lesser extent (5).

The correlation between T_4 and the FTE as a measure of thyroid hormone binding was found to be linear and close. Thus, alterations in hormone binding with changing T_4 levels are highly unlikely to account for that mechanism of maintaining T_3 normal.

The administration of high doses of T_4 is accompanied by a decrease in the T_3/T_4 ratio. This phenomenon is well known and has been recommended as a diagnostic criterion in thyrotoxicosis factitia (6). Braverman et al. (5) showed a reduction of T_4 to T_3 conversion in euthyroid volunteers treated with T_4. Administration of T_4 results in a reduction of the extrathyroidal T_4 to T_3 conversion rate, as demonstrated by Maguire et al. (7) in hypothyroid subjects. The findings of Shimizu et al. (8) suggest that the T_4 to T_3 conversion rate changes inversely with T_4 SC. The authors also found a good correlation of the serum T_3/T_4 ratios obtained by both chromatography and radioimmunoassay, if the T_4 SC was above the lower limit of detection of the T_4 assay. As demonstrated recently in man (9), the peripheral conversion rate appears to be independent of TSH SC. The results of the present study agree with recently reported findings of Nicoloff and colleagues (10,11). The current study provides some additional information with respect to rT_3 studied in the same subjects.

The above mentioned studies support the hypothesis that T_3 SC are maintained normal by altered peripheral T_4 conversion activity in states of T_4 deficiency or excess. From a teleological point of view, this mechanism can be considered another regulation system for the maintenance of normal thyromimetic activity. The peripheral system is subordinate to the pituitary-thyroid axis and operates independently from and complementary to the central system. The existence of two regulation mechanisms underlines the important physiologic role of thyroid hormones.

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REFERENCES

1. Larsen PR, Silva JE, and Kaplan MM. Endocrine Reviews 2: 87, 1981.
2. Lamberg B-A, Rantanen M, Saarinen P, et al. Acta Endocrinol (Copenh) 91: 248, 1979.
3. Inada M, Kasagi K, Kurata S, et al. J Clin Invest 55: 1337, 1975.
4. Nicoloff JT, Low JC, Dussault JH, et al. J Clin Invest 51: 473, 1972.
5. Braverman LE, Vagenakis A, Downs, P, et al. J Clin Invest 52: 1010, 1973.

6. Pearce CJ and Himsworth RL. N Engl J Med 307: 1708, 1982.
7. Maguire SB, Dennely A, and Cullen MJ. In J Robbins and LE Braverman (eds), Thyroid Research, Excerpta Medica, Amsterdam, 1976, p 259.
8. Shimizu T, Pittman CS, Chambers Jr JB, et al. In J Robbins and LE Braverman (eds), Thyroid Research, Excerpta Medica, Amsterdam, 1976, p 263.
9. Keck FS, Muller R, Duntas L, et al. Acta Endocrinol Suppl (Copenh) 270: 76, 1985.
10. Nicoloff JT, Lum SMC, Spencer CA, et al. Horm Metab Res Suppl 14: 74, 1984.
11. Lum SMC, Nicoloff JT, Spencer CA, et al. J Clin Invest 73: 570, 1984.

SITES OF T_4 TO T_3 CONVERSION IN THE RAT

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INTRODUCTION

Enzymatic T_4 5'-monodeiodination has been demonstrated in a variety of rat tissues (1,2) and may be an important factor in the regulation of T_3 concentrations in several target sites of thyroid hormone action. As a consequence, under various (patho)physiological conditions, the total T_3 content of tissues which exhibit T_4 to T_3 conversion in vivo may be affected to an extent not disclosed by alterations in circulating T_3 levels. In the present study, we evaluated the relationship between T_3 in several extrathyroidal tissues and plasma T_3 for both euthyroid and hypothyroid rats.

MATERIALS AND METHODS

The study was carried out with two groups of six adult male Wistar rats bred in our laboratory and weighing about 440 g at death. They received a modified AIN diet (3) and drank distilled water, both being available continuously. One group of rats was rendered hypothyroid by ^{131}I thyroid ablation (RTx) two months before the experiments began.

RTx rats received a continuous iv infusion of 0.75 µg T_4/day in order to create a defined hypothyroid state. Five days after the start of T_4 infusion, the animals also received ^{125}I-T_4 (26 µCi/day). On the fourth day of the ^{125}I-T_4 infusion, carrier-free ^{131}I-T_3 (40 µCi/day) was added to the infusion fluid. The same infusions, but without carrier T_4, were administered to intact, euthyroid rats. For both groups of rats, the ^{125}I-T_4 and ^{131}I-T_3 infusions lasted 10 and 6 days, respectively, which is sufficient to achieve isotopic equilibrium (4). To prevent re-use of $^{125}I^-$ or $^{131}I^-$ by eventual thyroid remnants in RTx rats and by the thyroid of intact rats, KI (400 µg/day) was added to the food the week before the infusion. During the iv infusion, KI was administered exclusively via the infusion. The infusions were prepared as described previously and administered iv continuously (4).

At the end of the infusion period, the rats were bled and perfused with saline containing 35 µM iopanoic acid and 0.1 mM PTU (5). The liver, kidneys, skeletal muscle (thigh), cerebral cortex, cerebellum, anterior pituitary gland, thymus, testis, and brown adipose tissue (interscapular) were

excised, weighed, and homogenized in ice-cold saline (0.1 mM PTU). Measured aliquots were taken of the plasma and each tissue homogenate, and their ^{125}I and ^{131}I contents counted. The ^{125}I-T_3 and ^{131}I-T_3 levels (expressed as the percentage of the daily infused dose of ^{125}I-T_4 and ^{131}I-T_3, respectively), and the ^{125}I-T_3/^{131}I-T_3 ratios for plasma and the tissue homogenates, were determined according to methods described previously in detail (4-6) involving extraction with ethanol/ammonia and thin layer chromatography. The corresponding concentrations (per g) of total T_3 and T_3 derived from local T_4 to T_3 conversion ($LcT_3(T_4)$) were calculated according to procedures used in our laboratory (4-6).

Table 1. Concentration of Total T_3 and T_3 Generated Locally from T_4 ($LcT_3(T_4)$), and the Percentage Contribution of $LcT_3(T_4)$ to Total Tissue T_3 in Various Tissues from Euthyroid (Eu) and Hypothyroid Rats (Hypo), as Determined at Isotopic Equilibrium

		T_3 (ng/g)		% Contribution
		Total	$LcT_3(T_4)$	$LcT_3(T_4)$
Liver	Eu	4.50 ± 1.00	1.76 ± 0.66	39.9 ± 10.6
	Hypo	0.91 ± 0.31[a]	0.14 ± 0.09[a]	15.0 ± 7.5[a]
Kidney	Eu	6.58 ± 1.66	0.63 ± 0.33	9.6 ± 5.0
	Hypo	1.22 ± 0.31[a]	0.05 ± 0.06[a]	3.6 ± 3.6[f]
Sk. Muscle	Eu	0.69 ± 0.10	0.04 ± 0.03	4.4 ± 3.4
	Hypo	0.19 ± 0.06[a]	0.01 ± 0.01[f]	1.5 ± 3.7
Ant. Pit.	Eu	5.67 ± 1.31	1.47 ± 0.57	23.4 ± 4.7
	Hypo	1.90 ± 0.38[a]	0.95 ± 0.13	49.8 ± 0.8[a]
Cx	Eu	1.68 ± 0.33	1.06 ± 0.26	65.3 ± 7.2
	Hypo	0.70 ± 0.16[a]	0.52 ± 0.13[b]	75.0 ± 1.8[c]
Ce	Eu	2.12 ± 0.65	1.09 ± 0.34	51.3 ± 3.4
	Hypo	1.02 ± 0.22[b]	0.62 ± 0.13[d]	61.1 ± 1.7[a]
Thymus	Eu	0.98 ± 0.16	0.19 ± 0.07	19.1 ± 5.9
	Hypo	0.30 ± 0.09[a]	0.09 ± 0.04[d]	30.7 ± 9.5[f]
Testis	Eu	0.43 ± 0.09	0.13 ± 0.05	29.1 ± 8.3
	Hypo	0.16 ± 0.04[a]	0.07 ± 0.03[f]	43.8 ± 8.4[d]
BAT	Eu	1.01 ± 0.23	0.28 ± 0.14	27.1 ± 8.7
	Hypo	0.68 ± 0.20[e]	0.46 ± 0.09[e]	65.3 ± 12.9[a]
Plasma	Eu	0.44 ± 0.05 (RIA)		
	Hypo	0.17 ± 0.04[a]		

Results are the mean ± SD, n=6 for both groups of rats. Ant. Pit. = anterior pituitary gland; Cx = cerebral cortex; Ce = cerebellum; BAT = brown adipose tissue. Eu vs Hypo: [a] $p < 0.001$; [b] $p < 0.005$; [c] $p < 0.01$; [d] $p < 0.02$; [e] $p < 0.025$; [f] $p < 0.05$.

The plasma T_4 and T_3 levels were determined by radioimmunoassay (RIA) using ^{131}I-labeled T_4 and T_3, respectively (4).

The results for euthyroid and hypothyroid rats were compared with the Student's two-tailed t test for unpaired variates.

RESULTS

At the end of the infusion period, the mean ± SD plasma T_4 and T_3 levels (RIA) found for T_4-maintained RTx rats (1.5 ± 0.8 μg/dl; 15 ± 5 ng/dl) were significantly lower ($p<0.001$) than those for euthyroid rats (5.2 ± 0.5 μg/dl; 44 ± 5 ng/dl). The plasma T_3 levels in hypothyroid rats, as calculated from the known SA of infused ^{125}I-T_4 (Table 1), were in close agreement with those derived from RIA.

Tables 1 and 2 summarize the results of our T_3 measurements. In both euthyroid and hypothyroid rats, T_3 was most abundant in the kidney and anterior pituitary gland, and least abundant in the testis and skeletal muscle. Hypothyroidism led to a substantial loss of total T_3 in the liver, kidney, and muscle. In the other tissues, the decrease in T_3 was less. In both experiments, locally produced T_3 was the main source of cellular T_3 in the cerebral cortex and cerebellum, while in the kidney and muscle, only a very small proportion of the total T_3 was derived from T_4 to T_3 conversion. The relative contribution of $LcT_3(T_4)$ to the total T_3 in the anterior pituitary gland, cerebral cortex, cerebellum, thymus, testis, and brown fat was greater in the hypothyroid than in the euthyroid state. The reverse was

Table 2. Tissue/plasma Ratios of Total T_3 and ^{131}I T_3 in Euthyroid (Eu) and Hypothyroid (Hypo) Rats

	T_3 (ng/g)		^{131}I T_3 (% daily dose/g)	
	Eu	Hypo	Eu	Hypo
Liver	9.35 ± 1.56	5.36 ± 0.53[a]	6.48 ± 1.21	4.54 ± 0.32[b]
Kidney	13.94 ± 5.46	9.43 ± 4.29	14.80 ± 3.00	9.34 ± 5.24
Sk. Muscle	1.58 ± 0.30	1.28 ± 0.06[e]	1.54 ± 0.25	1.28 ± 0.03[e]
Ant. Pit.	11.87 ± 4.26	12.70 ± 2.50	10.44 ± 3.29	8.70 ± 3.20
Cx	3.92 ± 0.69	4.86 ± 0.86	1.40 ± 0.30	1.26 ± 0.25
Ce	4.88 ± 1.43	7.13 ± 1.40[d]	2.38 ± 0.74	2.84 ± 0.60
Thymus	2.26 ± 0.37	1.68 ± 0.12[b]	1.83 ± 0.25	1.15 ± 0.15[a]
Testis	1.01 ± 0.24	0.86 ± 0.05	0.70 ± 0.10	0.47 ± 0.07[a]
BAT	2.34 ± 0.59	3.92 ± 1.20[c]	1.65 ± 0.39	1.22 ± 0.16[e]

Data represent the mean ± SD (n=6 for both groups of rats). Ant. Pit. = anterior pituitary gland; Cx = cerebral cortex; Ce = cerebellum; BAT = brown adipose tissue. Eu vs Hypo: [a]$p < 0.001$; [b]$p < 0.005$; [c]$p < 0.02$; [d]$p < 0.025$; [e]$p < 0.05$.

found for the liver and kidney. With the exception of the anterior pituitary gland and brown fat, the amount of $LcT_3(T_4)$ in the various tissues had decreased significantly during hypothyroidism.

Although the plasma and tissue ^{131}I-T_3 (infused as such) levels in hypothyroid rats were higher than in euthyroid animals (data not shown), the tissue/plasma ratios of ^{131}I-T_3 (Table 2) for liver, skeletal muscle, thymus, testis, and brown fat were lower in the hypothyroid animals. This would mean that during hypothyroidism, relatively less T_3 is taken up from the circulation which accounts, in part, for the lower T_3 content in these tissues.

DISCUSSION

It has been demonstrated repeatedly that cerebral cortex, cerebellum, anterior pituitary gland, and brown fat exhibit predominantly type II (i.e., PTU-insensitive) T_4 5'-monodeiodinating activity *in vitro*, which increases from the euthyroid to the hypothyroid state. In liver and kidney, both containing mainly a PTU-sensitive type I deiodinase, opposite changes in enzyme activity occur (1,7-9). Thus, our *in vivo* data complement the *in vitro* findings. The marked increment in the amount of $LcT_3(T_4)$ in brown fat of hypothyroid rats may support the concept that this tissue participates in the extrathyroidal production of plasma T_3 during hypothyroidism (10). The hypothyroid-induced increase in the relative contribution of $LcT_3(T_4)$ in the thymus and testis may have been secondary to an accelerated local T_3 production, which then would suggest that these tissues contain a type II T_4-converting enzyme too. In contrast to the liver, kidney, or anterior pituitary gland, no direct thyroid hormone-responsive mtabolic process has yet been identified in the brain tissue, thymus, or testis of adult rats. Hence, the eventual benefit from the increased contribution of $LcT_3(T_4)$ in the latter tissues remains unclear.

At present, we do not know which factors caused the decrease in the tissue/plasma ratios of ^{131}I-T_3 for liver, muscle, thymus, testis, and brown fat in hypothyroid rats. It could have been the result of a relative decrease in the number and/or affinities of cellular binding sites for T_3.

In summary, the data reported in this paper strongly suggest that hypothyroidism affects the source and quantity of T_3 in the various tissues differently, both quantitatively and qualitatively. Several tissues seem to control, at least in part, their own T_3 levels. Since the relationship between circulating T_3 and cellular T_3 appears to vary from one organ to the next, interpretation of measurements of plasma T_4 and T_3 concentrations should be approached with caution.

REFERENCES

1. Kaplan MM. Neuroendocrinology 38: 254, 1984.
2. Chopra IJ. Endocrinology 101: 453, 1977.
3. van Doorn J, van der Heide D, and Roelfsema F. Endocrinology 115: 705, 1984.
4. van Doorn J, van der Heide D, and Roelfsema F. J Clin Invest 72: 1778, 1983.
5. van Doorn J, van der Heide D, and Roelfsema F. Endocrinology 115: 174, 1984.
6. van Doorn J, Roelfsema F, and van der Heide D. Acta Endocrinol 101: 386, 1982.
7. Larsen PR, Silva JE, and Kaplan MM. Endocr Rev 2: 87, 1981.
8. Visser TJ, Kaplan MM, Leonard JL, et al. J Clin Invest 71: 992, 1983.

9. Leonard JL, Mellen SA, and Larsen PR. Endocrinology 112: 1153, 1982.
10. Silva JE, Gordon MB, Crantz FR, et al. J Clin Invest 73: 898, 1984.

EVALUATION OF OLEIC ACID, ARACHIDONIC ACID, AND VARIOUS DRUGS AS COMPETITORS FOR SERUM BINDING OF THYROXINE*

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Numerous drugs can displace thyroid hormones from plasma protein binding (1,2), and it has been suggested that substances of endogenous origin can also inhibit binding, particularly in severe non-thyroidal illness (3-5). The potency of a competitor is influenced by at least three factors: total circulating concentration, free fraction in undiluted serum, and relative affinity for T_4 binding sites. From these three factors, a prediction of inhibitory potency can be made. This prediction can be tested by measuring the effect of circulating concentrations of potential inhibitors on ^{125}I T_4 binding in undiluted serum at 37°C. In this study, we examine these predictions for furosemide, various nonsteroidal anti-inflammatory drugs, and free fatty acids (FFA), which have been assessed because they appear to be promising candidates as thyroid hormone-binding inhibitors (THBI) (3). Recent studies suggest that oleic acid may contribute more importantly to THBI of non-thyroidal illness than other FFA (4). In these studies (3-5), binding was assessed using diluted serum in a competitive ligand binding assay. We have examined the effect of serum dilution on the apparent inhibitory potency of added FFA in order to evaluate effects seen in such assays.

METHODS

Total plasma concentrations of drugs were taken from standard literature (6,7). The free fraction of drug competitors was established by equilibrium dialysis at 37°C of undiluted serum either using ^{14}C preparations, or by spectrophotometric measurement of drug transit from dialysate to serum. For long chain FFA, serum free fraction was measured by the heptane/H_2O partition method of Goodman (8). Relative competition for T_4 binding was measured in serum diluted 1:10,000 using ^{125}I T_4 $<10^{-11}$ M and dextran-charcoal separation at 4°C (2). At this serum dilution, >90% of competitor is unbound. Relative inhibitory potency was predicted from the product:

Circulating Concentration x Free Fraction x Relative Affinity

*This work was supported by grants from the National Health and Medical Research Council of Australia and the Australia-China Council.

Table 1. Potency Assessment of Various T_4 Binding Inhibitors

	[a]Circ Conc μM	[b]Free fraction percent	[c]Relative affinity	[d]Predicted potency	[e]Increase in ^{125}I T_4 free fraction percent
Fenclofenac	270	0.5	2.5	340	72
Mefenamic acid	80	0.7	5	280	22
Meclofenamic acid	60	0.2	14.3	170	27
Phenylbutazone	320	1.1	0.05	18	34
Aspirin	1800	7.5	0.001	14	29
Indomethacin	10	0.4	1.7	7	<5
Naproxen	200	0.5	0.01	1	<5
Furosemide	3	1.2	14.3	50	<5
Furosemide (high dose)	30	1.2	14.3	500	26
Oleic acid	300	0.010	0.04	<1	<5
Arachidonic acid	20	0.014	1.0	<1	<5

a,b,c,d: d = a x b x c; i.e., circ conc x free fraction x relative affinity. e: Effect of concentration a in undiluted serum at 37°C.

Equilibrium dialysis of ^{125}I T_4 in undiluted serum (2) was used to directly examine the effect of various competitors. Drugs were added to the dialysate in amounts calculated to achieve the intended serum concentrations at equilibrium. FFA was added in the serum compartment by appropriate dilution of FFA-enriched serum with normal serum. Oleic acid was added to serum by celite transfer from hexane (9). FFA transfer monitored using ^{14}C oleic acid was 78, 53, and 34% efficient from 20, 40, and 80 mM solutions of oleic acid in hexane. Measurement of total FFA was by the method of Novak (10). Oleic acid and arachadonic acid were from Sigma Chemical Co., St. Louis, MO. Results were comparable with sodium oleate from the same source.

RESULTS

The values for circulating concentration, free fraction, and relative affinity for T_4 binding sites of various drugs are shown in Table 1, predicted potency being calculated as the product of the three variables. The predicted potency has been compared to the percent increase in ^{125}I T_4 free fraction by equilibrium dialysis at 37°C at the appropriate final concentrations of inhibitor. While the general relationship is similar, there are substantial discrepancies. In particular, phenylbutazone and aspirin are more potent in undiluted serum at 37°C than predicted, while furosemide appears to be less potent.

At normal concentrations of oleic and arachidonic acids, both the prediction and direct dialysis at 37°C showed little inhibitory activity. A substantial increase in total concentration and/or free fraction would be required to achieve an inhibitory effect. The effect of increments of oleic acid, added to a normal serum pool, on free fraction of ^{125}I T_4 in undiluted serum at 37°C is shown in Fig. 1. There is only a slight increase in T_4 free fraction with addition of 1 and 2 mM oleic acid and a 2-3 fold increase

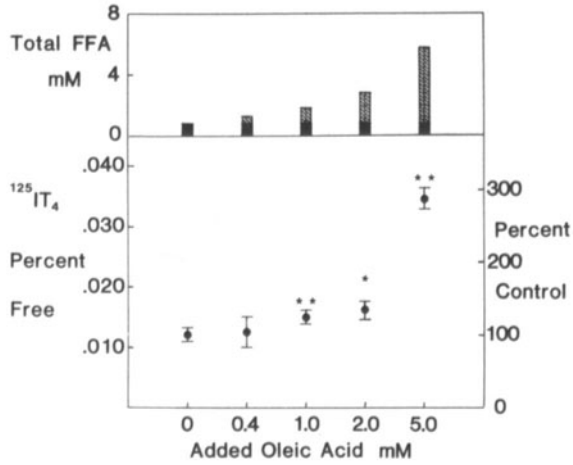

Fig. 1. Effect of added oleic acid on free fraction of ^{125}I T_4 in undiluted serum, measured by equilibrium dialysis at 37°C (n=6, mean ± SD). In the top panel, solid bars show endogenous total FFA (0.8 mM), the hatched bars show added oleic acid. *p<0.01, **p<0.001.

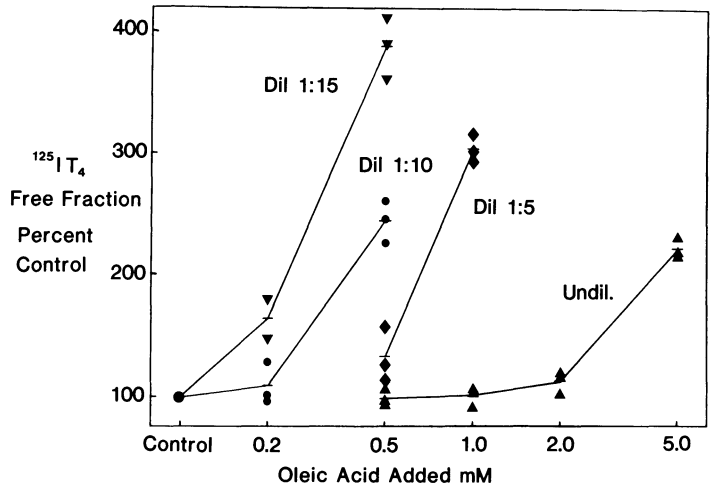

Fig. 2. Effect of various concentrations of oleic acid, added to undiluted serum and serum diluted 1:5, 1:10, and 1:15 on $^{125}I\ T_4$ free fraction by equilibrium dialysis at 37°C. Mean control free T_4 fraction was 0.011, 0.063, 0.109, and 0.173% for undiluted, 1:5, 1:10, and 1:15 serum, respectively.

at 5 mM. The marked influence of serum dilution on the increase in $^{125}I\ T_4$ free fraction produced by particular increments of added oleic acid in shown in Fig. 2. The effect of 5 mM addition to undiluted serum is reproduced by 0.5 mM addition to serum diluted 1:10.

DISCUSSION

In this study, we have evaluated a number of drugs by assessing their relative affinity for T_4 binding sites in a highly diluted system, using this together with an estimate of free fraction in undiluted serum and total circulating concentrations, to make a prediction of relative potency. While While the predictions are generally in accord with the effect on T_4 binding in undiluted serum at 37°C, some major discrepancies are seen, which are so far unexplained and require further study.

Binding competitors have often been assessed using diluted serum. The free fraction of competitor is then disproportionately high and the concentration of T_4 binding sites reduced by dilution. Such studies will generally over-estimate potency if interpreted without regard to dilution of the test serum.

The results for oleic and arachidonic acids, based both on predicted potency and direct addition experiments, suggest that concentrations well outside the physiological range are required in order to raise the T_4 free fraction in undiluted normal serum, a finding in accord with the previous study of Braverman et al. (11). The very high degree of albumin binding of oleic and arachidonic acids (8,12) is a key factor in limiting their potency as binding competitors *in vivo*. The competitive ligand-binding assay (3-5) used to assess inhibitors of T_4 binding in sera from patients with severe non-thyroidal illness gives a rapid and reproducible assessment of competitor activity, but suffers from the disadvantage that the serum used in the detection system must be diluted, (~1:17 in the system described

by Chopra). Measurement of the effect of added oleic acid on T_4 binding by undiluted serum suggests that the addition of >1 mM is required to displace T_4. Detection systems with diluted serum show an effect at much lower concentrations, but the significance of such effects remains in doubt. If FFA are responsible for the subnormal T_4 levels seen in non-thyroidal illness, they may exert this effect by actions unrelated to displacement of T_4 from serum binding sites.

REFERENCES

1. Cavalieri RR and Pitt-Rivers R. Pharmacol Rev 33: 55, 1981.
2. Stockigt JR, Lim C-F, Barlow JW, et al. J Clin Endocr Metab 60: 1025, 1985.
3. Chopra IJ, Huang T-S, Hurd RE, et al. J Clin Endocr Metab 58: 619, 1984.
4. Chopra IJ, Chua Teco GN, Mead JF, et al. J Clin Endocr Metab 60: 980, 1985.
5. Chopra IJ, Huang T-S, Beredo A, et al. Metabolism (in press).
6. Drug Information 85. Amer Soc Hosp Pharmacists 1985, p 705.
7. Martindale. The Extra Pharmacopoeia, 28th ed., 1982, p 234.
8. Goodman DS. J Amer Chem Soc 80: 3892, 1958.
9. Spector AA and Hoak JC. Analyt Biochem 32: 297, 1969.
10. Novak M. J Lipid Res 6: 431, 1965.
11. Braverman LE, Arky RA, Foster AE, et al. J Clin Invest 48: 878, 1969.
12. Spector AA. J Lipid Res 16: 165, 1975.

ALTERATIONS IN THYROID FUNCTION PARAMETERS FOLLOWING ADMINISTRATION OF FATTY ACIDS*

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Recent studies suggest that a thyroid hormone binding inhibitor (THBI) is detected frequently in sera of patients with systemic non-thyroidal illness (NTI) (1-3). There is at present a paucity of information concerning the role of THBI in thyroid hormone abnormalities observed commonly in NTI. THBI appears to be a lipid moiety and unsaturated fatty acids are promising candidate THBIs (1). Serum concentrations of some unsaturated fatty acids (e.g., oleic acid) are markedly increased in NTI (4). In this study, we have examined the effect of the parenteral administration of unsaturated fatty acids on serum thyroid hormone and TSH concentrations in the rat.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing about 150 g were maintained on Purina Rat Chow and water ad libitum. Fatty acids were obtained from Sigma Chemical Co. (St. Louis, MO). Arachidonic acid (sodium salt) was dissolved in saline and the resulting solution was clear. Other fatty acids were dissolved in ethanol and concentrations for injection in animals were diluted with water or saline; the injection material was in a fine suspension. Groups of rats were injected with vehicle or the solution of fatty acid in the vehicle. Animals were sacrificed by decapitation. Blood was collected from the trunk for measurement of thyroid hormones and TSH. Serum T_4, T_3, and TSH were measured by their respective radioimmunoassays (RIAs, 5-7). Reagents for rat TSH RIA were obtained from the National Institutes of Health, Bethesda, MD. Free fraction of T_4 (or T_3) was determined by equilibrium dialysis (8,9). Resin uptake of T_3 (RT_3U) was measured by Res-O-Mat kits available commercially from Mallinckrodt Inc., St. Louis, MO.

RESULTS

Fig. 1 shows the data on serum thyroid hormone and TSH concentrations for 18 h after intravenous administration of 10 mg sodium arachidonate. There was a progressive fall in serum concentration of total and free T_4. There was little or no change in the dialyzable fraction of T_4 (DFT_4).

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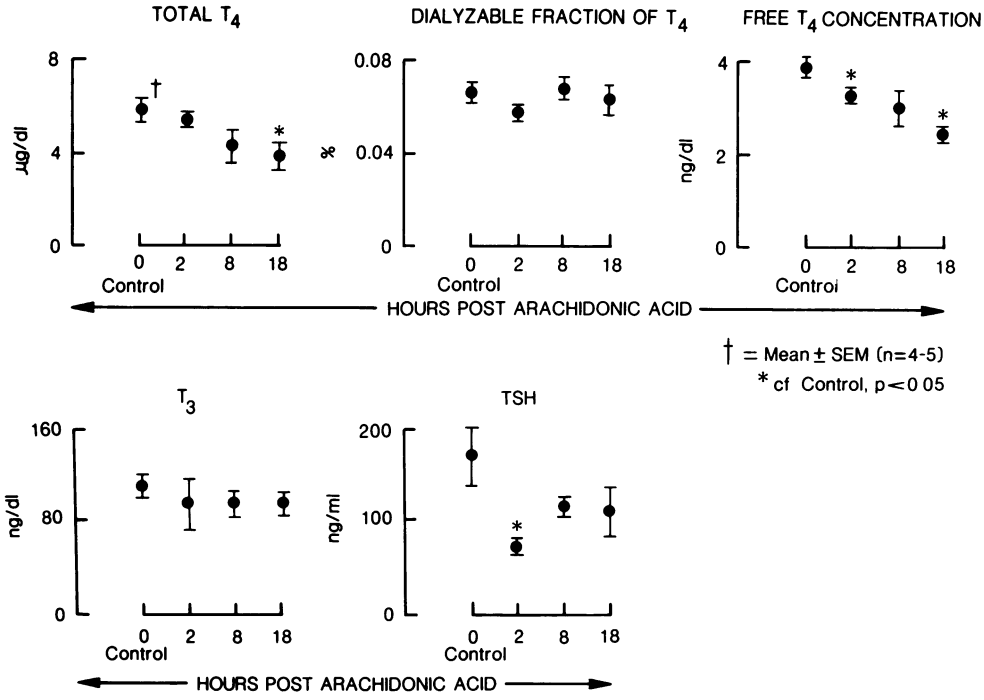

Fig. 1. Serum thyroid hormone and TSH levels during 18 hours after intravenous administration of sodium arachidonate (10 mg) to the rat.

Serum T_3 concentration showed a little fall, but it was not significant statistically. Serum TSH dropped significantly at 2 h and increased gradually thereafter to or toward baseline value. Similar changes were also observed after intravenous administration of 20 mg oleic acid, with the difference that serum T_3 concentration decreased significantly at 8 h after oleic acid administration (Fig. 2). Dose-response studies were conducted with 2.5-10 mg arachidonic acid and 3.12-25 mg oleic acid, showing that a significant ($p<0.05$) decrease in serum T_4 occurred 18 h after i.v. injection of all doses tested for both fatty acids. There was also a trend of increasing effect of increasing dose of fatty acids.

Fig. 3 depicts serum thyroid hormone and TSH levels at 18 h after intravenous administration of the vehicle (0.5 ml of 50% ethanol), or the unsaturated fatty acid, oleic acid (20 mg), or the saturated fatty acid, palmitic acid (20 mg) in the vehicle. Serum total and free T_4 concentrations decreased significantly after administration of oleic but not palmitic acid.

We next studied the response of serum TSH to thyrotropin-releasing hormone (TRH) 18 h after intravenous administration of either saline or arachidonic acid (10 mg) or oleic acid (20 mg) in saline. TRH (1 μg) or saline was injected i.p. and blood taken 30 min later. Neither the mean (± SE, ng/ml) serum TSH value of 773 ± 251 in arachidonic acid-injected rats nor that of 1010 ± 292 in oleic acid-injected rats differed significantly from the mean of 1025 ± 295 in control rats. We also studied the effect of two i.p. injections 12 h apart of saline, arachidonic acid (20 mg per injection) or oleic acid (40 mg per injection) on the TRH response 12 h after the last dose. The mean (± SE, ng/ml) serum TSH 30 min after 1 μg TRH i.p. was 1200 ± 166 in the control group, 876 ± 536 (N.S.) in the arachidonic acid-treated group, and 400 ± 333 (N.S.) in the oleic acid-treated group.

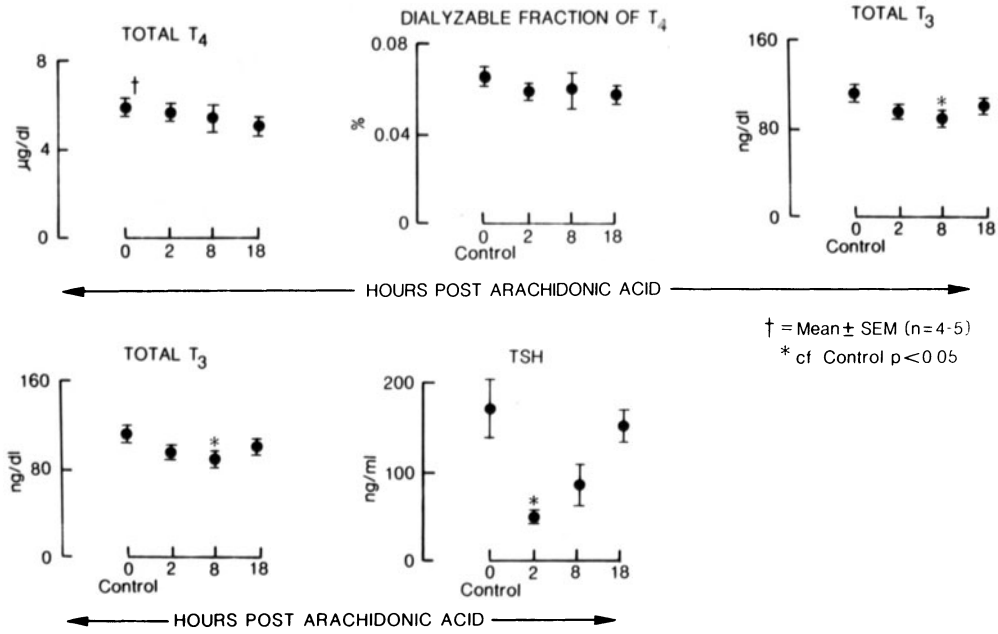

Fig. 2. Serum thyroid hormone and TSH levels during 18 hours after intravenous administration of oleic acid (20 mg) to the rat.

We next studied serum thyroid hormone and TSH levels for 24 hours after a single large i.p. dose of the unsaturated fatty acids, arachidonic or oleic (Fig. 4 for oleic acid). There was a significant fall in serum total and free T_4 16 to 24 h after oleic acid administration. The DFT_4, T_3, and TSH did not change. Following arachidonic acid administration

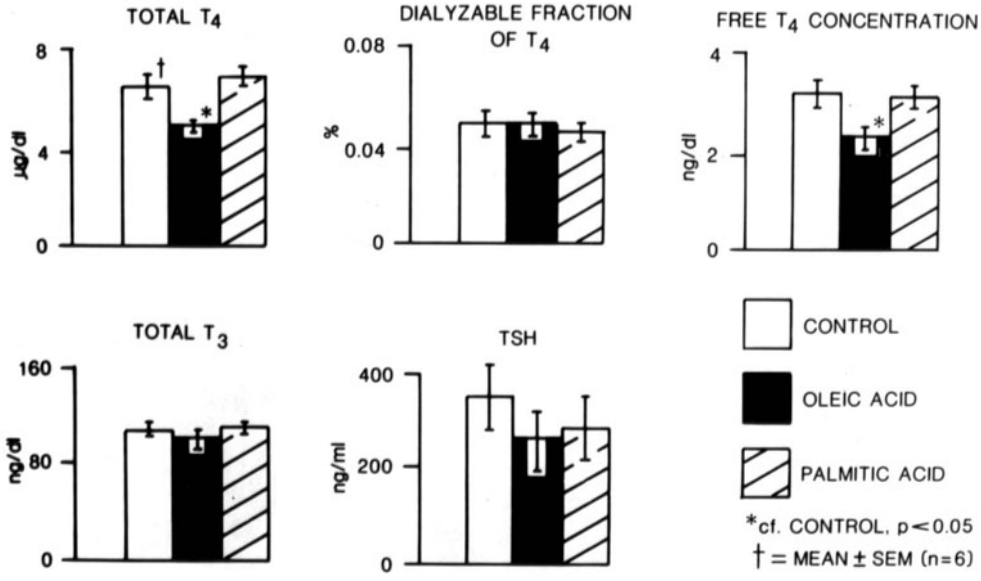

Fig. 3. Serum thyroid hormone and TSH levels at 18 hours after intravenous administration of vehicle (0.5 ml 50% ethanol, control) or oleic acid (20 mg) or palmitic acid (20 mg) in vehicle.

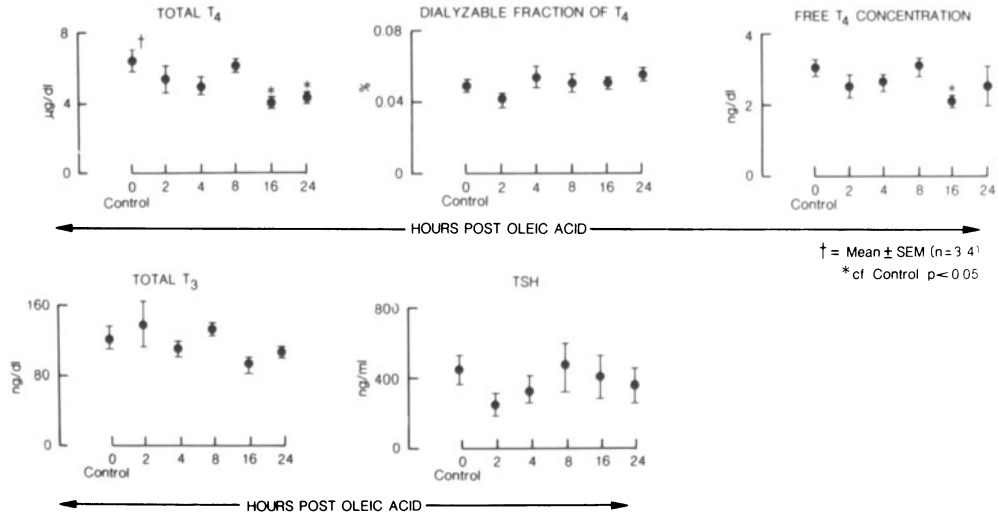

Fig. 4. Serum thyroid hormone and TSH levels during 24 hours after intraperitoneal administration of oleic acid (80 mg) to the rat.

(data not shown), we observed a significant fall in serum total T_4 and total T_3 at 24 h and a significant increase in DFT_4 at 16 h. Serum concentration of free T_4 and TSH decreased modestly, but the changes were not significant statistically.

We next studied serum thyroid hormone and TSH levels of rats given repeated doses of arachidonic acid (15 mg twice daily i.p. for 3 days). The results are shown in Fig. 5. There was a significant increase in DFT_4 and a significant fall in the serum concentration of T_4, free T_4, T_3, free T_3, T_3 resin uptake index, and free T_4 index, but serum TSH was not affected significantly.

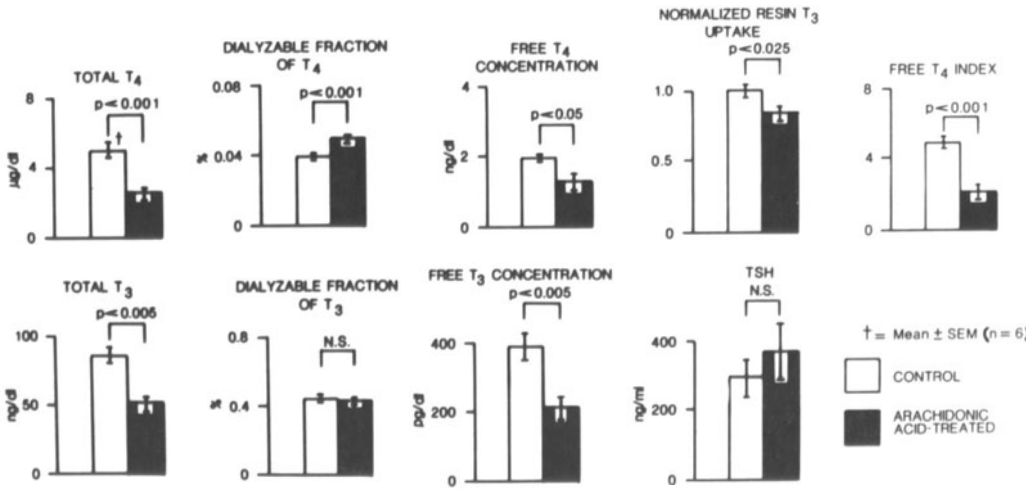

Fig. 5. Serum thyroid hormone and TSH levels in rats injected with sodium arachidonate (15 mg twice daily) for three days.

DISCUSSION

Thyroid function abnormalities in NTI include low or high T_4, low T_3, high DFT_4, high reverse T_3 (rT_3), high DFT_3, and a discrepancy between free T_4 index and free T_4 concentration (3,10-12). Serum TSH may be low in NTI and it may increase moderately during the recovery from NTI (13,14). Serum TSH response to TRH is often subnormal in NTI. We did not study serum rT_3, and we did not detect high T_4, high DFT_3, or high TSH in the present study. Serum TSH response to TRH was quite variable; the mean values in fatty acid-injected rats tended to be low but the decrease did not attain statistical significance. All the other abnormalities of NTI were demonstrable to a variable degree in rats injected with the polyunsaturated fatty acids, arachidonic acid and oleic acid (Fig. 1-5). The discrepancy between DFT_4 and RT_3U (i.e., a much greater increase in DFT_4 than RT_3U) was also similar to that observed in NTI patients. The smallest i.v. dose of oleic acid that was associated with a significant reduction in serum total T_4 was 3.12 mg. Assuming a plasma volume of a 150 g rat to be about 7.5 ml, the plasma concentration of oleic acid following i.v. administration of 3.12 mg oleic acid would approximate 416 μg/ml or about 1.39 mM, which is similar to the mean oleic acid concentration of 1.3 mM actually observed in a group of NTI patients (4). These data suggest that an elevation of polyunsaturated fatty acids could contribute to the thyroid functional abnormalities in NTI.

The mechanisms by which fatty acids may affect thyroid function tests are not known. Data showing a fall in serum TSH within 2 h after i.v. injection of oleic or arachidonic acids (Fig. 1 and 2) suggest that fatty acids importantly influence TSH secretion or release or both. It is likely that the fall in serum TSH led to the subsequent fall in serum T_4 which was maximal 16 to 24 h after the injection of fatty acids.

Oleic and arachidonic acids are known potent inhibitors of serum binding of T_4 *in vitro* in human (1) or rat serum (unpublished data). However, we were not able to demonstrate an increase in DFT_4 rats acutely injected with fatty acids. This may reflect avid binding of fatty acids by normal albumin in rat serum, a rapid clearance of fatty acids from the circulation or a combination of these effects. However, increased DFT_4 was demonstrated when fatty acids were injected twice daily for 3 days (Fig. 5). It is possible that persistent elevation of unsaturated fatty acids in circulation is associated with a compromise in the function of organs (e.g., liver) that clear fatty acids and this, in turn, may result in a sustained elevation of fatty acids and associated increase in DFT_4. Alternatively, fatty acids may not affect DFT_4 directly, and the increased DFT_4 observed after repeated fatty acid administration (Fig. 5) may be an effect of unknown factors that emerge as a result of systemic toxicity of intravenous unsaturated fatty acids. In either case, the available data are consistent with our impression that an elevation of unsaturated fatty acids contributes importantly to the thyroid functional abnormalities in NTI.

SUMMARY

Intravenous (i.v.) administration of arachidonic acid (10 mg) to the rat caused a progressive fall in serum total concentration of T_4, no significant change in dialyzable fraction of T_4 (DFT_4), and a progressive fall in free T_4 concentration. Serum total T_3 changed minimally, while serum TSH decreased significantly at 2 h but returned to normal thereafter. The i.v. administration of oleic acid (20 mg) produced similar effects. However, i.v. palmitic acid (20 mg) did not affect thyroid function. Serum T_4 fell significantly after i.v. administration of as little as 2.5 mg arachidonic acid or 3.12 mg oleic acid. The response of serum TSH to TRH

fell somewhat after parenteral administration of fatty acids, but not significantly. Intraperitoneal (i.p.) administration of arachidonic acid or oleic acid was associated with a progressive reduction in serum total T_4, no change in DFT_4, a moderate fall in free T_4, a significant fall in total and free T_3, and no change in TSH. Repeated i.p. administration of arachidonic acid (15 mg twice daily for three days) was associated with a rise in DFT_4, and a fall in total and free T_4, total and free T_3, a discrepancy between DFT_4 and RT_3 (i.e., a rise in DFT_4 and a fall in RT_3U) while serum TSH did not change significantly. The various alterations in thyroid function induced by injection of unsaturated fatty acid in rats resemble those observed in patients with systemic non-thyroid illnesses (NTI). These data suggest that high circulating levels of unsaturated fatty acids (e.g., oleic acid) may contribute importantly to the thyroid functional abnormalities of NTI.

REFERENCES

1. Chopra IJ, Huang TS, Hurd RE, et al. J Clin Endocrinol Metab 58: 619, 1983.
2. Oppenheimer JH, Schwartz HL, Mariash CN, et al. J Clin Endocrinol Metab 54: 757, 1982.
3. Chopra IJ, Huang TS, Beredo A, et al. Metabolism (in press).
4. Chopra IJ, Chua Teco GN, Mead JF, et al. J Clin Endocrinol Metab 66: 980, 1985.
5. Chopra IJ. J Clin Endocrinol Metab 34: 938, 1972.
6. Chopra IJ, Ho RS, and Lam R. J Lab Cln Med 80: 729, 1972.
7. Chopra IJ. Metabolism 29: 161, 1980.
8. Sterling K and Brenner MA. J Clin Invest 45: 153, 1966.
9. Chopra IJ, Van Herle AJ, Chua Teco GN, et al. J Clin Endocrinol Metab 51: 135, 1980.
10. Chopra IJ, Solomon DH, Hepner GW, et al. Ann Intern Med 90: 905, 1979.
11. Chopra IJ, Hershman JM, Pardridge WM, et al. Ann Intern Med 90: 946, 1983.
12. Wartofsky L and Burnam KD. Endocr Rev 3: 164, 1982.
13. Wehmann RE, Gregerman RI, Burns WH, et al. N Engl J Med 312: 546, 1985.
14. Bacci V, Schussler GC, and Kaplan TB. J Clin Endocrinol Metab 54: 1229, 1982.

A NEW FORM OF X-CHROMOSOME LINKED TBG ABNORMALITY WITH NO DEMONSTRABLE T_4-BINDING ACTIVITY*

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Diagnostic work-up for short stature uncovered TBG deficiency in an 11 year old girl (propositus) with XO Turner's syndrome. Studies on members of the family revealed that the two hemizygous affected subjects (propositus and father) had, compared to the mean normal levels, 1.5% native (n) TBG and 9-fold to 10-fold increased denatured (dn) TBG, both measured by specific RIAs. The heterozygous sister had 30% nTBG and 5.6-fold increased dnTBG, while the mother had normal levels (Table 1).

Characterization of the TBG abnormality gave the following results. First, nTBG in serum on the propositus and father showed: a) no demonstrable T_4-binding under conditions in which even lower concentrations of normal TBG exhibited binding; b) increased lability (initial t 1/2 of 3 hr at 37°C, a temperature not affecting normal TBG); c) failure of added T_4 to protect TBG denaturation at 56°C, when normal TBG demonstrated full protection; and d) when denatured *in vitro*, immunological identity with similarly denatured normal TBG. Secondly, nTBG in serum from the propositus and father: a) reacted only with anti-dnTBG serum; b) was immunologically indistinguishable from *in vitro* denatured normal TBG; c) did not bind T_4; and d) focused between pH 4.66 and 4.09 (4.18 and 4.54 for normal TBG) as shown by the Western blot technique. Finally, serum from the affected subjects did not alter the stability of added normal TBG.

These data are compatible with an inherited defect in TBG structure with loss of hormone binding and stability leading to its rapid *in vivo* denaturation and clearance. This defect is unique since sera from members of five families with inherited partial TBG deficiency had normal dnTBG levels.

*Supported in part by United States Public Health Service Grant AM 15070.

Table 1. Thyroid Function Tests in Members of the Family

Subject	Age	Karyotype	TT_4 (μg/dl)	FT_4I	TT_3 (ng/dl)	TSH (μU/ml)	nTBG (μg/dl)	dn TBG (μg/dl)
Propositus	11	XO	2.0	10.0	60	4.7	20	58
Father	33	XY	1.9	9.0	59	2.3	19	50
Sister	9	XX	3.4	8.3	84	2.5	502	34
Mother	32	XX	6.7	7.5	132	2.3	1490	2
Normal Range			5-12	6.4-10.5	90-175	<0.4-5	1100-2200	<2-10

NORMAL CELLULAR UPTAKE OF THYROXINE (T_4) FROM SERUM OF PATIENTS WITH FAMILIAL DYSALBUMINEMIC HYPERTHYROXINEMIA (FDH) OR ELEVATED THYROXINE-BINDING GLOBULIN (TBG): T_4 BOUND TO ALBUMIN IS NOT PREFERENTIALLY TRANSFERRED TO TISSUE*

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Only free thyroid hormone is thought to enter cells and be metabolically active while protein bound thyroid hormone, in equilibrium with the free fraction, remains in the intravascular compartment (1). Evidence that albumin-bound T_4 preferentially enters hepatocytes (2) and that subjects with FDH have increased hepatic T_4 delivery (3) have challenged this concept. We examined the in vitro cellular uptake of T_4 by human hepatocytes, Hep G2 (4), from serum of subjects with FDH. Results were compared to those from sera matched to the FDH sera by total T_4 concentrations and obtained from individuals with high TBG or thyrotoxicosis.

METHODS

Samples from nine FDH subjects were provided by Drs. L. Braverman and B. N. Premachandra. Samples from high TBG and thyrotoxic subjects were obtained from the clinical laboratory.

TT_4 and TBG were measured by RIA. A free T_4 index (FT_4I) using a resin T_4 uptake (RT_4U) (5), free T_4 by equilibrium dialysis (DT_4), and the uptake of T_4 by Hep G2 cells (CT_4) (4) were determined.

RESULTS

Group means are reported in Table 1. DT_4, CT_4, and FT_4I were normal in FDH and high TBG subjects and elevated in thyrotoxic patients. The relationship between DT_4 and CT_4 for all groups was similar to that in normal controls (4). The relationship between FT_4I and CT_4 was normal for FDH and thyrotoxic subjects, but FT_4I was lower in high TBG subjects. Despite similar DT_4 and CT_4 values, FT_4I values were lower in high TBG than FDH subjects.

DISCUSSION

The normal relationship between DT_4 and CT_4 indicates that T_4 uptake by liver cells in FDH and high TBG is related to the free T_4 concentration.

*Supported in part by United States Grant AM 15070.

Table 1. Results of Study Groups

	TT_4 (μg/dl)	TBG (mg/dl)	FTI	DT_4 (ng/dl)	CT_4 (pg/plate)
FDH	15.8 ± 2.1[a]	1.8 ± 0.4	10.3 ± 1.7	1.8 ± 0.4	29 ± 4
High TBG	15.6 ± 1.8	3.4 ± 0.2[b]	8.6 ± 1.7[c]	1.8 ± 0.2	28 ± 4
Thyrotoxicosis	15.9 ± 2.2	1.6 ± 0.2	17.2 ± 2.4[b]	3.4 ± 0.7[b]	48 ± 8[b]
Normal Range	5.5 ± 12.0	1.1 - 2.0	6.0 - 10.5	0.9 - 1.9	14 - 30

[a]Mean ± SD; [b]$p < 0.001$; [c]$p < 0.05$ vs FDH patients.

If protein-bound T_4 was preferentially taken up, the CT_4 value would have been high compared to the DT_4.

Unlike the RT_3U (6), the FT_4I value appears to be clinically useful in FDH. The lower RT_4I value in some subjects with high TBG suggests reduced affinity of TBG for T_4.

REFERENCES

1. Refetoff S. In LJ DeGroot, GF Cahill, and WD Odell (eds), Endocrinology, New York, 1979, p 348.
2. Pardridge WM and Mietus LJ. J Clin Invest 66: 367, 1980.
3. Premachandra BN, Nicola B, Pardridge WM, et al. Endocrinol 113 (suppl): T25, 1983 (abstract).
4. Sarne D, Refetoff S, Horwitz A, et al. In 7th International Congress of Endocrinology Abstracts, Excerpta Medica, Amsterdam, 1984, p 1360.
5. Refetoff S, Hagen SR, and Selenkow HA. J Nucl Med 13: 2, 1972.
6. Ruiz M, Rajatanavin R, Young RA, et al. N Engl J Med 306: 635, 1982.

CARRIER-MEDIATED TRANSPORT OF THYROID HORMONE (TH) INTO RAT HEPATOCYTES IS RATE LIMITING IN TOTAL CELLULAR UPTAKE AND METABOLISM

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TH has a wide spectrum of activities on several tissues, most importantly those related to the economy of energy production and expenditure. The main secretory product of the thyroid, thyroxine (T_4) has little, if any, intrinsic metabolic activity (1). Most of the in vivo effects of T_4 can be explained by its conversion to 3,3',5-triiodothyronine (T_3) in peripheral tissues (2). Another product of the peripheral deiodination of T_4 is the biologically inert 3,3',5'-triiodothyronine (reverse T_3, rT_3) (3). Normally, extrathyroidal conversion accounts for approximately 80% of total T_3 production and an even greater fraction of total rT_3 production in humans, as well as in rats (4,5). Most of the action of T_3 is initiated by binding to a nuclear, chromatic-associated receptor (6), although direct effects on mitochondria (7) and plasma membranes (8-10) have also been reported. Recent studies have pointed to differences between tissues with regard to the origin of nuclear T_3 (11). Thus, in the pituitary 50% and in the central nervous system 80% of nuclear T_3 is derived from local T_4 ---> T_3 conversion, the remainder being taken up from the circulation. Probably due to the rapid exchange between the plasma and tissue compartments, most of nuclear T_3 in the liver and the kidneys is derived from the circulation with a negligible contribution from local T_4 ---> T_3 conversion (11,12), despite the fact that the latter tissues are more important sites for the total body production of T_3 (13). Thus, passage of T_4 and T_3 through the plasma membrane plays an important part in the ultimate delivery of T_3 to nuclear receptors.

According to "the free hormone hypothesis" (14), the free hormone concentration in serum governs hormonal delivery to the cell and ultimately regulates hormonal action. Therefore, the free T_4 concentration should be more closely correlated with the T_4 turnover rate than the total concentration. This hypothesis has been challenged; however, as no correlation was found between serum free T_4 concentration and T_4 turnover in a group of healthy euthyroid individuals (15). Other examples are: normal T_4 turnover in non-thyroidal illness despite increased serum free T_4 levels (16, 17), and similarly normal T_4 turnover in the postoperative state in which decreased T_4-binding pre-albumin with elevated free T_4 concentrations can be encountered (18). Two non-related clinically euthyroid subjects have been reported who showed abnormal patterns of thyroid hormone levels.

Hyperthyroxinemia (both total and free hormone concentrations) was accompanied by elevated rT_3 and normal T_3 serum levels, while TSH responses to TRH were normal (19). In all these examples, it has been postulated that factors directly related to cellular handling of T_4 may be operative, such as plasma membrane translocation and intracellular degradation (e.g., deiodination).

Recently, we were able to demonstrate that in the state of caloric deprivation, T_4 transport into tissue cells is inhibited in humans (20). Consequently, T_3 production from T_4 and serum T_3 levels are lowered. In itself, the "low T_3 syndrome" during semi-starvation can be explained by diminished cellular T_4 uptake, especially in the liver. The hepatic clearance of plasma rT_3 - derived from other tissues - may be impaired similarly. Any decrease in intracellular deiodination of T_4 and rT_3 would potentiate the observed changes elicited by diminished uptake.

On the basis of circumstantial evidence, studies from different laboratories (21-31) have shown that iodothyronines are transported into tissues through saturable, energy-dependent systems. Direct proof for the involvement of a plasma membrane protein has been obtained only recently by the production of a monoclonal antibody (ER-22) directed against a plasma membrane protein with a molecular weight of 52000 dalton, which antibody inhibits the transport of iodothyronines into rat hepatocytes in monolayer (32).

The purpose of this study is to investigate the consequences on deiodination and conjugation, if iodothyronine transport is inhibited by ER-22.

METHODS AND MATERIALS

The source of most materials and procedures for the isolation and culture of rat hepatocytes have been previously described (24,25). Cultured rat hepatocytes in monolayer were incubated in the presence or absence of ER-22 (final dilution 1:1000), 100 µM PTU, 5 mM ouabain (uptake inhibitor), or ER-22 plus PTU (E+P) and tracer iodothyronine or iodothyronine sulfate (THS). Percentages of conjugated iodothyronines (sulfates (S) plus glucuronides (G)), iodothyronine and iodide in medium were analyzed by LH-20 chromatography (33) after acidification of the media. T_3-sulfate (T_3S) was obtained according to (34). Incubations were done for 20 h (T_3, T_4, T_3S) or 90 min (rT_3) at 37°C in culture medium plus 0.5% BSA. Number of experiments is 2-6, carried out at least in duplicate. Statistical analysis has been performed using one-way analysis of variance.

RESULTS AND DISCUSSION

The percentage distribution of conjugates (CJ), iodothyronine (TH) and I- in supernatants of primary cultures of rat hepatocytes incubated with various compounds as illustrated in Table 1. Uptake and metabolism of T_3S in our system is also markedly hampered by PTU or ouabain (data not shown). With these drugs, I- formation and T_3S clearance are significantly decreased, whereas intracellular formed T_3 by hydrolysis of T_3S is measurable in the medium.

Monoclonal antibody ER-22 inhibits the clearance of iodothyronines to the same extent as ouabain in a system with primary cultures of rat hepatocytes. Concomitantly, a decrease in I- production has been observed. Since ER-22 and ouabain do not affect deiodination per se (data not shown) and do specifically bind to membrane proteins, these data indicate that the uptake

Table 1. Distribution of Iodothyronines and Metabolites in Medium of Cultured Rat Hepatocytes; Effects of Inhibitors

	T_4			T_3			rT_3		
	I-	Conj	TH	I-	Conj	TH	I-	Conj	TH
Contr	12.7	1.9	82.9	51.5	10.3	32.0	54.1	1.8	45.8
ER-22	6.9*	1.3	90.8#	24.6*	9.8	64.8*	36.9*	1.9	62.8*
PTU	3.8*	3.8#	92.0*	12.2*	41.7*	46.2#	5.4*	5.4*	87.6*
Ouabain	6.9*	1.8	92.2#	21.5*	10.6	66.2*	41.0*	1.7	56.6*
ER-22 + PTU	4.0*	2.3	93.2*	8.0*	24.5*	65.1*	4.3*	5.5*	91.6*

*$p<0.001$, #$p<0.005$ vs. control

mechanism(s) of TH can regulate intracellular TH availability and, therefore, metabolism. Conjugates in the medium are not decreased by these compounds, probably due to inhibition of uptake of conjugates after cellular release, as has been found with labeled T_3S. PTU inhibits both deiodination (leading to conjugate accumulation) and clearance. As is illustrated in Fig. 1, inhibition of T_3 deiodination (via T_3S) should lead to a more pronounced conjugate accumulation as compared to T_4 or rT_3. Our data agree with this current concept of intracellular metabolism (33,34). The apparent diminished clearance of TH with PTU can be explained by 1) intracellular accumulation of TH (and therefore also in the medium) as a consequence of inhibition of deiodination (rT_3), 2) hydrolysis of intracellularly formed and accumulated conjugates with subsequent release of free TH into the medium (T_3) and 3) a combination of both (T_4). Hydrolysis of TSH to TH has indeed been found with T_3S (vide supra). Since 100 µM PTU almost completely blocks TH deiodination in this system, only slight effects have

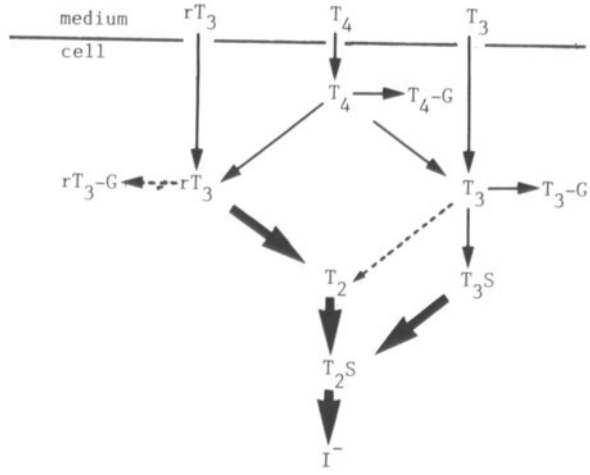

Fig. 1. Schematic representation of hepatic uptake and metabolism of iodothyronines by rat hepatocytes.

been found on I- formation by the addition of ER-22 to PTU (E+P). However, since only T_3 is predominantly conjugated prior to deiodination (Fig. 1), the addition of ER-22 (thus inhibiting T_3 uptake) to incubation with PTU results intracellularly in a decreased substrate availability and subsequently diminished T_3S production.

It can be concluded that inhibition of carrier-mediated transport of TH in equilibrium is a rate-limiting process for total TH cellular uptake and metabolism.

REFERENCES

1. Surks MI and Oppenheimer JH. J Clin Invest 60: 555, 1977.
2. Braverman LE, Ingbar SH, and Sterling K. J Clin Invest 49: 855, 1970.
3. Jorgensen EC. Pharmacol Ther (B) 2: 661, 1976.
4. Chopra IJ. J Clin Invest 58: 32, 1976.
5. Chopra IJ, Solomon DH, Chopra U, et al. Recent Prog Horm Res 34: 1, 1978a.
6. Oppenheimer JH, Schwartz HL, Surks MI, et al. Recent Prog Horm Res 32: 529, 1976.
7. Sterling K and Milch PO. Proc Natl Acad Sci USA 72: 3225, 1975.
8. Goldfine ID, Smith GJ, Simons CG, et al. J Biol Chem 251: 4233, 1976.
9. Segal J, Schwartz H, and Gordon A. Endocrinology 101: 143, 1977.
10. Blanchard RF and Davis PJ. Endocrinology 103 (suppl): T-19, 1978.
11. Larsen PR. N Engl J Med 306: 23, 1982.
12. Van Doorn J, van der Heide D, and Roelfsema F. J Clin Invest 72: 1778, 1984.
13. Chopra IJ. Endocrinology 101: 453, 1977.
14. Robbins J and Rall JE. Recent Prog Horm Res 13: 161, 1957.
15. Hennemann G, Docter R, and Dolman A. J Clin Endocrinol 33: 63, 1971.
16. Inada M and Sterling K. J Clin Invest 46: 1275, 1967.
17. Bellabarba D, Inada M, Varsamo-Aharon N, et al. J Clin Endocrinol Metab 28: 1023, 1968.
18. Bernstein G, Hasen J, and Oppenheimer JH. J Clin Endocrinol Metab 27: 741, 1967.
19. Jansen M, Krenning EP, Oostdijk W, et al. The Lancet ii: 849, 1982.
20. Docter R, Heyden van der JTM, Krenning EP, et al. Ann d'Endocrinol 45: 136, 1984.
21. Rao GS, Eckel J, Rao ML, et al. Biochem Biophys Res Comm 73: 98, 1976.
22. Parl F, Korcek L. Siegel JS, et al. FEBS Letters 83: 145, 1977.
23. Krenning EP, Docter R, Bernard HF, et al. FEBS Letters 91: 113, 1978.
24. Krenning EP, Docter R, Bernard HF, et al. Biochem Biophys Acta 676: 314, 1981.
25. Krenning EP, Docter R, Bernard HF, et al. FEBS Letters 140: 229, 1982.
26. Krenning EP, Docter R, Visser TJ, et al. J Endocrinol Invest 6: 59, 1983.
27. Cheng SY, Maxfield FR, Robbins J, et al. Proc Natl Acad Sci USA 77: 3425, 1980.
28. Horiuchi R, Cheng SY, Willingham M, et al. J Biol Chem 257: 3139, 1982.
29. Halpern J and Hinkle PM. Endocrinology 110: 1070, 1982.
30. Holm AC, Wong KY, Pliam NB, et al. Acta Endocrinol 95: 350, 1980.
31. Krenning EP, Docter R, Bernard HF, et al. Ann d'Endocrinol 45: 59, 1984.
32. Mol JA, Krenning EP, Docter R, et al. Endocrinology (suppl) 115: T-17, 1984.
33. Otten MH, Mol JA, and Visser TJ. Science 221: 81, 1983.
34. Mol JA and Visser TJ. Endocrinology 117: 1, 1985.

ENHANCED RATE OF CELLULAR UPTAKE OF THYROXINE (T_4) FROM SERA OF SUBJECTS WITH FAMILIAL DYSALBUMINEMIC HYPERTHYROXINEMIA (FDH)

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Recent investigations in our laboratory have shown that in several individuals with familial dysalbuminemic hyperthyroxinemia (FDH) and with normal serum free thyroxine (FT_4) concentration, sex hormone-binding globulin (SHBG) levels were significantly elevated in relation to normal subjects (1). The elevation in serum SHBG was not an estrogen-mediated effect, as these subjects were either in post-menopausal state or otherwise had normal levels of serum cortisol-binding globulin (CBG). Since SHBG is sensitive to thyroid hormone (TH) action and has been observed to increase in hyperthyroidism (2), the SHBG increase in FDH subjects suggested the possibility that an enhanced rate of T_4 uptake might occur in this syndrome, particularly in those tissues which have direct access to protein-bound T_4 (e.g., liver). To further test the hypothesis of increased T_4 tissue availability in FDH syndrome, T_4 uptake from normal and FDH sera studied using red blood cells, lymphocytes, and an anionic resin sponge.

METHODS

The techniques used to characterize sera for dysalbuminemic hyperthyroxinemia syndrome by conventional and reverse flow electrophoresis have been previously published from our laboratory (3,4). The use of the resin sponge for thyroid hormone (TH) uptake measurements has also been previously noted (5). Briefly, in the present investigations, 1 ml of appropriately diluted (1/10 or 1/25) serum was equilibrated with ^{125}I-T_4 and was incubated at 37° with a resin sponge (Abbott Laboratories) ranging from 1 min to 30 min in separate experiments. The radioactivity in a few tubes was counted to represent initial activity. At the end of the incubation, the sponges were washed with deionized water 4 times as previously described, and the radioactivity in washed sponges determined (final activity). The percent sponge uptake of radioactivity was obtained by dividing final by initial X 100.

Cellular Uptake of ^{125}I-T_4

For measuring red cell uptake of ^{125}I-T_4 from sera, out-dated whole blood was obtained from the American Red Cross and the cells were washed 4 times with saline. Equal volumes of washed cells were then incubated

with 1/25 diluted normal or FDH sera that had been previously equilibrated with ^{125}I-T_4. The incubation was allowed to continue either for 1 min or 2 hr. After incubation, the cells were washed again 4 times with saline and radioactivity of the cell pellet was measured in a scintillation counter. Similar procedures were used to determine the ^{125}I-T_4 uptake from labeled normal or FDH sera utilizing freshly isolated lymphocytes obtained by the Ficoll-Hypaque technique. Sera from 10 FDH subjects representing 4 families were included in this study.

RESULTS

Sera used for the studies of cellular uptake of ^{125}I-T_4 were appropriately characterized for FDH as previously published (3,4). In FDH sera, the T_4 concentrations varied from 12.0 to 21.6 µg/dl (normal 5.5 - 12.0 µg/dl), whereas FT_4 concentration ranged from 1.4 to 2.1 ng/dl (normal = 0.9 - 2.3 ng/dl).

The percent, as well as absolute resin sponge T_4 uptake from normal and FDH sera at various intervals after incubation, are noted in Table 1. The absolute T_4 uptake from FDH sera was significantly higher than normals at all intervals after incubation (Table 1).

Similarly, when washed red blood cells were incubated with ^{125}I-T_4 labeled sera, the 1 min and 2 hr absolute T_4 uptakes from FDH sera were significantly higher than that noted in normals (Table 2). Although there was a decrease in percent T_4 uptake in FDH sera because of the presence of abnormal T_4 binding albumin, it was not commensurate with the increase in total T_4, so that the absolute T_4 uptake was greater than that observed in normal sera. Similar results were also noted when T_4 uptake from FDH sera was examined using lymphocytes. The absolute T_4 uptake from FDH sera both at 1 min and 2 hr after incubation was significantly greater than that observed in normal sera (Table 2). The relative differences in lymphocyte ^{125}I-T_4 uptake between normal and FDH sera were also confirmed using varying lymphocyte cell populations. Finally, that the cellular and resin sponge uptake of T_4 was sensitive to variations in FT_4 in the system was documented by the demonstration of the expected increase in both percent and absolute T_4 uptake from hyperthyroid sera in comparison to FDH sera (Tables 1 and 2).

DISCUSSION

The results presented clearly indicate that the rate of T_4 dissociation from FDH sera, as manifested by absolute T_4 uptake, is greater than that noted from control sera. This was demonstrated using red cells and lymphocytes, as well as in a cell-free system (anionic resin sponge). It should be underscored that the greater rate of cellular T_4 uptake from FDH sera relative to controls 1 min after incubation occurred in a time interval during which T_4 egress from the cell can be considered negligible.

The observations suggesting supranormal T_4 extraction by red cells and lymphocytes from patients with FDH syndrome are significant and challenging in relation to the currently accepted ideas concerning cellular hormone transport. As FT_4 concentrations in FDH and normal human sera are not significantly different, the increase in celluar T_4 uptake from FDH sera suggests the possibility that there is an increase in T_4 flux from TBP toward serum. The elevated T_4 flux would allow for local increase in cellular uptake without change in serum free thyroxine concentration. It has been noted recently that T_4 bound to normal albumin (6) and human prealbumin (7) is available for hepatic uptake in the rat. It has also recently been hypothesized that in tissues served by capillaries which are

Table 1. Resin Sponge Uptake of ^{125}I-T_4 from FDH and Other Sera at Various Intervals after Incubation

	1 minute		5 minutes		10 minutes		20 minutes	
	% Uptake	Absolute uptake*	% Uptake	Absolute uptake*	% Uptake	Absolute uptake*	% Uptake	Absolute uptake*
Normal Sera								
#1	6.1	0.51	14.7	0.90	24.4	2.07	41.4	3.52
#2	6.4	0.46	15.2	1.09	20.8	1.77	36.3	2.61
#3	6.3	0.57	13.2	2.20	21.4	1.95	39.4	3.58
#4	6.1	0.42	14.4	0.99	22.4	1.54	39.8	2.75
#5	9.2	0.72	18.4	1.43	23.2	1.81	39.8	3.10
Mean ± S.D.	6.8 ± 1.34	0.54 ± 0.12	15.2 ± 1.94	1.32 ± 0.53	22.4 ± 1.43	1.83 ± 0.20	39.3 ± 1.86	3.1 ± 0.44
FDH Sera								
#6	6.6	0.96	12.6	1.83	17.2	2.49	34.3	4.97
#7	5.1	0.83	12.5	2.52	18.6	3.01	33.0	5.35
#8	6.6	1.03	12.7	1.98	18.2	2.84	29.6	4.62
#9	5.8	0.93	11.6	3.14	16.9	2.70	29.4	4.70
#10	6.1	0.87	13.1	1.76	15.4	2.19	28.7	4.07
Mean ± S.D.	6.0 ± 0.63	0.92† ± 0.08	12.5 ± 0.55	2.25† ± 0.58	17.3 ± 1.25	2.65† ± 0.32	31.0 ± 2.45	4.74† ± 0.47
Hyperthyroid Sera								
#11	8.0	1.44	20.1	3.62	24.6	4.43	47.3	8.51

*% X total T_4; †$p<0.05$

Table 2. RBC and Lymphocyte Uptake of T_4 from FDH and Other Sera

Source of sera	Serum Total T_4 (μg/dl)	Serum Free T_4 (ng/dl)	RBC Uptake				Lymphocyte Uptake			
			Fractional (%)		Absolute (μg/dl)*		Fractional (%)**		Absolute (μg/dl)*	
			1 Minute	2 Hour	1 Minute	2 Hour	1 Minute	2 Hour	1 Minute	2 Hour
Normal	8.1 ±	1.6 ±	7.0 ±	29.6 ±	0.5 ±	2.4 ±	3.3 ±	9.5 ±	0.3 ±	0.8 ±
(10)	1.4	0.3	0.7	2.7	0.1	0.3	0.4	1.6	0.01	0.08
FDH	16.2 ±	1.9 ±	5.8 ±	21.5 ±	0.9 ±	3.5 ±	6.7 ±	7.0 ±	1.1 ±	1.3 ±
(10)	1.9	0.4	0.3	1.1	0.2†	0.2†	0.2	1.4	0.02†	0.5†
Hyperthyroid	17.1 ±	4.6 ±	7.6 ±	34.4 ±	1.3 ±	5.9 ±	8.2 ±	12.5 ±	1.4 ±	2.1 ±
(4)	2.1	0.6	0.5	1.9	0.2††	0.7††	0.5	2.1	0.3††	0.3††

*Total T_4 X %

**Two different lymphocyte populations employed for 1 minute and 2 hour T_4 uptake measurements

†Values significantly greater than normal uptakes ($p<0.01$)

††Values significantly greater than FDH uptakes ($p<0.05$)

permeable to TBPs, hormone delivery rate is markedly dependent on the serum level of bound hormone (8). In the case of elevated TBG in pregnancy, increased concentration of bound T_4 may allow more T_4 for placental passage (8). As in FDH with increased plasma serum T_4 binding, elevated T_4 binding in inherited TBG excess (or in pregnancy) does not render the subject hyperthyroid since enhanced availability of T_4 occurs only in tissues whose capillaries are highly permeable to TBP.

The observations of elevated cellular T_4 uptake from FDH sera (as determined by total T_4 X % uptake) are of particular interest in light of elevated SHBG concentrations found in some FDH subjects. If these findings can be confirmed by additional *in vivo* and *in vitro* indices of TH action, they could then be of importance for further clarification of the FT_4 hypothesis of T_4 availability for cellular uptake. It might even be that the nonequilibrium measures of T_4 uptake (Tables 1 and 2) more accurately reflect TH action in some tissues than equilibrium dialysis serum FT_4 concentration in subjects with FDH.

SUMMARY

In view of our previous observations of elevated sex hormone-binding globulin (SHBG) in some clinically euthyroid subjects with familial dysalbuminemic hyperthyroxinemia (FDH), the possibility of selective increase in tissue uptake of T_4 was examined by determining T_4 extraction from normal and FDH sera using red blood cells, lymphocytes and an anionic resin sponge. Red blood cells were incubated at 37°C with ^{125}I-T_4 equilibrated FDH sera and the 1 min and 2 hr cellular T_4 uptakes (T_4 concentration X % uptake) were 0.95 $\pm$ 0.18 and 3.54 $\pm$ 0.67, respectively, and these values were significantly higher ($p<0.01$) than respective control values (0.52 $\pm$ 0.08 and 2.4 $\pm$ 0.32). Similar differences in T_4 uptakes between normal and FDH sera were also noted when lymphocytes were incubated with ^{125}I-T_4 equilibrated sera (the 1 min and 2 hr cellular uptake of T_4 = 1.08 $\pm$ 0.019 and 1.35 $\pm$ 0.5, respectively; the control T_4 uptakes at 1 min and 2 hr = 0.27 $\pm$ 0.01 and 0.76 $\pm$ 0.08, respectively). In addition, when an anionic resin sponge was incubated with ^{125}I-T_4 equilibrated FDH sera, T_4 uptakes at 1 min and 30 min were 0.92 $\pm$ 0.08 and 4.74 $\pm$ 0.47, respectively, which were also significantly higher ($p<0.01$) than the control values of 0.54 $\pm$ 0.12 and 3.1 $\pm$ 0.44. These results indicate that the presence of excess serum T_4 protein binding in FDH may allow an increased cellular uptake of T_4 through enhanced net dissociation of albumin-bound T_4, despite a normal serum FT_4 concentration. Absolute T_4 uptake is probably accentuated in tissues which have direct access to protein-bound T_4.

REFERENCES

1. Premachandra BN, Nisula, B, Pardridge, WM, et al. 59th Meeting Amer Thyroid Assoc, New Orleans, LA, 1983, p T-25.
2. Tulchinsky D and Chopra IJ. J Clin Endocrinol Metab 37: 873, 1973.
3. Borst GC, Premachandra BN, Burman KD, et al. Amer J Med 73: 283, 1982.
4. Silverberg JDH and Premachandra BN. Annals Intern Med 96: 183, 1982.
5. Premachandra BN and Ibrahim II. Clinica Chimica Acta 70: 43, 1976.
6. Pardridge WM and Mietus LJ. J Clin Invest 66: 367, 1980.
7. Pardridge WM, Premachandra BN, and Fierer G. Amer J Physiol 248: G545, 1985.
8. Ekins R. Lancet 1: 1129, 1985.

ELEVATION OF SEX HORMONE BINDING GLOBULIN (SHBG) LEVELS IN SOME SERA WITH INHERITED INCREASE IN THYROXINE BINDING GLOBULIN (TBG)

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Previous observations reported from this laboratory have shown that there is often an elevation of sex hormone-binding globulin (SHBG) in sera with excess thyroxine (T_4) protein binding (e.g., familial dysalbuminemic hyperthyroxinemia (FDH) (1). The elevation of sex hormone transport protein was apparently not an estrogen-mediated effect, as cortisol-binding globulin (CBG) levels in sera with elevated SHBG were normal. Since SHBG is a thyroid hormone (TH) sensitive protein, the possibility was entertained that the elevation of SHBG in subjects with excess T_4 protein binding (as in FDH) may have been due to the availability of a greater than normal amount of T_4 for tissue uptake (despite a normal level of free thyroxine (FT_4)). In view of these observations, SHBG determinations, as well as in vitro T_4 uptakes from sera, were studied in another hyperthyroxinemic syndrome caused by an excess of T_4 transport protein, viz. inherited TBG increase.

METHODS

The presence of high TBG in sera was suggested by an elevated total T_4 and low T_3 uptake, and was further characterized by conventional and reverse flow electrophoresis techniques, as well as by measuring TBG-RIA concentrations. For the determination of red cell uptake of ^{125}I-T_4 from sera, out-dated whole blood was obtained from the American Red Cross, and the cells were washed 4 times with saline. Equal volumes of washed cells were then incubated with 1/25 diluted normal or high TBG sera that had been previously equilibrated with ^{125}I-T_4. The incubation was allowed to continue for either 1 min or 2 hr. After incubation, the cells were washed again 4 times with saline and radioactivity of the cell pellet was measured in a scintillation counter. T_4, T_3, as well as TSH measurements, were performed by conventional RIAs. SHBG and CBG concentrations were also determined by RIA procedures. T_3 resin uptakes were measured in a clinical laboratory. FT_4 was determined by equilibrium dialysis techniques.

RESULTS

The data on thyroid parameters in subjects with TBG excess from six different families are shown in Table 1. As expected, individuals positive

Table 1. Thyroid Function Data in Sera from Six Families with High TBG Inheritance

Sample Number	FT_4 (ng/dl)	T_4 (µg/dl)	T_3 (ng/dl)	Resin Uptake (%)	TSH (µunits/ml)
Family #1					
1	1.6	14.5	237	20	1.5
2	1.8	15.0	184	21	1.5
Family #2					
3	2.0	26.2	452	16	1.4
Family #3					
4	2.1	15.5	224	21	1.3
5	-	7.5	159	30	1.9
6	-	7.8	118	29	1.3
7	1.8	20.0	318	16	4.7
8	-	7.2	119	29	1.1
Family #4					
9	1.6	13.7	186	31	< 1
10	1.5	14.1	180	29	< 1
Family #5					
11	1.8	19.0	304	17	2.3
Family #6					
12	1.3	11.0	206	25	3.1
Normal range	(0.9 - 2.3)	(5 - 12)	(80 - 200)	(25 - 35)	(0 - 10)

for the TBG excess trait exhibited the typical pattern of elevated total T_4 low T_3 resin uptake, T_3 levels in the upper normal or hyprthyroid range, and normal concentrations of TSH and FT_4. In five out of nine female subjects with TBG excess, SHBG concentrations (116 $\pm$ 42 nM) were elevated. Two of these subjects had an SHBG elevation 100% above the normal mean. In the one male subject with TBG excess, SHBG concentration (57 nM) was above the normal range (12-52 nM). These data suggest that there might be sub-groups of subjects with TBG excess exhibiting normal or significantly elevated serum SHBG concentration. Although SHBG concentrations in sera with TBG excess were elevated, CBG levels were in the normal range with one exception (Table 2).

Despite a significant decrease in fractional T_4 uptake because of excess protein binding, the absolute RBC T_4 uptake (total T_4 X % uptake) in high TBG sera was greater than in normals after a 2 hr incubation, but not at 1 min due presumably to a higher degree of statistical variability (Table 3) in the latter. The relative differences in RBC uptake of ^{125}I-T_4 between normal and TBG excess sera were also sustained when varying red cell populations were utilized in the assay. The red blood cell assay system

Table 2. SHBG Concentrations in Sera of Subjects with Inherited or Congenital TBG Excess

Sera	Sex	TBG (mg/dl)	SHBG (nM)	CBG (nM)
1	F	6.6	57	808
2	F	4.4	80	898
3	F	5.6	169	652
4	F	7.9	131	953
5	F	3.4	40	707
6	F	3.8	52	-
7	F	3.5	58	-
8	F	6.7	80	-
9	F	4.6	70	-
Mean		5.1 ± 1.6	81.9 ± 41.8	804 ± 126
10	M	7.0	57	-
Normal Range:				
	Females	2.0 ± 0.3	64 ± 15	415 ± 941
	Males	1.8 ± 0.3	32 ± 10	415 ± 941

used for studying T_4 extraction was sensitive to variations in available FT_4 in the system as demonstrated by the expected marked increase in both fractional and absolute T_4 uptakes from hyperthyroid sera in comparison to FDH sera.

DISCUSSION

The elevation in SHBG concentration in sera with TBG excess is intriguing and represents the second instance where SHBG elevations are observed in sera with excess T_4 binding (the other case being FDH) (1). The observed increase in SHBG in subjects with TBG excess was apparently not the result of an increase in circulating estrogen, since these patients were either post-menopausal, or otherwise had normal CBG levels. These observations are of interest, as they suggest that in subjects with TBG excess, there might be a common genetic abnormality affecting both of the hepatic glycoproteins, TBG and SHBG; or, alternately, in view of the demonstrated sensitivity of SHBG to thyroid hormone (TH), the increase in SHBG in inherited TBG excess may have been due to a greater than normal amount of thyroid hormone available (i.e., through increased TBG-T_4 dissociation) for hepatocyte uptake than can be gauged from serum free thyroxine concentration alone which is usually in the normal range in subjects with T_4 transport protein-induced hyperthyroxinemia. That this indeed might be the case was shown in preliminary RBC uptake measurements which were greater in TBG excess sera in comparison to controls, despite the fact that serum FT_4 concentration in the former was in the normal range.

The observations indicating that there might be increased T_4 cellular uptake from sera with TH transport protein excess take on added signif-

Table 3. RBC Uptake of T_4 from Sera of Controls and Subjects with Inherited or Congenital TBG Excess

	Serum Total T_4 (μg/dl)	Serum Total T_4 (ng/dl)	% Uptake		Absolute Uptake* (μg/dl)	
			1 Min	2 Hr	1 Min	2 Hr
Normal (10)	8.1 ± 1.4	1.6 ± 0.3	7.03 ± .57	29.6 ± 2.73	0.52 ± 0.08	2.40 ± .32
TBG excess (4)	16.0 ± 2.1	1.8 ± 0.3	4.65 ± .35†	18.4 ± 1.30†	0.72 ± 0.28	2.94 ± 0.42†
Hyperthyroid (4)	17.1 ± 2.1	4.6 ± 0.6	7.56 ± .47	34.4 ± 1.91	1.31 ± 0.19	5.92 ± .72

* Total T_4 X %

† $p < 0.05$

icance in view of the recent observations which suggest that T_4 bound to albumin (2), as well as pre-albumin (3), is readily extractable by hepatocytes in vivo. Ekins (4) has suggested that in tissues served by capillaries which are permeable to thyroxine binding proteins, hormone delivery rate is markedly dependent on the serum level of bound hormone. In the case of elevated TBG in pregnancy, increased concentration of bound T_4 may allow more T_4 for placental passage. As FT_4 concentrations are normal in TBG excess sera, this suggests that there is an increase in the net dissociation of T_4 from T_4 binding proteins. As in FDH with increased serum T_4 binding, elevated T_4 binding in inherited TBG excess (or in pregnancy) does not render the subject hyperthyroid, since enhanced availability of T_4 probably occurs only in tissues whose capillaries are highly permeable to TBP.

SUMMARY

Previous observations from our laboratory have shown that in sera with excess thyroxine (T_4) protein binding (as in familial dysalbuminemic hyperthyroxinemia) there is often a corresponding elevation in serum SHBG. In further studies, sera from ten subjects with TBG excess were examined for serum SHBG levels. The TBG excess subjects represented one male and nine females with TBG-RIA concentration varying from 3.4 - 7.9 mg/dl (normal range for both male and female = 1.2 - 2.6 mg/dl). Total T_4 concentration varied from 11.0 - 26.2 µg/dl (normal 5.5 - 12.0 µg/dl); whereas, free thyroxine (FT_4) concentration (1.3 - 2.1 ng/dl) was within the normal range (0.9 - 2.3 mg/dl). In five out of nine female subjects, SHBG concentration was 106 $\pm$ 42 nM which was higher than normal controls (64 $\pm$ 15 nM). In the one male subject with TBG excess, SHBG concentration was 57.5 nM which was above the normal range for males (12 - 52 nM). The parallel increase of SHBG and TBG concentrations does not appear to be an estrogen-mediated effect, as this correlation was also noted in a male and in two post-menopausal women. The increase in SHBG level in six out of ten sera with TBG excess may have been due to enhanced hepatic T_4 uptake due to increased net T_4 dissociation from serum T_4 binding proteins, despite normal serum FT_4 concentration. Alternately, the parallel increase in transport proteins might repreent a genetic abnormality affecting both of the hepatic glycoproteins TBG and SHBG.

REFERENCES

1. Premachandra BN, Nisula B, Pardridge WM, et al. 59th Meeting Amer Thyroid Assn, New Orleans, LA, 1983, p T-25.
2. Pardridge WM and Mietus LJ. J Clin Invest 66: 367, 1980.
3. Pardridge WM, Premachandra BN, and Fierer G. Amer J Physiol 248: G545, 1985.
4. Ekins R. Lancet I: 1129, 1985.

IDENTIFICATION AND REGULATION OF THYROPEROXIDASE: USE OF SPECIFIC ANTISERUM

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Thyroid peroxidase is the hemoprotein enzyme involved in catalyzing the iodination and subsequent coupling of tyrosyl residues in thyroglobulin (Tg) to form thyroid hormone (1-13).

Proteolytic purification of this tightly membrane-bound enzyme has yielded fragments whose characteristics, including molecular weight, have depended on the method of solubilization (4,13,15). Molecular weight estimations of the trypsinized enzyme have ranged from 45,000 in beef (4) to 64,000 in hog (6) to larger fragments of molecular weight of 92,000 and greater in both beef and hog thyroid preparations (5,12,16).

Since thyroperoxidase is clearly an essential enzyme in the biosynthesis of thyroid hormone, we have attempted a more accurate characterization of this hemoprotein enzyme by immunoprecipitation. To this end, we have obtained anti-thyroperoxidase from Dr. A. Taurog at the University of Texas Health Science Center. There has not been a successful preparation of this antisera prior to this because of the problems associated with purification of thyroperoxidase, as well as lack of sufficient quantities for immunization purposes. The antiserum was made against a partially purified highly active preparation of thyroperoxidase from a porcine source and was found to react with the immunizing antigen in an Ouchterlony immunodiffusion assay at a dilution of 1:64. We performed three different experiments to assess the specificity of the antiserum. The first involved a test of its ability to immunoprecipitate the iodinating activity from a crude solubilized thyroperoxidase enzyme preparation. Tg was used as a substrate and the results were expressed in the form of acid precipitable counts of ^{125}I incorporated into Tg. When thyroperoxidase antibody was used to immunoprecipitate the active enzyme from these assays, the amount of iodinated Tg was reduced 80% to background level (Table 1). Antibodies against intestinal peroxidase, horseradish peroxidase, and normal rabbit serum did not remove this activity. A second experiment was then performed in which poly $(A)^+$ RNA from sheep thyroid (i.e., mRNA) was used to program the cell-free translation of thyroid specific polypeptide products. When these polypeptides (products) were reacted with anti-thyroperoxidase serum and electrophoresed, a single band of molecular weight 77,000 was detected which confirmed that the antiserum has a single detectable specificity (Fig. 1). Finally, since Tg is the major protein constituent of the thyroid, we also considered the reactivity of

Table 1. Immunoprecipitation of Iodinating Activity from a Crude Solubilized Thyroperoxidase Preparation

Antiserum	CPM x 10^{-3} ± S.D.	
Intestinal peroxidase	10.162	1.299
Horseradish peroxidase*	8.892	.732
Thyroperoxidase	2.614	.086
NRS	9.195	.143
Pansorbin only	9.465	.618
No serum	12.758	.849
No enzyme	3.785	.318

*This antiserum is directed against the active site of horseradish peroxidase.
The ability of various antisera against peroxidase to immunoprecipitate iodinating activity from a crude solubilized TPO preparation was tested. Non-serum controls were included. Values represent the mean of triplicate determinations from trichloracetic acid precipitable counts incorporated into substrate Tg.

anti-thyroperoxidase with Tg which may have been a contaminant in the immunizing antigen but we found no reactivity between thyroperoxidase antiserum or normal rabbit serum with ^{125}I-Tg (Table 2).

We then used the antiserum to study the immunoreactive products present in the ovine thyroid cell culture. For these experiments we collected two-week cultures of thyroid cells which had been labeled overnight with ^{33}S-methionine. These cells' membranes were lysed and the membrane and cytosolic proteins were incubated with anti-thyroperoxidase. Immunoprecipitates were collected and electrophoresed under reducing conditions on an SDS-polyacrylamide gel and subjected to autoradiography. Anti-thyroperoxidase precipitated a protein with molecular weight 104,000 which was most abundant in those cells cultured in the presence of TSH (Fig. 2). The difference in molecular weight between the translation product and the native enzyme can be explained by the fact that thyroperoxidase is glycosylated and contains a heme prosthetic group. Also noted on this autoradiograph was a doublet of proteins with molecular weights of 58,000 and 60,000 which were of greatest intensity in the lane containing immunoprecipitate from cells cultured without TSH. Since the appearance of the higher molecular weight form of the enzyme in TSH-stimulated cultures corresponds well with the ability of these cells to iodinate thyroglobulin, we have concluded that this represents the active form of the enzyme. Furthermore, the presence of the two lower molecular weight species, most prevalent in unstimulated cells, and their concurrent decrease in iodinating activity suggests that thyroperoxidase is indeed synthesized in these cultures but may be rapidly broken down into two smaller, possibly inactive species. The molecular weight of the active enzyme agrees well with that determined by other investigators. We have have detected the smaller molecular weight species because our detection was based on immunoreactivity and not peroxidase activity. The 58,000 and 60,000 molecular weight species are unlikely to be non-related contaminants identified by the antiserum due to their presence in the antigen preparation because of their TSH dependence, as well as the singular

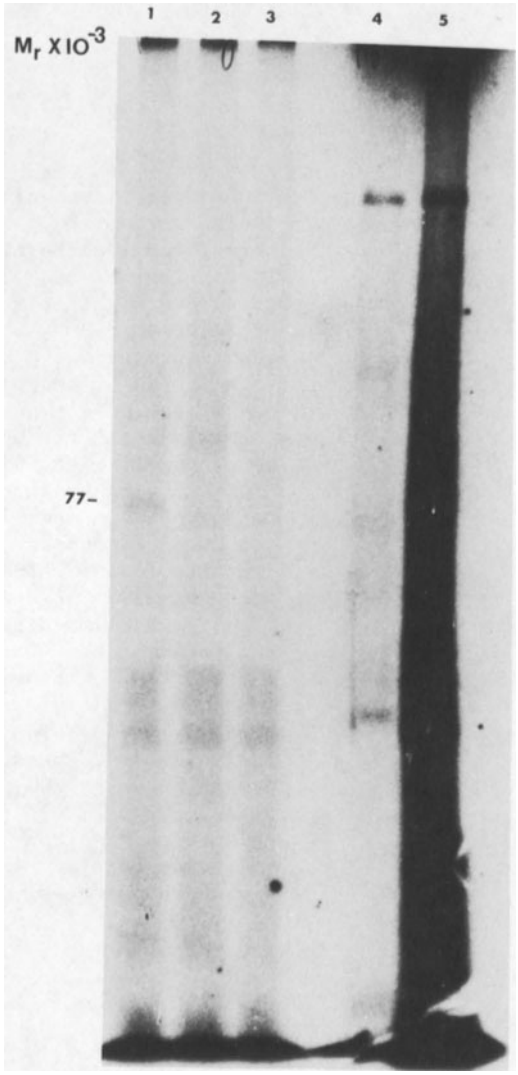

Fig. 1. Autoradiogram depicting the cell-free translation products of sheep thyroid poly (A^+) RNA. RNA was prepared from frozen glands and poly (A^+) RNA selected on oligo dT cellulose then translated in a rabbit reticulocyte lysate in the presence of ^{35}S-methionine. Aliquots were reacted with antibody then subjected to SDS-PAGE, dried, and the autoradiogram obtained. Lane 1: immunoprecipitation with anti-TPO antiserum. Lane 2: antithyroglobulin immunoprecipitate. Lane 3: NRS. Lane 4: reticulocyte lysate endogenous background. Lane 5: total poly (A^+) translation products. Nonspecific bands determined by reference to lane 3 are associated with the serum components.

Table 2. Reactivity of Antithyroperoxidase with ^{125}I-thyroglobulin

	Antiserum			
	TPO	NRS	Tg*	Total (TCA)
cpm	369	233	5686	6110

*Tg-thyroglobulin.
Thyroglobulin was iodinated in vitro by incubation of ^{125}I with TSH-stimulated sheep thyroid cells in 1° culture then immunoprecipitated with the indicated antiserum. Total cpm represents the mean TCA precipitable ^{125}I available in each immunoprecipitate (n=3).

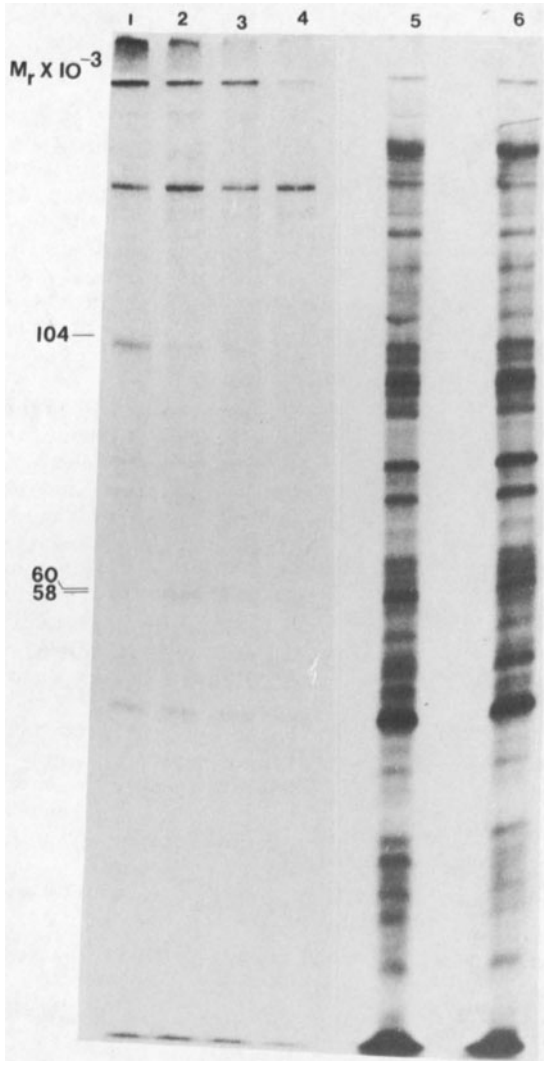

Fig. 2. TPO reactive cytosolic proteins from sheep thyroid cell culture. Thyroid cells were grown overnight in the presence of ^{35}S-methionine and with or without TSH. Immunoprecipitation was performed and the products were subjected to SDS-PAGE followed by antoradiography. Lanes 1 and 2: TPO immunoprecipitate (+ and - TSH, respectively). Lanes 3 and 4: NRS immunoprecipitate (+ and - TSH, respectively). Lanes 5 and 6: total cytosolic protein (+ and - TSH, respectively). Background bands at 200,000 and 43,000 are cell culture protein contaminants of all immunoprecipitates.

gen preparation because of their TSH dependence, as well as the singular specificity demonstrated by the translation product immunoprecipitation. This experiment also shows that native peroxidase is a single polypeptide with no intermolecular disulphide bonds.

In order to study the accumulation of anti-thyroperoxidase reactive products over time, we performed a Western immunoblot of electrophoresed TSH-stimulated cell lysates from two-week ovine thyroid cell cultures. When electrophoresis was performed under non-reducing conditions, poorly resolved material was evident (probably thyroperoxidase aggregates) in the region at and above 100,000 daltons. Also present were two sharp bands of molecular weight, 54,000 and 58,000. The aggregates almost completely disappeared with reduction, leaving only a band at about molecular weight 100,000 and increasing the intensity of the two smaller species. We have interpreted these results as signifying a basal level of synthesis and breakdown of thyroperoxidase both in the TSH-stimulated and unstimulated cells. The inducibility of iodination in thyroid cell cultures upon addition of TSH may involve not only an increase in the synthesis of the enzyme, but also its stabilization. Such an effect would explain the apparent short life

span of active thyroperoxidase in the absence of TSH in the cell cultures and would provide the basis for a finely tuned response to TSH modulation of the synthesis of thyroid hormone.

REFERENCES

1. Remy L, Michel-Bechet M, Athouel-Haon AM, et al. Acta Histochem 67: 159, 1980.
2. Strum JM and Karnovsky MJ. J Cell Biol 44: 655, 1970.
3. Bjorkman U, Ekholm R, Ericson LE, et al. Mol Cell Endocrinol 5: 3, 1976.
4. Ljungren JC and Akeson A. Arch Biochem Biophys 127: 346, 1968.
5. Hosoya T and Morrison M. J Biol Chem 242: 2828, 1967.
6. Coral M and Taurog A. J Biol Chem 242: 5510, 1967.
7. Taurog A. Recent Prog Horm Res 26: 189, 1970.
8. Taurog A, Lothrop ML, and Estabrook RW. Arch Biochem Biophys 139: 221, 1970.
9. Krinsky MM and Alexander NM. J Biol Chem 246: 4755, 1970.
10. Yip CC. Biochem Biophys Acta 96: 75, 1965.
11. DeGroot LJ and Thompson JE. Endocrinology 76: 632, 1965.
12. Alexander NM. Endocrinology 100: 1610, 1977.
13. DeGroot LJ and Nagasaka A. In K Fellinger and R Hoffer (eds), Further Advances in Thyroid Research, Vienna Academy of Medicine, Vienna, Austria, 1971, p 413.
14. Eggo MC and Burrow GN. Endocrinology 113: 1655, 1982.
15. Rawitch AB, Taurog A, Chernoff SB, et al. Arch Biochem Biophys 194: 244, 1979.
16. Nakashima T and Taurog A. Clinica Chimica Acta 83: 129, 1978.

IRREVERSIBLE INACTIVATION OF LACTOPEROXIDASE BY EXCESS HYDROGEN PEROXIDE INVOLVING CLEAVAGE OF THE CATALYTIC HEME MOIETY*

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Iodination of tyrosyl residues and the coupling reaction to produce the thyroid hormones are catalyzed by one single enzyme, the thyroid peroxidase. In addition to the two substrates, tyrosine and I^-, the presence of H_2O_2 is required. Though the overall pathway for the two reactions has been known for a long time, the molecular mechanism, and especially the regulatory functions of I^- and H_2O_2, are still a matter of debate. In this report we present evidence that H_2O_2 and I^- are not merely essential substrates but, in addition, they may be involved in the regulation of enzyme activity.

RESULTS AND DISCUSSION

Since thyroid peroxidase and lactoperoxidase (LPO) have been shown to behave very similarly (1,2), we have used LPO as a model enzyme in all experiments. The prosthetic group of this hemoprotein has been reported to be Fe-protoporphyrin IX (3). In the ground state the iron is in the ferric (+III) form. A catalytic cycle proceeds as follows (4).

$$\text{LPO} + H_2O_2 \longrightarrow \text{cpd I} \qquad (1)$$

$$\text{cpd I} + \text{AH} \longrightarrow \text{cpd II} + \text{A}\cdot \qquad (2)$$

$$\text{cpd II} + \text{AH} \longrightarrow \text{LPO} + \text{A}\cdot \qquad (3)$$

$$\text{A}\cdot + \text{A}\cdot \longrightarrow A_2 \qquad (4)$$

Oxidation with H_2O_2 results in formation of cpd I (reaction 1). Although opinions on the exact chemical nature of this compound are still controversial, it is generally accepted that it retains the two oxidizing equivalents from H_2O_2, thus being capable of accepting two electrons from an electron donor (2). If cpd I reacts with a 1-electron-donor (reaction 2), cpd II and an A· radical are produced. In a second 1-electron transfer (reaction 3), cpd II is reverted to the ground state and the two A· radicals may terminate the cycle as shown in reaction 4. In the absence of an exogenous electron donor, H_2O_2 may function as a reductant. This

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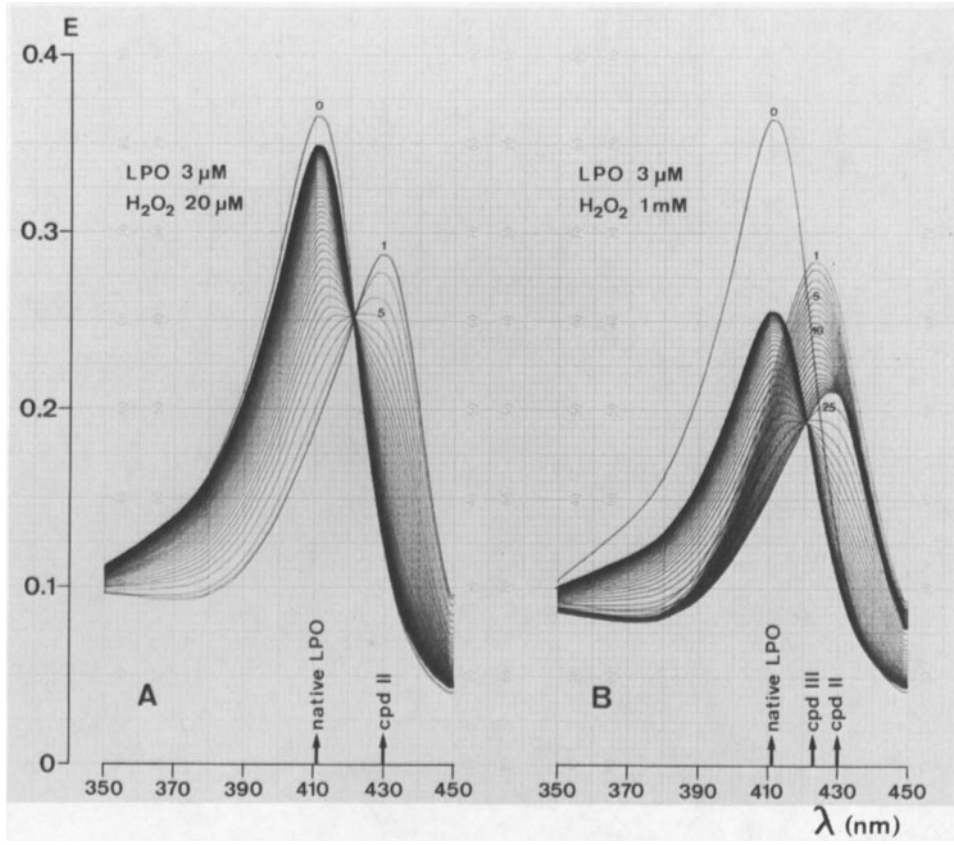

Fig. 1. Formation and decay of cpd II (A) and cpd III (B) from LPO in the presence of excess H_2O_2 were followed by serial overlay recordings of spectrophotometric rapid scans in the Soret region. Time intervals between scans: 1 min. Some of the cycle numbers are indicated in the figure.

is shown in Fig. 1A, where LPO by addition of 20 µM H_2O_2 is converted into cpd II. Cpd I cannot be detected under these experimental conditions due to a rapid spontaneous conversion into cpd II (2). The peak of cpd II then decreases slowly with a simultaneous recovery of the native enzyme. The curves going through an isosbestic point on the original peak (421 nm) suggest nearly full recovery of the native enzyme. If the H_2O_2 concentration is increased to 1 mM, cpd II reacts with excess peroxide whereby cpd III is formed (Fig. 1B). The peak of cpd III decreases very sluggishly with time. However, in contrast to the decomposition of cpd II, no compensatory recovery of the native enzyme can be observed during that time. Only when almost all of the excess H_2O_2 is used up does cpd III shift back into cpd II which is then reconverted into native peroxidase. The difference between original and final peak heights is directly proportional to the loss of enzyme recovery (5).

Finally, if the H_2O_2 concentration is increased to 10 mM, cpd III completely disappears over a period of several hours without any recovery of the native enzyme (Fig. 2). This total loss of absorption in the Soret region suggests destruction of the heme moiety corresponding to irreversible inactivation of LPO.

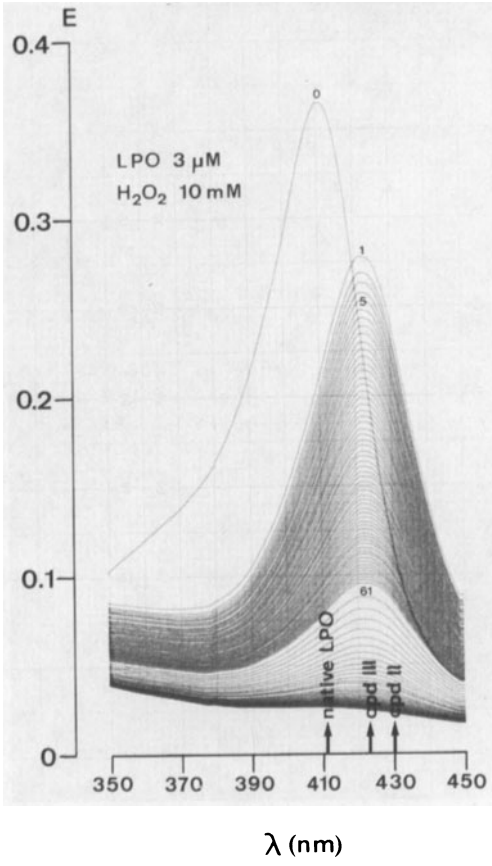

Fig. 2. Total decay of cpd III (3 µm) in the presence of 10 mM H_2O_2. No compensatory formation of native LPO occurs.

This hypothesis was experimentally confirmed by determining enzyme activity which was zero after loss of the Soret spectrum (5). Moreover, when such a sample was treated with trichloracetic acid and the fractions analyzed by atomic absorption spectroscopy, the heme iron was detected in the supernatant, whereas it was found in the precipitate when native LPO was treated similarly. All experiments presented so far were carried out in the absence of an exogenous electron donor. In order to evaluate the effect of such a reductant on compound formation and enzyme inactivation, I^- was added to the otherwise unchanged incubation mixture. A catalytic cycle in the presence of iodide proceeds as follows:

$$\text{LPO} + H_2O_2 \longrightarrow \text{cpd I} \qquad (5)$$

$$\text{cpd I} \xrightarrow{H_2O_2} \text{cpd II} \xrightarrow{H_2O_2} \text{cpd III} \qquad (6)$$

$$\text{cpd I} \xrightarrow{I^-} \text{LPO} + I^+$$

$$I^+ \xrightarrow{I^-} I_2 \xrightarrow{I^-} I_3^- \qquad (7)$$

$$I_2/I^+ + H_2O_2 \longrightarrow \begin{matrix}\text{molecular } O_2 \\ \text{superoxide anion } O_2^{\cdot-} \\ \text{singlet oxygen } {}^1\Delta g\ O_2\end{matrix} \qquad (8)$$

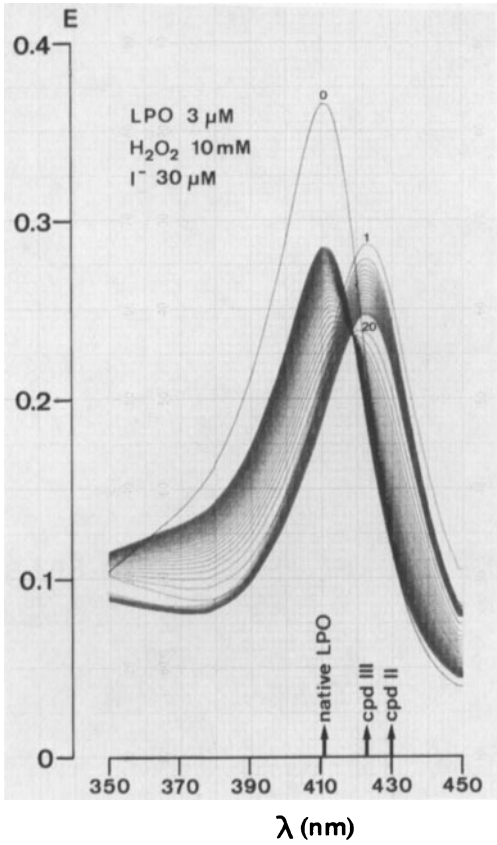

Fig. 3. Addition of 10 µM I^- to an otherwise identical incubation mixture as described in Fig. 2 accelerated the overall reaction rate. After about 20 minutes, cpd III shifted back via cpd II into native LPO.

The following reactions deserve special attention. First, reaction 6 indicates that H_2O_2 and I^- may compete for the active site of cpd I. H_2O_2 produces cpd II in a 1-electron transfer, which is converted into cpd II in a 1-electron transfer, which is converted into cpd III with additional H_2O_2, whereas I^- reacts in a 2-electron transfer with cpd I resulting in the production of iodinium cation I^+ and native enzyme (4). These two pathways do not exclude each other, but may occur simultaneously. One or the other reaction may be favored depending on the concentrations of the reactants. The second point to consider is that I^+ and/or I_2, but not I_3^-, can oxidize H_2O_2 and produce molecular oxygen, singlet oxygen, or superoxide anions as indicated in reaction 8 (6).

In Fig. 3 the results of a typical experiment are shown. Whereas in the absence of I^- cpd III was degraded slowly but completely (Fig. 2), even small amounts of I^- accelerated the reaction including inactivation as shown in separate experiments (5). However, after about 20 minutes, the peak of cpd III shifted back via cpd II to the ground state and thus preserved the enzyme activity to some extent.

Experiments to establish the nature of the substance which irreversibly inactivates peroxidase are presently underway. All data obtained so far indicate that the damaging component is not H_2O_2, as often assumed, but a reaction product thereof such as superoxide radicals or singlet oxygen, which are produced possibly in a catalatic or pseudocatalatic pathway from LPO + H_2O_2 or from H_2O_2 and I_2 (6). Although superoxide anions or radicals respectively (pK_a 4.8) are not believed to be directly involved in the process of irreversible enzyme inactivation, they may react with H_2O_2 in the following way:

$$O_2^{\cdot-} + H_2O_2 \longrightarrow O_2 + OH^- + OH\cdot \qquad (9)$$

In this pathway, known as the Haber-Weiss reaction, hydroxyl radicals OH· are produced (7). Whether this radical is the actual damaging species, however, remains to be established.

SUMMARY

We have presented evidence that:

1. Excess H_2O_2 converts LPO into cpd III followed by irreversible inactivation of the enzyme.

2. I^- in low concentrations accelerates the overall reaction rate including inactivation but partially preserves enzyme activity.

3. Irreversible inactivation of peroxidase involves decomposition of the heme moiety and liberation of heme iron.

4. Although the exact nature of the damaging substance is not yet established, there is strong evidence that it is not H_2O_2, but a reaction product thereof which is generated in a catalatic or pseudocatalatic reaction.

REFERENCES

1. Taurog A, Dorris ML, and Lamas L. Endocrinology 94: 1286, 1974.
2. Ohtaki S, Nakagawa H, Nakamura M, et al. J Biol Chem 257: 761, 1982.
3. Sievers G. Biochim Biophys Acta 579: 181, 1979.
4. Ohtaki S, Nakagawa H, Kimura S, et al. J Biol Chem 256: 805, 1981.
5. To be published.
6. Huwiler M, Burgi U, and Kohler H. Eur J Biochem 147: 469, 1985.
7. Haber F and Weiss J. Proc R Soc London 147: 332, 1934.

RESIDUAL CAPACITY FOR IODIDE ORGANIFICATION IN HUMAN THYROID SLICES PROVOKED BY HYDROGEN PEROXIDE

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Iodination of tyrosyl residues in thyroglobulin is one of the essential steps of biosynthesis of thyroid hormones. In 1944, Dempsy demonstrated a peroxidase in the thyroid histochemically (1). Since then, the study concerned with thyroid hormone production has progressed greatly. So far, evidence has been provided, indicating that thyroid peroxidase is the key enzyme for iodide organification in the thyroid. Subsequently, it was also demonstrated that the process of thyroid hormone synthesis involves hydrogen peroxide.

Ahn and Rosenberg observed H_2O_2 generation in canine thyroid, which was increased by adding TSH (2), whereas Johansson and Bjorkman provided cytochemical evidence of H_2O_2 generation (3). Benard and Brault showed that 100 mg of hog thyroid slices generated 7 ng of H_2O_2 per min by a method using homovanilic acid (4,5). Thus, there have been several reports demonstrating that the thyroid itself generates hydrogen peroxide, which is a sine qua non for hormone synthesis. Yet, we are not certain as to whether hydrogen peroxide generation is the rate-limiting step of hormone synthesis in the human thyroid, as suggested by results from Chiraseveenuprapund and Rosenberg using calf thyroid (6). The purpose of the present study, therefore, was to further investigate this problem.

MATERIALS AND METHODS

Thyroid tissues were obtained at surgery. The thyroid tissue was cut immediately with an ice cooled slicer. Two thyroid slides weighing 20-40 mg were placed in a prewarmed 100 ml Erlenmeyer flask for 60 min preincubation at 37°C, which contained 5 ml of Eagle's solution with 5 mU of TSH and 500 pmole of iodide.

After preincubation, the tissues were transferred into another flask containing a solution similar to that for preincubation but with addition of 5 μCi of ^{125}I and hydrogen peroxide. Postincubation was performed for another 60 min at 37°C. Each tissue was weighed after incubation and homogenized in a glass homogenizer with 1 ml of 0.067 M phosphate buffer, pH 7.0, containing 1 μmole of methimazole. 0.8 ml of homogenate was precounted in

a γ counter and mixed with 2 ml of 10% TCA solution. The mixture was centrifuged at 3,000 rpm for 5 min. The supernatant was discarded, and the activity of the precipitate counted. In the case of medium, 0.3 ml of medium was precounted and added with 0.1 ml of carrier serum for TCA precipitation. The precipitate was washed twice with TCA solution. In order to observe the effect of hydrogen peroxide, 2.5 μmole (1.7 μg/0.1 ml/hr) of H_2O_2 was added to the postincubation medium. The duplicated thyroid tissues were then pooled for homogenization, and 2 ml of the methimazole phosphate buffer was used instead of 1 ml of the buffer as in the case mentioned above. TCA precipitable iodine (considered as PBI) was expressed in pmole/0.1 g thyroid tissue/hr.

The effect of 500 nmole/ml of H_2O_2 was examined with 13 surgical specimens; 9 Graves' disease, 2 perinodular, and 2 adenoma tissues.

RESULTS

Incubation Method

The aim of the present experiment, as indicated above, was to demonstrate that hydrogen peroxide is the rate-limiting step of thyroid hormone synthesis, by showing a residual capacity for iodide organification in human thyroid slices upon the addition of hydrogen peroxide. To achieve this aim, the incubation technique in this type of experiment must be excellent, i.e., yielding thyroid hormone production close to an *in vivo* pattern. Our data with the present incubation method already described (7), is shown in Fig. 1. The pattern and yield of iodoamino acid in rat thyroid by this *in vitro* technique are fairly close to those obtained by *in vivo* injection of iodide. Thus, this incubation technique appears to be appropriate to conduct this type of experiment.

Effect of Various Doses of H_2O_2 (0-5 x 10^5 nmole/ml) on Iodination

Thyroid tissue was obtained from a 12 year old female patient with Graves' disease, who was treated with 10 mg of methimazole and 30 mg of propranolol per day for a long time, and with I_2 administration during the last nine days. Various doses of H_2O_2 were added to the postincubation media. The highest PBI was obtained with 5 x 10^2 nmole/ml of H_2O_2, which was approximately 450% that of without H_2O_2. On the other hand, PBI with 10^4 nmole/ml of H_2O_2 was still 60% above the value of the control. However, more than 5 x 10^4 nmole/ml of H_2O_2 depressed the PBI, suggesting that the thyroid might be damaged by a high dose of H_2O_2 (Fig. 2).

Effect of 0-10^4 nmole/ml of H_2O_2 of Iodination (Fig. 3)

The tissue was obtained from a 13 year old female patient with Graves' disease, who was treated with 10 mg of methimazole and 20 mg of propranolol per day for a long time, and with I_2 administration for the last 12 days. Total PBI and intrathyroidal PBI with 5 x 10^2 and 10^3 nmole/ml of H_2O_2 made a plateau. However, the PBI or TCA precipitable iodine found in the medium after incubation did not make a peak. The precipitable iodine in the medium seemed to rise gradually with graded doses of H_2O_2, implying that higher doses of H_2O_2 might damage the tissue. These data, therefore, suggest that intrathyroidal PBI reflects the synthesis of thyroid hormone better than total PBI, including PBI in the medium.

PBI without H_2O_2 and that with 10 nmole/ml of H_2O_2 showed a good agreement between duplicated samples. Otherwise, there were fairly large discrepancies between duplicated values with high doses of H_2O_2. No PBI at the higher doses of H_2O_2 was lower than the values with 0 or 10 nmole/ml

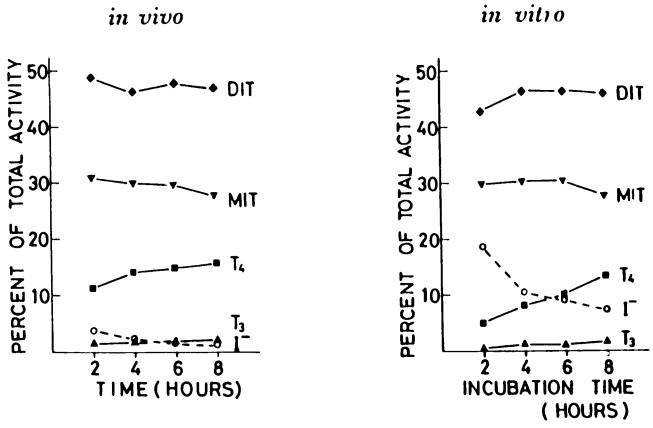

Fig. 1. Iodoamino acid synthesis in rat thyroid lobe by an in vitro method (comparison with in vivo synthesis). In vivo and in vitro synthesis of iodoamino acid was followed for 8 hrs. The pattern and the yield of iodoamino acid by both methods were very similar.

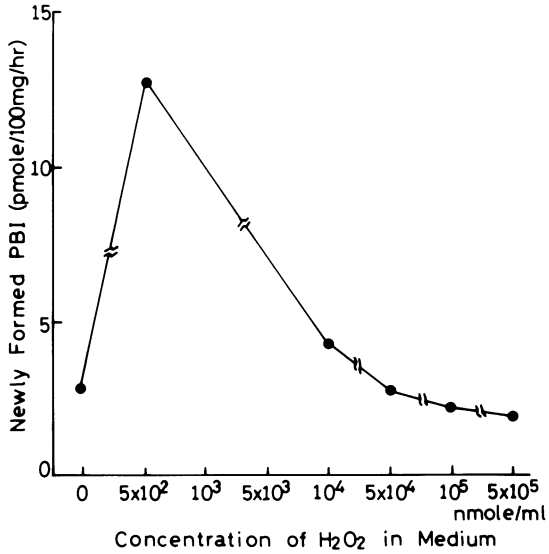

Fig. 2. Effect of various doses (0-5 x 10^5 nmole/ml) of H_2O_2 on iodination in the thyroid of a 12 y.o. female patient with Graves' disease. Patient treated with MMI, propranolol, and I_2. The highest PBI was obtained with 5 x 10^2 nmole/ml of H_2O_2. PBI was depressed by very high doses of H_2O_2, probably through its destructive effect on the tissue.

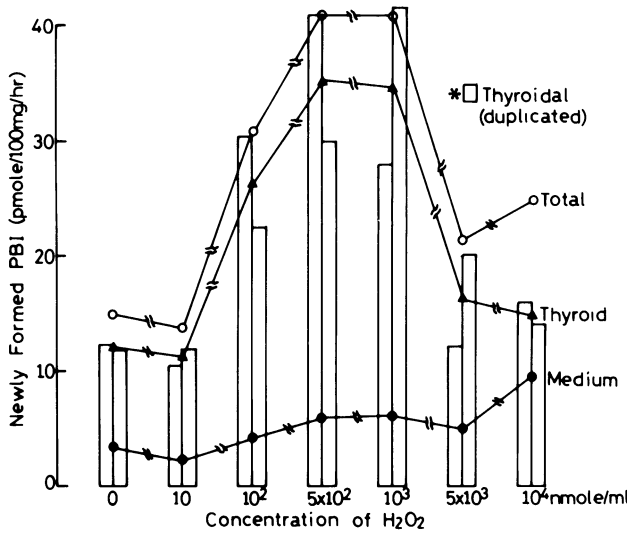

Fig. 3. Effect of various doses (0-10^4 nmole/ml) of H_2O_2 on iodination in the thyroid of a 13 y.o. female patient with Graves' disease. Patient treated with MMI, propranolol, and I_2. Total PBI and intrathyroidal PBI with 5 x 10^3 and 10^4 nmole/ml of H_2O_2 makes a plateau, but the PBI in medium does not make any peak, which seems to rise gradually with graded dose of H_2O_2.

of H_2O_2. As mentioned above, 10 nmole/ml of H_2O_2 was not sufficient to overcome a catalatic activity in the thyroid to stimulate iodination. Based on the above data, 5 x 10^2 nmole/ml of H_2O_2 was used in subsequent experiments with patients, and duplicated samples were pooled for homogenization to minimize fluctuation of data.

Study of Clinical Cases

Effect of H_2O_2 on iodination of Graves' disease thyroid (Fig. 4). Nine patients with Graves' disease were treated with MMI, propranolol, and I_2 except for one case treated only with 20 mg of KI per day. All patients were euthyroid at surgery. Exogenous hydrogen peroxide significantly enhanced PBI synthesis in all nine cases with Graves' disease. PBI expressed in percent of the control ranged from 123% to 1,245%. Thus, residual capacity for iodination is demonstrated in the thyroid of patients with Graves' disease.

Effect of H_2O_2 on iodination of two perinodular and two adenoma tissues (Fig. 5). Two morphologically normal perinodular tissues showed significant increase in PBI formation by exogenous H_2O_2, but the adenoma tissues did not respond to H_2O_2. Results indicate that perinodular thyroid tissue, as well as Graves' thyroids, have a residual capacity for hormone production, which can be elicited by adding H_2O_2, but adenoma tissues do not.

DISCUSSION

In the present experiment, less than 10 nmole/ml of H_2O_2 did not at all enhance iodination, implying that this concentration of H_2O_2 in the

medium is too low to reach an iodination site beyond an H_2O_2 destructive system in the thyroid. Since the work of Leblond and Gross in 1948 on the iodination site in the thyroid (8), many studies have been published. Most reports recognized morphological evidence of iodination in the follicular lumen close to the apical plasma membrane in the very early phase of the experiments, so that H_2O_2 added to the medium must cross the follicular cell against decomposing activity. However, 10^2 - 10^3 nmole/ml of H_2O_2 in Fig. 3 seems to overcome a catalatic activity in the thyroid to stimulate PBI production. On the other hand, PBI or TCA precipitable iodine in the medium does not seem to reflect synthetic ability of the thyroid, but to be derived from destructed tissue. Thus, in the in vitro system including H_2O_2, intrathyroidal PBI should reflect better the PBI synthetic activity of the thyroid than PBI in the medium or total PBI.

Hydrogen peroxide enhanced iodination in all cases with Graves' disease, as well as in normal tissues. This result suggests that the H_2O_2 supply is the rate-determining step in thyroid hormone synthesis and that the peroxidase enzyme is used for the reaction repeatedly, while the H_2O_2 generated is probably decomposed in a very short time.

In contrast to tissues from Graves' disease or normal tissues, adenoma tissues did not respond at all to H_2O_2. One possible explanation is that H_2O_2 could not reach the iodination site due to strong decomposing activity in the adenoma tissue. The other is that the adenoma tissue generates large amounts of H_2O_2, stimulating iodination to a maximum level. If H_2O_2 would be supplied excessively, the rate-determining material could be iodide rather than H_2O_2, which might be trapped or restrictedly by the adenoma tissue. Attention should be paid then to H_2O_2 generation as an important factor in the mechanisms of excessive hormone production of thyrotoxicosis in Graves' disease.

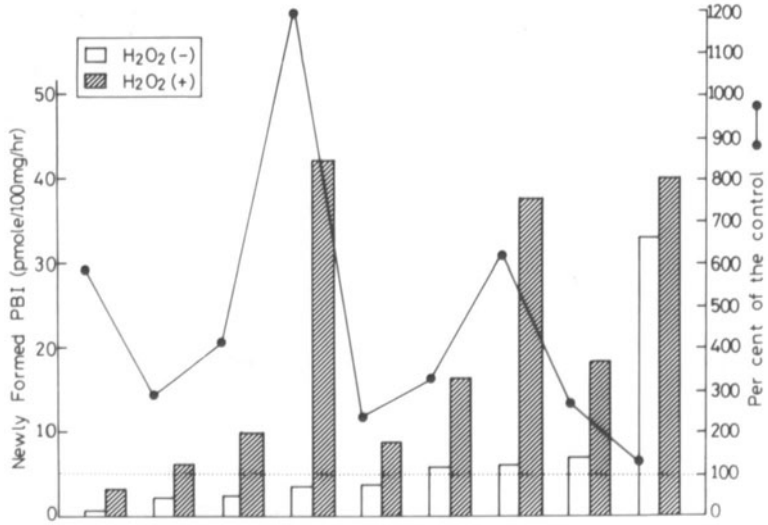

Fig. 4. Effect of 5 x 10^2 nmole/ml of H_2O_2 on iodination of Graves' disease thyroids. Nine patients with Graves' disease were treated with MMI, propranolol, and I_2 except for one case treated with KI only. PBI was enhanced by H_2O_2 in all nine cases. Unshaded bars: newly formed PBI without H_2O_2. Shaded bars: newly formed PBI with H_2O_2. ●——●: newly formed PBI with H_2O_2 expressed in percent of PBI without H_2O_2.

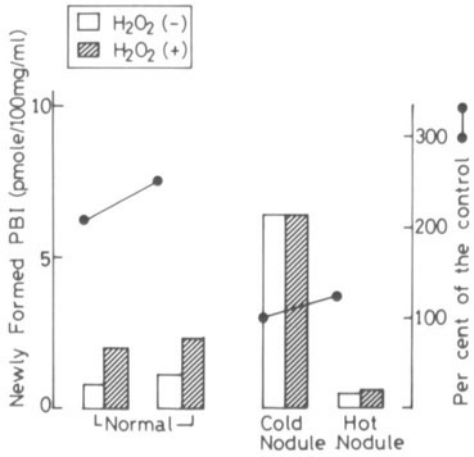

Fig. 5. Effect of H_2O_2 on iodination of normal and adenoma tissues. Unshaded bars: newly formed PBI without H_2O_2. Shaded bars: newly formed PBI with H_2O_2. ●——●: newly formed PBI with H_2O_2 expressed in percent of PBI without H_2O_2, the control. PBI was raised by H_2O_2 in two normal thyroids, but not at all in adenoma tissues.

In conclusion, H_2O_2 generation seems to be the rate-determining step of hormone synthesis of the thyroid which has sufficient amounts of the other materials for hormone production, namely iodide, peroxidase and thyroglobulin sufficiently.

REFERENCES

1. Dempsy EW. Endocrinology 34: 27, 1944.
2. Ahn CS and Rosenberg IN. Endocrinology 86: 396, 1970.
3. Johansson BR and Bjorkman U. Cell Tissue Res 228: 337, 1983.
4. Bernard B and Brault J. Union Med Can 100: 701, 1971.
5. Bernard B and Brault J. Further Advances in Thyroid Research, 1971, p 771.
6. Chiraseveenuprapund P and Rosenberg IN. Endocrinology 109: 2095, 1981.
7. Okamura K, Inoue K, Nakashima T, et al. Acta Endocrinol 92: 286, 1979.
8. Leblond CP and Gross J. Endocrinology 43: 306, 1948.

T_3 AND rT_3 GENERATION FROM EXOGENOUS AND ENDOGENOUS T_4 IN PERFUSED DOG THYROIDS

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In previous studies, we found evidence for intrathyroidal deiodination of T_4 released from thyroglobulin (endogenous T_4) to T_3 and rT_3 by iodothyronine deiodinases similar to those found in the liver (1). This is the major mechanism behind the relative hypersecretion of T_3 and rT_3 compared to T_4 observed in thyroid perfusion studies (1).

The aim of the present study was to evaluate if the thyroid may also produce T_3 and rT_3 from exogenous T_4, i.e., T_4 entering the thyroid gland via the vascular bed.

MATERIAL AND METHODS

Purified T_4 was obtained from Henning, West Berlin, FRG. Simultaneous perfusions of the two separate thyroid lobes of dogs (2) were employed in all experiments. One lobe served as a control. T_4, T_3, and rT_3 were measured by radioimmunoassays (3-5). Special precautions were taken when measuring T_3 and rT_3 in samples containing serum or high T_4 combinations. Iodothyronine-free dog serum was prepared by treatment with charcoal (6). Student's t test for paired comparisons was employed for statistical evaluation using a 5% limit of statistical significance.

RESULTS

In a series of experiments, both lobes were perfused with buffer medium (Krebs-Ringer bicarbonate buffer with 5 nmol/l glucose, 4.6% dextran, and 0.2% bovine serum albumin) for 200 min. One lobe received, in addition, 200 ng/ml of T_4 during the period 60-200 min. This gave a gradual increase in T_3 and rT_3 concentrations in effluents to levels several times higher than the controls. Results from individual experiments are shown in Table 1.

To investigate the effect of the presence of thyroid hormone binding proteins in perfusates on T_3 and rT_3 generation, one lobe was perfused with the buffer medium, and the other with iodothyronine-free dog serum during the period 60-200 min. Both lobes received 200 ng/ml T_4 during the period 60-200 min. Perfusion with serum caused a decrease in T_3 and rT_3 generation from exogenous T_4 (Fig. 1).

Table 1. T_3 and rT_3 Production from Exogenous T_4 in Perfused Dog Thyroid Lobes

Exp.		T_4 ng/ml 30-60*	T_4 ng/ml 170-200*	T_3 ng/ml 30-60*	T_3 ng/ml 170-200*	rT_3 ng/ml 30-60*	rT_3 ng/ml 170-200*
1	+T_4	23.0	193.0	3.59	7.00	0.57	1.87
	Control	15.0	4.5	2.69	1.39	0.60	0.22
2	+T_4	3.3	158.5	0.67	12.62	0.11	2.39
	Control	4.6	4.9	1.04	1.03	0.16	0.18
3	+T_4	3.6	152.2	0.76	9.81	0.08	1.45
	Control	3.3	3.1	1.34	0.97	0.11	0.12
4	+T_4	15.4	174.1	2.51	6.39	0.35	0.83
	Control	28.0	12.3	3.59	2.47	0.37	0.27
5	+T_4	29.0	231.7	3.38	5.97	0.72	1.72
	Control	24.9	18.4	3.35	2.21	0.68	0.43
6	+T_4	42.6	169.9	3.80	1.27	0.92	0.88
	Control	69.2	6.5	5.43	0.97	1.60	0.30
Mean	+T_4	19.5	179.9	2.45	7.18	0.46	1.52
± SE		6.2	11.9	0.58	1.57	0.14	0.25
Mean	Control	24.2	8.3	2.91	1.51	0.59	0.25
± SE		9.9	2.4	0.66	0.27	0.22	0.04
P%		NS	--	NS	1.8	NS	0.4

*Mean of three effluent samples obtained during the minute interval indicated. Both lobes were perfused for 200 min. One lobe (+T_4) received 200 ng/ml T_4 during the period 60-200 min.

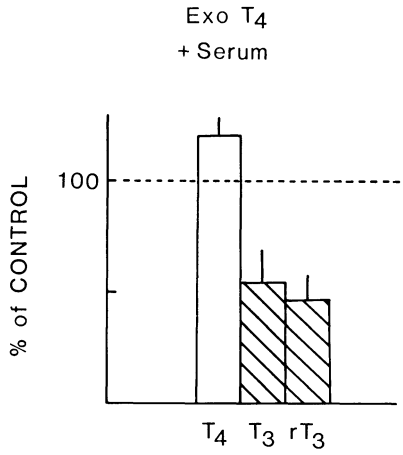

Fig. 1. Effect of perfusion with serum on T_3 and rT_3 generation from exogenous T_4 in perfused dog thyroid lobes. Both lobes were perfused with buffer medium for 60 min. From 60-200 min, one lobe received 200 ng/ml T_4 in the buffer medium (control), and the other received 200 ng/ml T_4, added to iodothyronine-free dog serum. Shown are the concentrations of iodothyronines in effluent from lobes perfused with serum in percent of corresponding control lobes at the end of the perfusion (170-200 min); mean + SE, n = 6. Hatched columns: $p<0.05$ vs control.

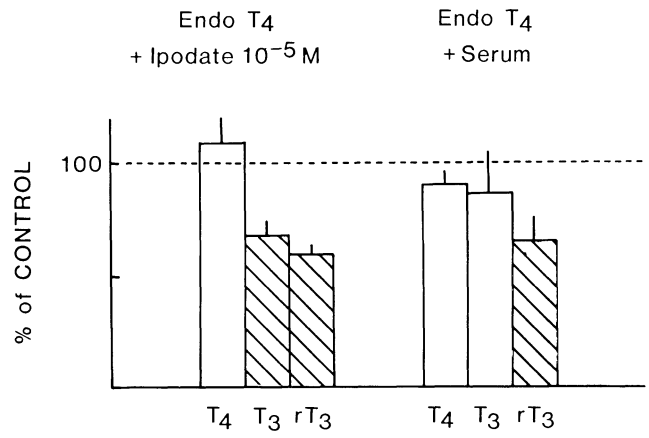

Fig. 2. Left: one lobe received 10^{-5} M iopdate during the interval 60-200 min. Both lobes received 100 μU/ml TSH during the interval 100-200 min. Ipodate lobe in percent of control lobe (results from (7)).
Right: one lobe was perfused with iodothyronine-free dog serum during the interval 60-200 min. Both lobes received 100 μU/ml TSH during the interval 100-200 min. Serum lobe in percent of control lobe (170-200 min). Mean ± SE, n = 5. Hatched columns: $p<0.05$ vs control.

To evaluate if some of the intrathyroidal generation of T_3 and rT_3 from endogenous T_4 (liberated from thyroglobulin) originated from T_4 reuptake following secretion, one lobe was perfused with buffer medium for 200 min and the other with iodothyronine-free dog serum during the period 60-200 min. Both lobes received 100 μU/ml bovine TSH during the period 100-200 min (Fig. 2). Perfusion with iodothyronine-free dog serum did not influence the generation of T_3 from endogenous T_4, while the generation of rT_3 was significantly lowered. For comparison, the secretion pattern observed when deiodination of endogenous T_4 to both T_3 and rT_3 is inhibited by 10^{-5} M ipodate, as studied previously (7), are shown (Fig. 2).

DISCUSSION

Thyroid perfusion studies in rats (8) and dogs (1) have shown that thyroid secretions contain relatively more T_3 and rT_3 compared to T_4 than should be expected from the iodothyronine content of thyroglobulin. The major mechanism behind this disparity is that some of the T_4 released from thyroglobulin (endogenous T_4) is deiodinated to T_3 and rT_3 before leaving the thyroid (1). This deiodination is catalyzed by enzymes similar to those found in some other organs such as the liver (9-11). The enzyme capacity is increased by prolonged hyperstimulation of the thyroid (11-13). This, however, influences only slightly the T_4/T_3 ratio in thyroid effluent from perfused dog thyroids (14). *In vivo* studies in humans employing measurements of the venous-arterial difference in iodothyronine concentrations across the thyroid, and the T_4 and T_3 content of thyroglobulin, have shown a relatively high rate of release of T_3 from the intact human thyroid (15). Such a relatively high T_3 difference across the thyroid, compared to T_4, could be due

to deiodination of both endogenous T_4 from thyroglobulin, and of exogenous T_4 taken up from the blood passing through the gland and released again as T_3.

The present study shows that the thyroid can produce T_3 from circulating T_4. This T_3 production was inhibited considerably by the presence of T_4 binding proteins, probably due to a decrease in thyroidal uptake of T_4. The production of T_3 from endogenous T_4 was not influenced by the presence of T_4 binding proteins in the perfusate. This may suggest that *in vivo* in the normal thyroid, T_3 production from endogenous T_4 is more important than T_3 production from plasma T_4. However, in the hyperplastic, hyperstimulated thyroid, T_3 production from plasma-borne T_4 may well be of considerable quantitative importance.

CONCLUSION

The thyroid may produce T_3 and rT_3 by several mechanisms, including synthesis in thyroglobulin, deiodination of T_4 released from thyroglobulin, and deiodination of T_4 entering the gland via the vascular bed. The T_3 production from plasma-borne T_4 may be of considerable quantitative importance in certain types of goiter.

REFERENCES

1. Laurberg P. Metabolism 33: 379, 1984.
2. Laurberg P. Acta Endocrinol (Copenh) suppl. 236, 1980.
3. Weeke J and Orskov H. In KGMM Alberti (ed), Recent Advances in Clinical Biochemistry, Churchill Livingstone, Edinburgh, London and New York, 1978, p 111.
4. Weeke J and Orskov H. Scand J Clin Lab Invest 35: 237, 1975.
5. Laurberg P. Endocrinology 102: 757, 1978.
6. Laurberg P. Endocrinology 109: 1569, 1981.
7. Laurberg P. Endocrinology 111: 1904, 1982.
8. Greer MA and Haibach H. In RO Greep, EB Astwood, MA Greer, et al. (eds), Handbook of Physiology, section 7: Endocrinology, volume III. Thyroid, American Physiological Society, Washington, 1974, p 135.
9. Laurberg P and Boye N. Endocrinology 110: 2124, 1982.
10. Erickson VJ, Cavalieri RR, and Rosenberg LL. Endocrinology 108: 1257, 1981.
11. Wu SY. Endocrinology 112: 417, 1983.
12. Erickson VJ, Cavalieri RR, and Rosenberg LL. Endocrinology 111: 434, 1982.
13. Ishii H, Inada M, Tanaka K, et al. J Clin Endocrinol Metab 52: 1211, 1981.
14. Laurberg P. Endocrinology 109: 1560, 1981.
15. Tegler L, Anderberg B, and Smeds S. Horm Metab Res 14: 593, 1982.

EFFECTS OF SYNTHETIC IODOLIPIDS ON THYROID FUNCTION "IN VITRO": POSSIBLE ROLE IN THYROID REGULATION*

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The inhibitory effects of iodine on the thyroid are mediated by an iodine organic intermediate (see review, 1), whose chemical nature is not known. Although thyroid hormones have been proposed to be such intermediates, their role is still controversial (see ref. 2 for discussion). It is known that, in addition to thyroid hormones, the gland is capable of synthesizing iodolipids (see review, 3), another type of organic iodocompounds. We have recently shown that iodinated free fatty acids comprise most of the iodolipids (4). The results obtained with different inhibitors of arachidonic acid metabolism suggested that iodinated derivatives of arachidonic acid may be a component of iodinated free fatty acids. Moreover, recent data from our laboratory has shown that in calf thyroid slides incubated with ^{125}I, the incorporation of radioiodine into 15-iodo-14-hydroxy- and 15-hydroxy-14-iodo-eicosatrienoic acid (I-OH-A) and the corresponding lactones was observed (to be published). Boeynaems and Hubbard (5) have also shown that rat thyroid produces the acid 5-iodo-6-hydroxy-eicosatrienoic 8,11,14 delta lactone (IL-δ). In a previous publication, we have demonstrated that a semi-purified preparation of iodoarachidonate inhibits in vitro different thyroid parameters (6). We have also shown that studies with pure preparations of I-OH-A and another derivative with an iodine atom in position 6 (IL-δ).

Calf thyroid slices were incubated as already described (6,7), and the following parameters were determined:

Iodine uptake: T/M ratios were measured in slices incubated with 1 mM MMI and pulse-labeled with ^{125}I. Addition of 10^{-4} M I-OH-A caused a 69% inhibition, while no change was observed in slices treated with 10^{-4} M IL-δ. Dose-effect studies showed that I-OH-A was effective up to 10^{-6} M.

Time-course studies showed that the inhibitory effect of I-OH-A was evident when slices were preincubated for only 15 min with this compound. Arachidonic acid at 10^{-4} also inhibited iodine uptake.

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†Established investigators from the CONICET.

Iodine organification: The PB ^{125}I was measured under similar conditions as above, except that MMI was omitted. Incubation with 10^{-4} M I-OH-A caused a 64% inhibition, while 10^{-6} M decreased PB by 12%. IL-6 was again less potent, since at 10^{-4} M it impaired this parameter by 45%. Arachidonic acid, 10^{-4} M, had no effect. TSH (20 mU/ml) caused a 35% stimulation, while simultaneous incubation of this hormone with 10^{-4} M and 10^{-5} M I-OH-A decreased this value to 100% and 75%, respectively. Iodothyronines failed to change iodine organification.

In order to further evaluate the possible mechanisms involved, the influence of actinomycin D and puromycin on I-OH-A action was assessed. Incubation with these compounds failed to alter the inhibitory effect of I-OH-A.

Total RNA biosynthesis: Slices were labeled with ^{3}H-uridine and its incorporation into total RNA was measured as already described (8). Incubation with 10^{-4} M I-OH-A caused a 52% inhibition in ^{3}H-uridine uptake by the slices. A similar degree of inhibition was observed when RNA specific activity was measured. Therefore, we have concluded that I-OH-A mainly affects the uptake of this RNA precursor, and arachidonic acid was without effect.

RNA transcription was measured by the incorporation of ^{3}H-UTP into RNA by purified thyroid nuclei. Total activity, alpha amanitin sensitive (RNA polymerase II) and resistant activities (RNA polymerases I + II) were determined (9). Two experimental protocols were utilized: a) incubation of slices $\pm$ I-OH-A, isolation of nuclei and determination of the activities; and b) preincubation of purified nuclei with I-OH-A and measurement of transcription thereafter. No influence of I-OH-A was observed under these conditions.

Cyclic AMP formation: As already described, TSH (10 mU/ml) caused a significant stimulation of cAMP formation by calf slices. Addition of 10^{-5} M I-OH-A blocked this action by 41%.

Uptake of ^{14}C-AIB: Incubation with 10^{-5} M I-OH-A induced a significant decrease in the entry of the label to a greater degree than with KI. Addition of MMI did not influence this inhibition. Arachidonic acid had no significant effect.

The present data demonstrate that an iodinated derivative of arachidonic acid exerts a direct inhibitory effect of different thyroid parameters, thereby mimicking the effect of iodine. Although these findings strongly support our hypothesis that such compounds play a role in the mechanism of action of iodine and thyroid autoregulation, further studies will be required to determine their precise physiological significance.

REFERENCES

1. Pisarev MA and Kleiman de Pisarev DL. J Endocrinol Invest 3: 317, 1980.
2. Pisarev MA. J Endocrinol Invest, in press.
3. Pisarev MA, Burton G, Busse Grawitz P, et al. Ninth International Thyroid Congress, 1985.
4. Chazenbalk GD, Pisarev MA, Juvenal GJ, et al. Acta Endocrinol (Kbh) 108: 72, 1985.
5. Boeynaems JM and Hubbard WC. J Biol Chem 255: 9001, 1980.
6. Chazenbalk GD, Pisarev MA, Krawiec L, et al. Acta Physiol Pharmacol Latinoamer. 34: 367, 1984.

7. Pisarev MA and Aiello LO. Acta Endocrinol (Kbh) 82: 298, 1976.
8. Kleiman de Pisarev DL, Pisarev MA, and Juvenal GJ. Acta Endocrinol (Kbh) 89: 316, 1978.
9. Kleiman de Pisarev DL, Pisarev MA, and Spaulding SW. Endocrinology 104: 634, 1979.

FORMATION OF DIIODOTYROSINE (DIT) BY ETHER-LINK CLEAVAGE OF T_4 IN RAT LIVER HOMOGENATE AS MEASURED BY DIT RADIOIMMUNOASSAY

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Recent studies clearly demonstrate that extrathyroidal formation of diiodotyrosine (DIT) by cleavage of the ether bridge of T_4 is an existing pathway of T_4 metabolism. Tracer techniques demonstrated DIT production by this pathway *in vitro* in phagocytosing human leukocytes (1) and both *in vitro* and *in vivo* in the rat (1,2). Further evidence was provided by very recent *in vivo* studies on the turnover of DIT and T_4 in thyroidectomized T_4-substituted rats (3). The present paper describes studies on this metabolic process in rat liver homogenate using a sensitive radioimmunoassay for direct measurement of T_4-derived DIT. Initial preliminary results have been reported elsewhere (4).

METHODS

Livers of female Wistar rats were homogenized in four volumes of ice-cold 0.12 M Tris buffer, pH 7.4, containing 0.03 M tetraethylethylendiamine. After addition of one volume of buffer, used for washing the homogenizer, the crude homogenate was centrifuged at 1500 g for 30 min. The supernatant, referred to hereafter as the homogenate, was used for subsequent incubations. Protein concentration was determined by the method of Bradford (5) using the Bio-Rad protein assay kit. Homogenates not used immediately after preparation were stored at -20°C.

In a typical experiment, 100-150 µl of homogenate, containing 2-6 mg protein, were incubated in a final volume of 0.5 ml with 1 µM T_4 as the substrate in the absence or presence of 50 mM 3-amino-1,2,4-triazole (AT) and other additives at 37°C for 60 min under oxygen. Reactions were stopped by adding 1 ml of ice-cold ethanol. After centrifugation, 20-50 µl of ethanol extracts were directly assayed for DIT. For DIT measurement, the RIA method described previously was used without prior immunoextraction of DIT from the sample (6). Control experiments were performed by the incubation of T_4 without homogenate and homogenate without T_4, as well as the addition of T_4 to ice-cold homogenate without incubation (time-zero tubes). DIT concentrations measured in test samples were corrected for the amount of DIT in the time-zero tubes.

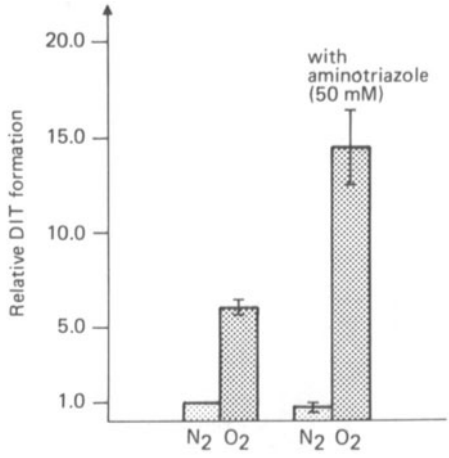

Fig. 1. DIT formation from T_4 in rat liver homogenate under anaerobic (N_2) and aerobic (O_2) conditions.

RESULTS

A significant generation of DIT from its substrate T_4 was observed only under aerobic conditions (Fig. 1). In an atmosphere of nitrogen, DIT production was undetectable or slightly above the detection limit of our assay system, even in the presence of the catalase inhibitor aminotriazole (AT). Fig. 2 presents data about the influence of the incubation time on the production of DIT from T_4. Without AT, DIT increased up to 30 min and then remained unchanged up to 90 min of incubation. In the presence of 50 mM AT, DIT increased progressively and, after 90 min, reached a mean value approximately 5-fold higher than the plateau values observed without AT. As shown in Fig. 3, a rise in AT concentration of up to 30 mM was associated with an increased formation of DIT. DIT production was constant at AT concentrations ranging from 30 to 60 mM and tended to decrease at higher AT levels. Preincubation of homogenate at temperatures above 22°C resulted in a marked deactivation of the DIT-forming system. Preincubation for one hour at 37°C reduced DIT production to about 1/2, at 46°C to 1/10, and at 56°C to almost undetectable values in comparison to samples preincubated at 0°C (Fig. 4). T_4 conversion to DIT was optimal at pH 7 to 8. The recovery of known amounts of DIT (5 and 12.5 nM) added to homogenate before incubation

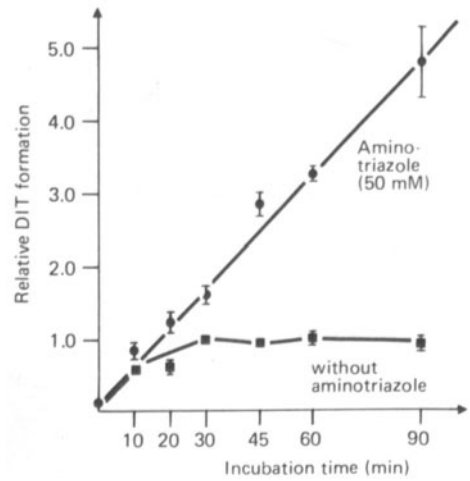

Fig. 2. Effect of incubation time on DIT formation from T_4 in rat liver homogenate. Relative DIT formation = 1.0 at an incubation time of 60 min, without aminotriazole, 37°C, pH 7.4, O_2. Mean $\pm$ SD, n = 4.

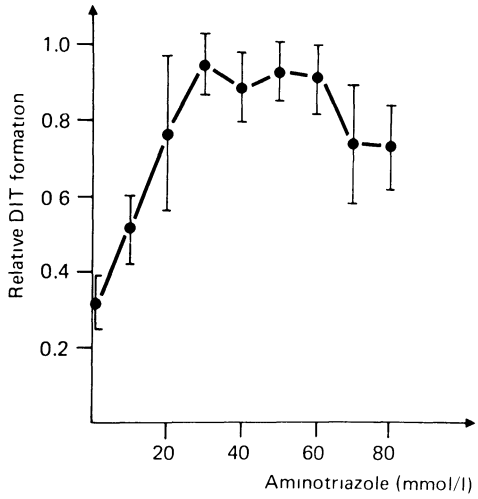

Fig. 3. DIT formation from T_4 in rat liver homogenate at varying concentrations of aminotriazole. Maximum DIT formation of each experiment = 1.0 (60 min, 37°C, O_2, mean ± SD, n = 3).

ranged between 91.2% and 96.0%. There was no statistically significant degradation of DIT (0.1 and 10 nM) added at varying incubation periods from 10 to 120 minutes.

Table 1 presents data on the effects of metal ions and the chelating agent EDTA on AT-stimulated DIT formation. Two mM concentrations of calcium, magnesium, and zinc did not markedly influence DIT production, whereas bivalent manganese ions caused an almost complete inhibition of the reaction. A strong inhibition of the DIT-forming process was also observed after addition of 5 mM EDTA. Studies on the effects of propylthiouracil (PTU) yielded non-uniform results. Initial experiments using relatively low substrate (0.3-0.7 µM T_4) and high protein concentrations (6 mg per incubation vial) showed a dose-dependent inhibition of DIT formation. In later experiments (Table 2), PTU had no clear effect on DIT production either in the absence or in the presence of AT. Addition of the H_2O_2-generating system glucose and glucose oxidase (GOD) surprisingly led to a reduction of basal, as well as AT-stimulated DIT formation. As shown in Table 2, this inhibitory effect was completely abolished by combination of glucose and GOD with PTU in the presence, but not in the absence, of AT stimulation.

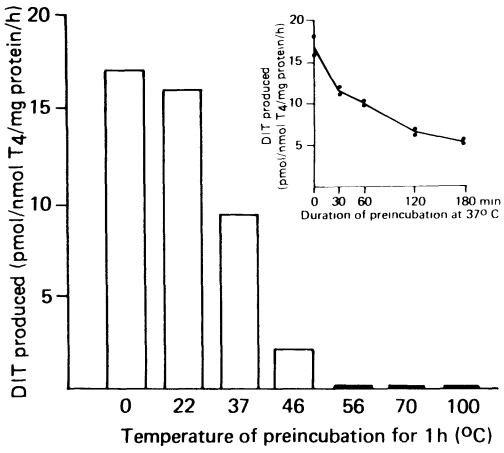

Fig. 4. Effects of temperature and preincubation time on the viability of the DIT-forming activity. Homogenates were preincubated without T_4 for 1 h at varying temperatures. Thereafter, incubation was carried out in the presence of the substrate T_4 for 1 h at 37°C. Inset: preincubation of homogenates for varying periods at 37°C followed by incubation with T_4 substrate as above.

Table 1. Effects of Metal Ions and EDTA on the Aminotriazole-stimulated DIT Formation from T_4

Additives[a]	DIT production (pmol/nmol T_4/mg protein/h) without EDTA	with EDTA
Control	18.3	1.7
Ca^{2+}	22.4	2.2
Mg^{2+}	18.6	3.9
Mn^{2+}	0.7	1.4
Zn^{2+}	22.4	8.7

a)Concentration of additives: Aminotriazole 50 mM, EDTA 5 mM, and metal ions 2 mM.

Studies using iodothyronines other than T_4 as substrates for DIT formation by ether-link clevage in the rat liver yielded the following results (values as pmol DIT/nmol substrate/mg protein/h): T_4 10.2; T_3 6.3; 3,5-T_2 3.0; 3,3'-T_2, and rT_3 undetectable.

DISCUSSION

Our results confirm recent findings by Balsam et al. (2) that the rat liver degrades T_4 to DIT *in vitro*. Balsam and co-workers (2), as well as Burger et al. who utilized human leukocytes as the *in vitro* system (1), used tyrosyl ring-labeled ^{125}I-T_4 as the precursor and chromatographic techniques

Table 2. Effects of Propylthiouracil (PTU), Glucose/glucose Oxidase (GOD), and Aminotriazole (AT) on the DIT Production from T_4 in Rat Liver Homogenate

Additives	Experiment	DIT production (pmol/nmol T_4/mg protein/h) without AT	with AT[a]
Control	I	6.9	46.2
	II	8.6	38.2
PTU[b]	I	5.4	46.2
	II	9.8	82.0
Glucose/GOD[c]	I	2.5	5.5
	II	2.4	6.0
Glucose/GOD and PTU	I	1.7	52.2
	II	3.3	63.2

a)Aminotriazole 50 mM, b)PTU 1 mM, c)Glucose (2 g/l)/GOD (1500 U/l).

for separation of reaction products and detection of ^{125}I-labeled DIT. However, the present study has employed a sensitive radioimmunoassay for direct quantification of DIT generated from its unlabeled substrate T_4 by the rat liver. In agreement with observations of the above mentioned authors, in vitro DIT formation from T_4 was found to be an oxygen-dependent and temperature-sensitive process. Heat inactivation of the DIT-forming system indicated its enzymic nature. Inhibition of the H_2O_2-decomposing liver enzyme catalase by aminotriazole consistently led to a profound enhancement of DIT production. DIT generating activity seems to require polyvalent metal ions, since it was markedly inhibited by the chelating agent EDTA.

PTU is known to inhibit most peroxidase-catalyzed reactions, although its activity is not limited to this effect (7). The substance has been shown to inhibit ether-link cleavage of T_4 in phagocytosing leukocytes (1). In the present study, PTU in combination with AT abolished the suppressive effect of the H_2O_2-generating system glucose/GOD on DIT formation. This preliminary finding is difficult to interpret and, although obtained in two independent experiments, requires further investigation for confirmation and elucidation of the underlying mechanism. Apart from the unclear effect of PTU, our results support the suggestions of Burger et al. (1) and Balsam et al. (2) that DIT formation by clevage of the diphenyl ether bridge of the T_4 molecule is a peroxidase-mediated enzymatic process.

REFERENCES

1. Burger A, Engler D, Buergi U, et al. J Clin Invest 71: 935, 1983.
2. Balsam A, Sexton F, Borges M, et al. J Clin Invest 72: 1234, 1983.
3. Meinhold H and Buchholz R. In Abstracts of the 7th International Congress of Endocrinology, Excerpta Medica, Amsterdam, Oxford and Princeton, 1984, p 1085.
4. Meinhold H and Schwander J. Acta Endocrinol 108 Suppl 267: 76, 1985.
5. Bradford MM. Anal Biochem 72: 248, 1976.
6. Meinhold H, Beckert A, and Wenzel KW. J Clin Endocrinol Metab 53: 1171, 1981.
7. Klebanoff SJ and Green WL. J Clin Invest 52: 60, 1973.

IS IODIDE REQUIRED FOR PEROXIDASE-CATALYZED COUPLING IN THYROGLOBULIN?

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There is controversy regarding the possible role of iodide in the coupling reaction. We first reported (1,2) that chemically iodinated thyroglobulin (Tgb), containing almost no iodothyronines and dialyzed to remove the remaining excess of iodide, would give rise to a significant amount of thyroid hormones when incubated in the presence of a peroxidase system (thyroid or lactoperoxidase and glucose, glucose oxidase as H_2O_2 generating system) in the absence of added iodide, presumably by coupling of the preformed iodotyrosines. It was subsequently reported (3) that chemically iodinated Tgb releases iodide when incubated with peroxidase, suggesting that iodide is needed for coupling to occur and that maximal coupling rates are obtained with an iodide concentration as high as 100 μM. More recently (4), it has been reported that free iodide is an inhibitor of iodothyronine synthesis *in vitro* and that, in the presence of limiting H_2O_2, it actively regulates the efficiency of coupling.

Thus, it was of interest to study the subject further. Since, *in vitro*, Tgb iodination and coupling take place almost simultaneously, one must first completely separate both reactions. We have been able to do so by either chemical iodination, which yields a very little amount of iodothyronines, or by enzymatic iodination with low iodide concentrations (5). By so doing, two phases can be distinguished: 1) iodination proceeds rapidly until completion and coupling also takes place, and 2) coupling alone proceeds once iodination has been completed, followed by peroxidase-catalyzed coupling with or without prior removal of the remaining iodide by either gel filtration or dialysis, and comparison of the efficiency of T_4 formation.

The results obtained show that coupling takes place with similar efficiency in samples where the iodide has or has not been removed. However, when iodide is removed prior to incubation with the peroxidase system, the enzyme regenerates minute amounts of iodide during the incubation, most likely by deiodination of Tgb (3). This very low iodide concentration may be required for coupling, though the exact mechanism is still not clear.

MATERIALS AND METHODS

Experimental design. Fig. 1 shows the approach followed to answer the question whether or not iodide is required for coupling. Iodine poor

EXPERIMENTAL DESIGN

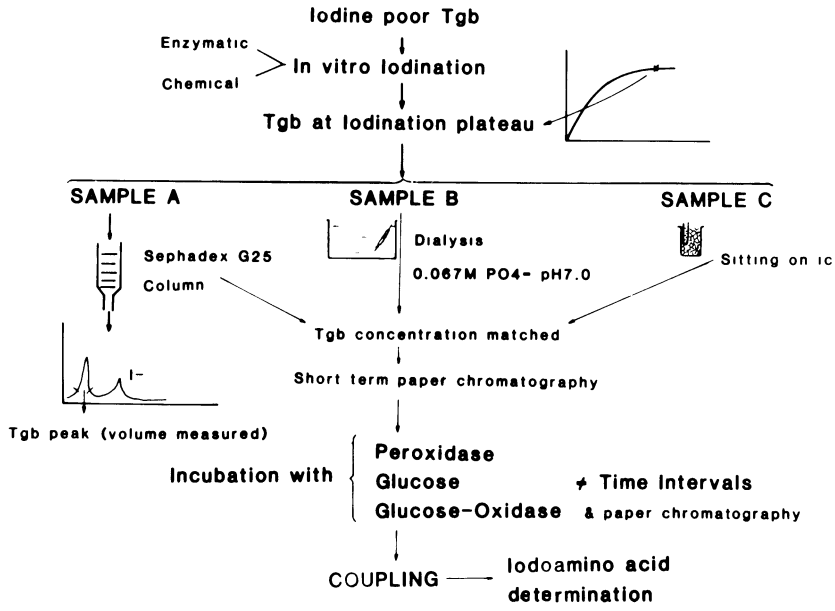

Fig. 1. Outline of procedure for testing whether or not iodide is required for the coupling reaction. See text for more details.

(0.04% I) human Tgb (1 nmole/ml), purified from a multinodular goiter kindly provided by Dr. Geraldo Medeiros-Neto (Sao Paulo, Brazil), was iodinated in vitro at a level of 25 atoms I per mole of Tgb, either enzymatically with iodide labeled with radioiodine (Amersham, SA 1 μCi/ atom), lactoperoxidase (Sigma, 1 μg/ml), glucose (Merck, 1 mg/ml), and glucose oxidase (Boehringer, grade I, 1.5 μg/ml) in phosphate buffer, 0.067 M, pH 7.0, at 37°C for increasing periods of time, or chemically with a triiodide solution (0.08 M 12 in 0.25 M I^-, labeled with radioiodine to a SA of approximately 25 μCi/ μatom) in the same buffer at room temperature for increasing periods of time. Once the iodination was completed, the labeled Tgb was divided into three samples: A) which was passed through a Sephadex G-25 column to separate any remaining iodide from the Tgb, B) which was dialyzed extensively against 0.067 M phosphate, pH 7.0, to dialyze out the remaining iodide once the iodination stopped, and C) which was left on ice until the Tgb peaks from A and B were processed (the Tgb peaks pooled and the volume measured). Once the Tgb concentrations had been matched and aliquots taken for short-term paper chromatography to determine the iodide concentration still remaining in each sample, they were incubated at 37°C with lactoperoxidase (1 μg/ml), glucose (1 mg/ml), and glucose oxidase (1.5 μg/ml) in the absence or presence of 10^{-7} M I^- (final concentration) for increasing periods of time at which both short-term and long-term (preceded by pronase digestion) paper chromatography were carried out to calculate the number of atoms of iodine and the number of residues of each iodoamino acid bound per mole of protein as previously described (1,6).

RESULTS AND COMMENTS

Fig. 2 shows the rate of in vitro enzymatic iodination and coupling of Tgb (left panel) and the increase in T_4 formation after incubation, with

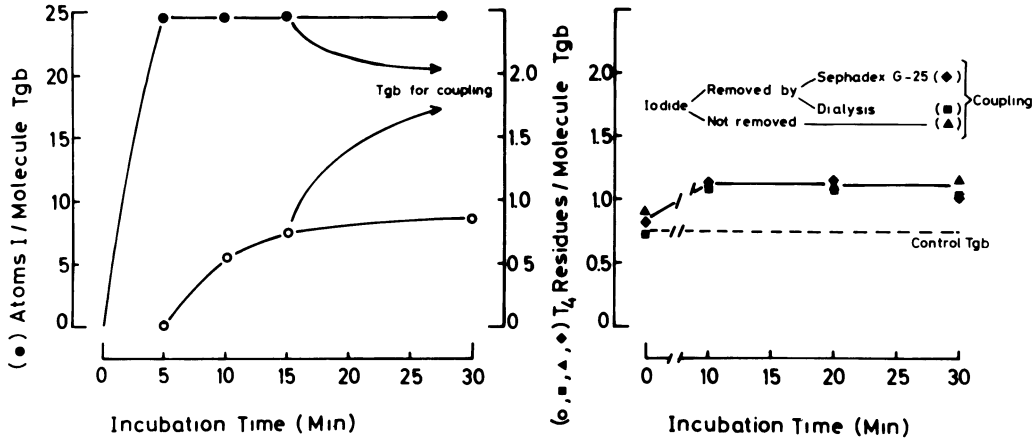

Fig. 2. Atoms of iodine (•) and residues of T_4 (o) per molecule of Tgb formed by in vitro enzymatic iodination of iodine poor Tgb (left panel) and increase of T_4 in the prelabeled Tgb containing 24.4 atoms I and 0.83 residues T_4 per molecule at onset (right panel), by incubation with the peroxidase system with and without prior removal of free iodide. See text for more details.

and without prior iodide removal, with the peroxidase system in the absence of any added iodide. As seen in the left panel, iodination proceeded very rapidly and was completed in five minutes, while coupling was still increasing. At 15 minutes, Tgb was separated and iodide was removed by gel filtration or dialysis in some samples but not in other samples. The right panel shows that coupling increased from the control Tgb by further incubation with the peroxidase system. No differences were seen between the Tgb in which iodide had been removed and that in which the iodide had not been removed.

Fig. 3 shows the T_4 residues formed after incubation with peroxidase at different time intervals in three different samples, the free iodide present immediately after iodination (point out of the scale), and at different periods after the onset of incubation with the peroxidase system. It is of interest to note that the free iodide concentration dropped almost to zero following gel filtration or dialysis, but remained constant in the sample left on ice. However, the number of atoms of iodine found as iodide increased during incubation with the peroxidase system in the two samples where iodide had been removed, supposedly by deiodination of Tgb by the enzyme (4), while it remained constant in the one where iodide had not been removed. Though not shown, addition of 10^{-7} M iodide to the samples where iodide had been removed prior to the coupling experiment did not result in any further increase in T_4 formation.

Since in the in vitro enzymatic iodination iodide and peroxidase are in close contact in the incubation mixture, iodide could play a role in the coupling reaction (3) by interacting with the peroxidase system and forming compound II, which presumably catalyzes only the coupling reaction (7) but, even after removal of any free iodide, this compound could already be present and catalyze the coupling between preformed iodotyrosines when incubated with peroxidase. If this is true, the approach using enzymatic iodination would not be adequate. In order to avoid this complication, a similar experiment was performed using chemically iodinated Tgb. As shown in Fig. 4, coupling also increased by increasing the incubation time of all samples

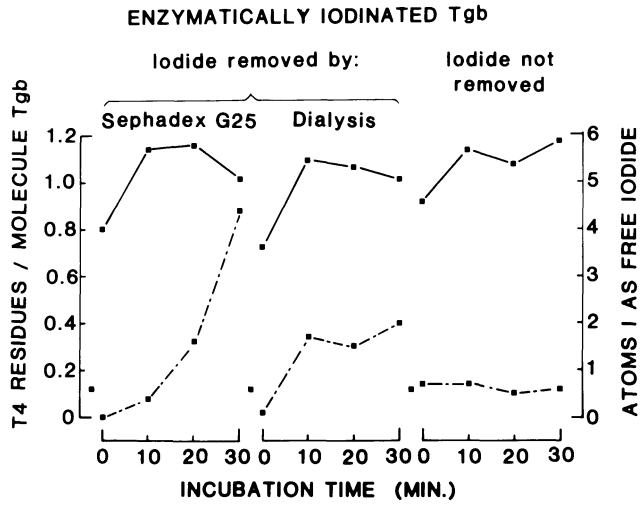

Fig. 3. T_4 formation (■—■) and iodide generation (■---■) during incubation with the peroxidase system of Tgb in Fig. 2. The point out of scale corresponds to the free iodide present in Tgb after iodination and prior to iodide removal. See text for more details.

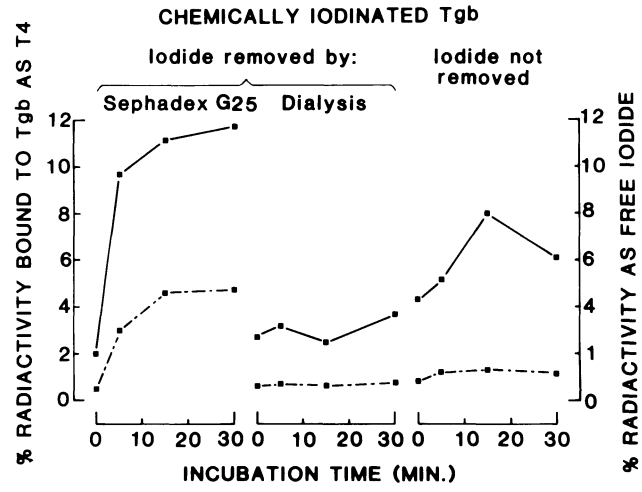

Fig. 4. T_4 formation (■—■) and iodide generation (■---■) in chemically prelabeled Tgb, containing 24.7 atoms I and 0.12 residues of T_4 per molecule at onset, by incubation with the peroxidase system with and without prior iodide removal. See text for more details.

while, during incubation with the peroxidase system, the iodide concentration increased only in the Tgb·sample passed through Sephadex G-25. It is clear, however, that the iodide concentration in all Tgb samples remained very low (10^{-7} to 5 x 10^{-7} M) which, again, is compatible with the concept that iodide plays a role in the coupling reaction.

In summary: 1) in vitro iodinated Tgb contains minute amounts of iodide in the reaction mixture. 2) Iodide removal from both chemically and enzymatically iodinated Tgb, prior to incubation with a peroxidase system in the absence of added iodide, does not lead to a decrease in the efficiency of coupling. 3) Addition of small iodide concentrations 10^{-7} M) to iodinated Tgb, after removal of iodide and prior to incubation with a peroxidase system, does not change the coupling efficiency. 4) Iodinated Tgb from which iodide has been removed, regenerates minute amounts of iodide during incubation with a peroxidase system. From these results, we conclude that iodide, in very small concentrations (about 5 x 10^{-7} M), may be required for the coupling reaction though the exact mechanism is not yet clear.

REFERENCES

1. Lamas L, Dorris ML, and Taurog A. Endocrinology 90: 1417, 1972.
2. Taurog A, Dorris ML, and Lamas L. Endocrinology 94: 1286, 1974.
3. Deme D, Pommier J, and Nunez J. Eur J Biochem 70: 435, 1976.
4. Wildberger E, Von Gruenigen C, Kohler J, et al. Eur J Biochem 130: 485, 1983.
5. Lamas L, Santisteban P, and Turmo C. Annales d'endocrinologie 45: 125A, 1984.
6. Lamas L. Eur J Biochem 96: 93, 1979.
7. Nunez J. In F Wold and K Moldave (eds), Methods of Enzymology vol. 107, Academic Press, Inc., 1984, p 476.

INHIBITION OF THYROID PEROXIDASE (TPO) AND LACTOPEROXIDASE (LPO) BY GOITRIN AND RICININE

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SUMMARY

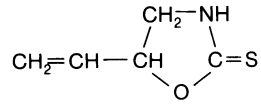
The plant toxins, goitrin and ricinine, inhibit TPO and LPO. Goitrin (0.3 mM) completely inhibits the peroxidase-catalyzed oxidation of iodide and is nine times more potent than ricinine. Both toxins also inhibit the peroxidase-catalyzed oxidation of guaiacol, although less efficiently than iodide oxidation. Goitrin is oxidized by H_2O_2 in the presence, but not in the absence, of peroxidase, whereas ricinine is not oxidized. Iodide stimulates (twofold) the rate of goitrin oxidation. Spectra obtained with mixtures of LPO, H_2O_2, and goitrin indicate that LPO_{oxid} oxidizes goitrin and is converted back to native LPO. Thus, both LPO_{oxid} and $LPO\text{-}I_{oxid}$ oxidize goitrin but $LPO\text{-}I_{oxid}$ is more effective. Because of their well known toxicity, neither of these toxins derived from Brassicae plants (goitrin) and castor beans (ricinine) is considered a serious dietary problem.

INTRODUCTION

Goitrin, L-5-vinyl-2-thiooxazolidone (Fig. 1), is an antithyroid compound found in Brassicae plants (turnips, cabbage, etc.) that inhibits the organification of iodine in the thyroid gland (1-3). The purpose of this study was to determine the effect of goitrin on thyroid peroxidase (TPO) activity, a heme-enzyme that oxidizes iodide to "active iodine" and couples iodotyrosines to form thyroxine and 3,5,3'-triiodothyronine (4,5). In addition, we studied the effect of ricinine (1,2-dihydro-3-cyano-4-methoxy-1-methyl-2-pyridone), a toxin derived from castor beans (Fig. 2), on TPO activity. Because of its ready availability and very similar catalytic properties to TPO, highly purified lactoperoxidase (LPO) was also utilized to obtain kinetic and spectral data in the presence of the plant toxins.

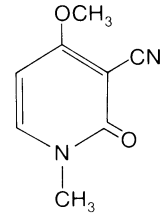
MATERIALS AND METHODS

Goitrin (RS-goitrin) was synthesized by a modification of the method of Ettlinger (6) and ricinine was purchased from K and K Laboratories, Plainview, NY. Glucose oxidase and LPO ($A_{410}:A_{280}$ = 0.59) were obtained from Sigma Chemical Co., St. Louis, MO, and TPO was purified from beef thyroid glands with an $A_{410}:A_{280}$ = 0.55 as previously described (7). All other reagents and chemicals were the highest purity available from commercial



Goitrin
(L-5-Vinyl-2-Thiooxazolidone)

Fig. 1. Chemical structure of goitrin.



Ricinine
(1,2-Dihydro-3-Cyano-4-methoxy-1-methyl-2-pyridone)

Fig. 2. Chemical structure of ricinine.

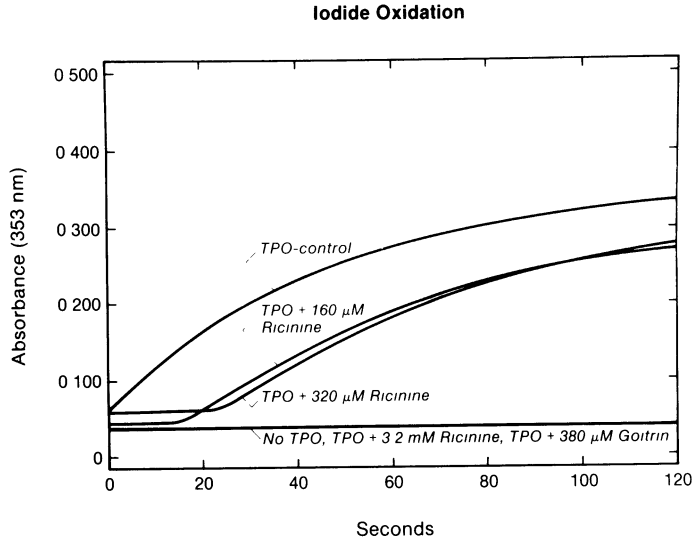


Fig. 3. Inhibition of TPO-catalyzed guaiacol oxidation by goitrin and ricinine. Conditions: The reactions were performed at ambient temperature (23°C) in a final volume of 3 ml that contained 0.1 M sodium phosphate buffer, pH 7.4, 40 μmoles potassium iodide, 550 ng bovine TPO, and 0.4 μmole H_2O_2. The reaction mixtures were continuously stirred with a magnetic stirring bar and the reactions were initiated by adding 10 μl of 0.04 M H_2O_2.

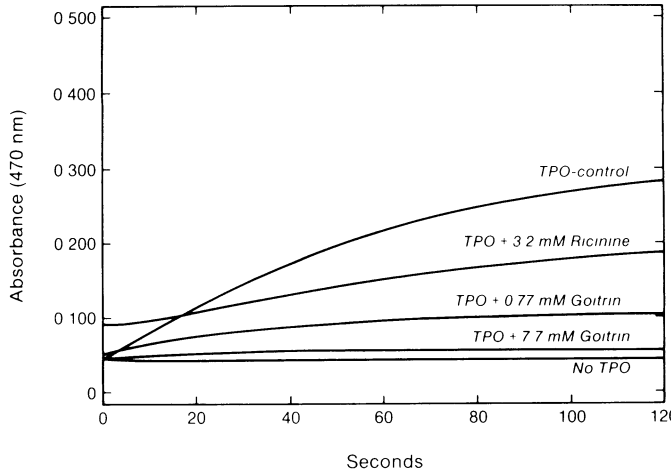


Fig. 4. Inhibition of TPO-catalyzed guaiacol oxidation by goitrin and ricinine. Reaction conditions were the same as in Fig. 3 except that 100 μmoles guaiacol and 0.8 μmole H_2O_2 were present.

sources. Spectra and kinetic analyses were obtained with a Hewlett-Packard 8450 UV/VIS spectrophotometer equipped with a cuvette stirring bar and constant temperature cell holder. Iodide oxidation was measured at 353 nm (8) and guaiacol oxidation at 470 nm (7).

RESULTS

Inhibition of the TPO-catalyzed oxidation of iodide by goitrin and ricinine is shown in Fig. 3. Goitrin at a concentration of 380 μM completely inhibited iodide oxidation and was about nine times more effective than ricinine (3.2 mM). With lesser concentrations of ricinine (160 and 320 μM), iodide oxidation proceeded at the same rate as the TPO-control (no inhibitor present) after a lag phase of 15-20 seconds. This lag phase

Table 1. Inhibition of TPO by Goitrin and Ricinine

Inhibitor	Iodide oxidation (Percent inhibition)	Guaiacol oxidation (Percent inhibition)
Goitrin	100*	58*
Ricinine	37+	59++

Inhibitor concentrations: *380 μM, **770 μM
+320 μM, ++3,200 μM

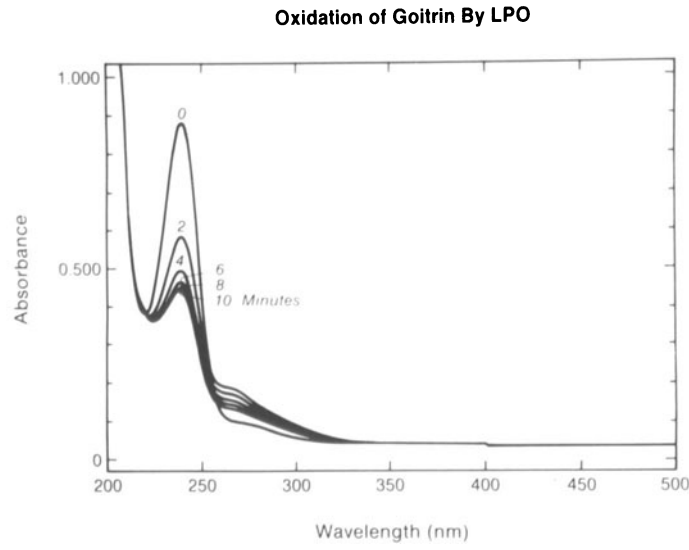


Fig. 5. Oxidation of goitrin by lactoperoxidase and H_2O_2. The reaction mixture contained 0.1 M sodium phosphate buffer, pH 7.4, 50 µM goitrin, 20 µg LPO, 5.6 mM D-glucose, and 10 µg glucose oxidase in a total volume of 3.0 ml. The reaction was performed at ambient temperature (23°C) and was initiated by adding glucose oxidase while continuously stirring with a magnetic stirring bar. Spectra were obtained every two minutes as indicated.

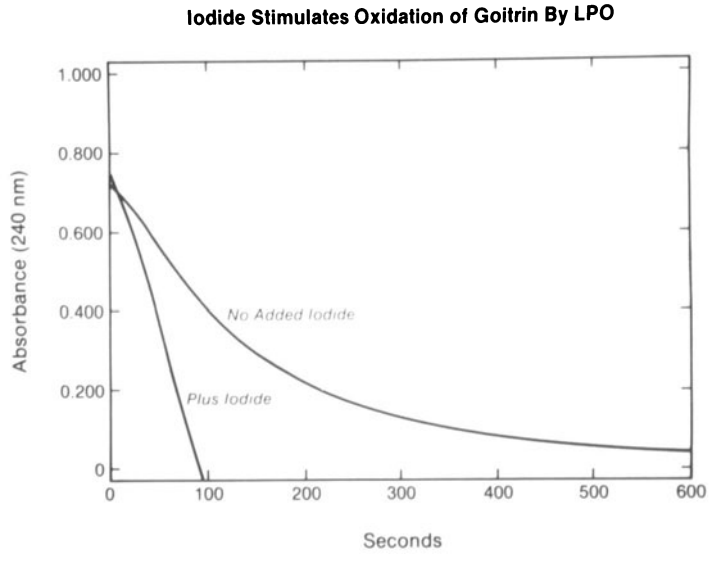


Fig. 6. Stimulation of the rate of LPO catalyzed oxidation of goitrin by iodide. Reaction conditions were the same as in Fig. 5 except that 10 µM potassium iodide was added where indicated.

was probably due to the reaction of "active iodine" with ricinine, similar to the reaction of other goitrogens with iodine (9).

When guaiacol was used as the substrate, both plant toxins inhibited its oxidation by TPO (Fig. 4), though less effectively than with iodide. TPO activity towards guaiacol was completely inhibited by 7.7 mM goitrin, while 50% inhibition was observed with 770 μM goitrin. Ricinine was less effective than goitrin in inhibiting guaiacol oxidation by TPO, similar to the results with iodide oxidation. A summary of the relative inhibitory effects of goitrin and ricinine on the TPO-catalyzed oxidation of iodide and guaiacol is presented in Table 1.

In other experiments not shown, we found that the plant toxins inhibited LPO in essentially the same manner as TPO. Therefore, because lactoperoxidase was available in a high degree of purity and in relatively large quantities, the remainder of our studies were performed with this enzyme.

LPO oxidized goitrin with H_2O_2 that was generated by glucose-glucose oxidase (Fig. 5). Goitrin possesses an absorption spectrum with a peak of 240 nm that disappeared after 10 min reaction with H_2O_2 and LPO. The addition of iodide stimulated the rate of oxidation of goitrin about twofold (Fig. 6). Other data demonstrated that H_2O_2 (glucose-glucose oxidase) in the absence of LPO did not oxidize goitrin, and that ricinine was not oxidized by H_2O_2 and LPO.

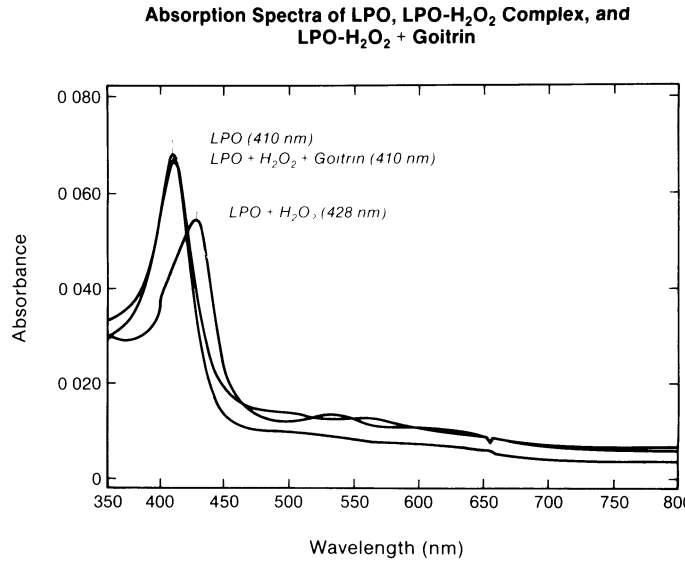


Fig. 7. Effects of H_2O_2 and goitrin on the absorption spectrum of LPO. The spectrum of native LPO (410 nm peak) was obtained with a solution containing 0.1 M sodium phosphate buffer, pH 7.4, and 150 μg LPO in a total volume of 1.5 ml. After the addition of H_2O_2 (final concentration of 29 μM), the spectrum shifted within 2 minutes with a peak at 428 nm. After 2 minutes, goitrin was added (final concentration of 66 μM) to the LPO-H_2O_2 mixture, and the spectrum was essentially converted back to the spectrum of native LPO.

After mixing LPO with H_2O_2, the Soret spectrum of the native enzyme with a peak at 410 nm was shifted with a peak at 428 nm (Fig. 7), typical of LPO_{oxid} (10,11). When goitrin was added to LPO_{oxid}, the Soret spectrum reverted back to that of native LPO, indicating that goitrin had been oxidized and LPO was regenerated.

DISCUSSION

The mechanism of inhibition of TPO and LPO by several common antithyroid drugs (1-methyl-2-mercaptoimidazole (MMI), 6-n-propylthiouracil (PTU), and 2-thiouracil) has been extensively studied and clarified during the past few years (10-17). The results have demonstrated that TPO-I_{oxid} oxidized these antithyroid drugs, and that inhibition of iodination by the drugs in the thyroid gland was due to competition between the thioureylene drugs and tyrosyl residues in thyroglobulin (11,13-15). Moreover, MMI and PTU were firmly bound (presumably covalently) to TPO when incubated with the enzyme and H_2O_2, and iodide prevented the inhibition of TPO by the antithyroid agents (11,13).

Our findings indicated that LPO_{oxid} oxidized goitrin, and that native LPO was formed (Fig. 7) in the absence of added iodide, although iodide stimulated the oxidation of goitrin (Fig. 6). Hence, it is concluded that goitrin was oxidized by both LPO_{oxid} and LPO-I_{oxid}, and that oxidized goitrin was not bound to the heme moiety on the enzyme. The inhibition of peroxidase activity in vitro by the plant toxins is in good agreement with the in vivo goitrogenic effectiveness of goitrin (2,3) and ricinine (18).

REFERENCES

1. Chesney AM, Clawson TA, and Webster B. Johns Hopkins Hosp Bull 43: 201, 1928.
2. Astwood EB, Greer MA, and Ettlinger MG. J Biol Chem 181: 121, 1949.
3. Astwood EB. Ann Intern Med 30: 1087, 1944.
4. Alexander NM. J Biol Chem 234: 1530, 1959.
5. Taurog A. In SC Werner and SH Ingbar (eds), The Thyroid, Harper and Row, New York, 1978, p 31.
6. Ettlinger MG. J Am Chem Soc 72: 4792, 1950.
7. Alexander NM. Endocrinology 100: 1610, 1977.
8. Alexander NM. Anal Biochem 4: 341, 1962.
9. Langer P and Michajlovskij N. Endocrinologia Experimentalis 6: 97, 1972.
10. Ohtaki S, Nakagawa H, Nakamura M, et al. J Biol Chem 257: 761, 1982.
11. Engler H, Taurog A, and Nakashima T. Biochem Pharmacol 31: 3801, 1982.
12. Davidson B, Soodak M, Strout HV, et al. Endocrinology 104: 919, 1979.
13. Neary JT, Soodak M, and Maloof F. In F Wold and K Moldave (eds), Methods in Enzymology, Academic Press, New York, 1984, p 445.
14. Engler H, Taurog A, Luthy C, et al. Endocrinology 112: 86, 1983.
15. Taurog A. Endocrinology 98: 1031, 1976.
16. Shiroozu A, Taurog A, Engler H, et al. Endocrinology 113: 362, 1983.
17. Edelhoch H, Irace G, Johnson ML, et al. J Biol Chem 254: 11822, 1979.
18. Pahuja DN, Gavnekar SV, Shah DH, et al. Biochem Pharmacol 28: 641, 1979.

SELECTIVE INHIBITION BY MONOAMINE OXIDASE (MAO) INHIBITORS OF THE IODOTYROSINE FORMATION INDUCED BY MAO SUBSTRATES IN BOVINE THYROID TISSUE

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Monoamine oxidase (EC 1.4.3.4) (MAO) may probably be involved in the hydrogen peroxide generation required for the iodination process in the thyroid tissue (1,2). Biogenic amines have been demonstrated to be able to generate hydrogen peroxide and to increase iodine organification in thyroid tissue, and these effects were decreased by MAO inhibitors (3,4). On the other hand, it was observed that rat thyroid MAO activity was increased by TSH administration and reduced after hypophysectomy (2).

In several tissues, MAO occurs in two functional forms, A and B, which exhibit different specificities for their substrates and inhibitors (5-8). The A form is selectively inhibited by clorgyline, and preferentially deaminates 5-hydroxytryptamine (5-HT), whereas the B form deaminates β-phenylethylamine (PEA) and is sensitive to deprenyl inhibition. Tyramine is a common substrate for both forms of the enzyme.

In this study, the forms of MAO involved in the iodotyrosine formation induced by those monoamines that are MAO substrates were investigated.

MATERIALS AND METHODS

A particulate fraction (30,000 xg) of bovine thyroid tissue was used. Particulate fraction preparation and iodotyrosine formation assay were carried out as previously described (4). The peroxidase activity was evaluated by the tyrosine-iodinase method (9).

Clorgyline (N-methyl-N-propargyl-3 (2,4-dichlorophenoxy) propylamine hydrochloride, M & B 9302) was kindly donated by May and Baker, Ltd., Dagenham, Essex, England.

l- Deprenyl (phenyl-isopropyl-methyl-propinylamine) was a generous gift from Prof. J. Knoll, Department of Pharmacology, Semmelweis University of Medicine, Budapest, Hungary.

RESULTS AND DISCUSSION

The effect of graded concentrations of two inhibitors of MAO, clorgyline (selective of A form) and diprenyl (selective of B form), on the

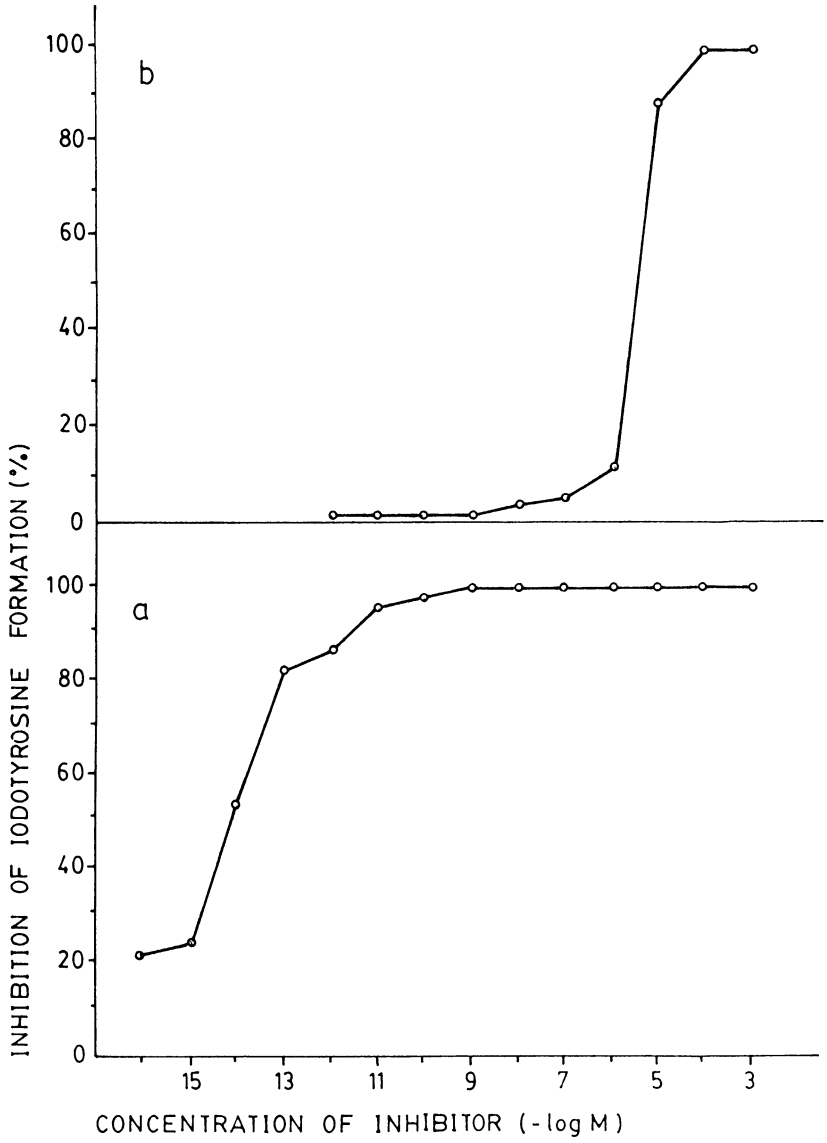

Fig. 1. Effect of clorgyline (a) and deprenyl (b) on the iodotyrosine formation induced by 5-hydroxytryptamine (5-HT) in bovine thyroid tissue. The assay system contained 300-400 µg protein (30,000xg fraction), 10^{-3} M tyrosine, 10^{-5} M KI, 0.1 µCi ^{131}I, and 0.1 M phosphate buffer, pH 7.2, in a volume of 0.5 ml. Particulate fraction was preincubated with the MAO inhibitors for 15 min at 25°C. At time zero, the substrate (10^{-4} M) was added. Incubation was done for 20 min at 37°C. The results are expressed as percent of the control activity.

iodotyrosine formation induced by the MAO substrates 5-HT (preferred of A form), PEA (preferred of B form), and tyramine (both borms) was investigated.

The iodotyrosine formation induced by 5-HT was markedly reduced at low clorgyline concentrations indicating that 5-HT was deaminated by the A form

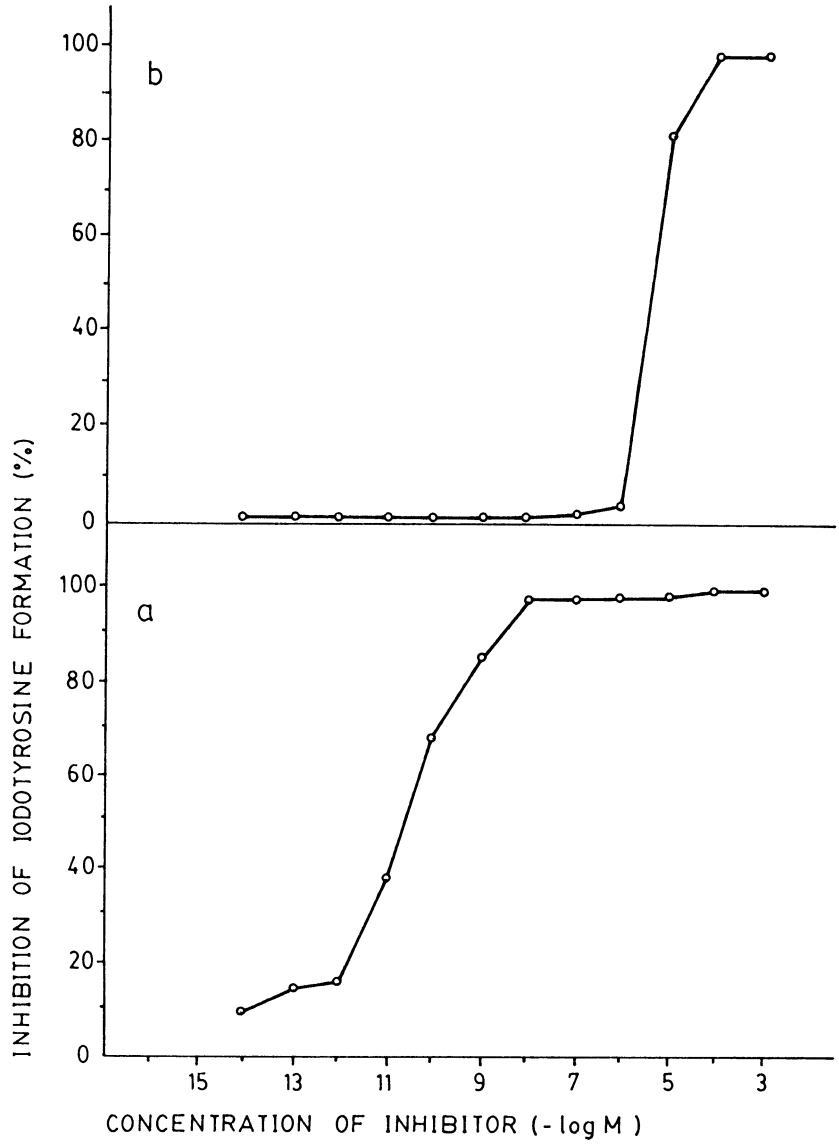

Fig. 2. Effect of clorgyline (a) and deprenyl (b) on the iodotyrosine formation induced by tyramine. The assay and expression of the results were as in Fig. 1.

of MAO (Fig. 1a). Fig. 1b shows that iodotyrosine formation was weakly sensitive to deprenyl except at high inhibitor concentrations, which is consistent with a limited amount of the B form of MAO.

When tyramine was the substrate, a strong decrease of iodotyrosine formation was also obtained at low clorgyline concentrations. More than 90% inhibition was already produced by 10^{-8} M clorgyline, a concentration where MAO A is selectively inhibited without any significant effect on the B form (10) (Fig. 2a). This indicates that tyramine was metabolized mainly by the A form of MAO. Deprenyl scarcely reduced the iodotyrosine formation (Fig. 2b).

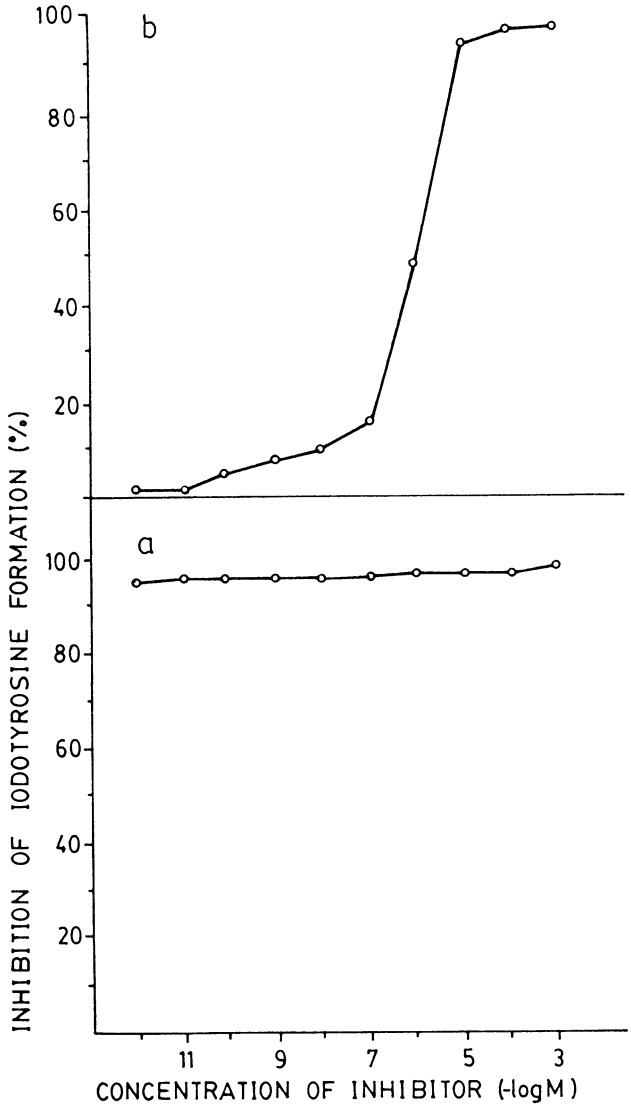

Fig. 3. Effect of clorgyline (a) and deprenyl (b) on the iodotyrosine formation induced by tyramine. The assay and expression of the results were as in Fig. 1.

Fig. 3a shows that iodotyrosine formation was highly sensitive to clorgyline when PEA was the substrate, whereas it was only weakly inhibited by deprenyl (Fig. 3b).

An additional effect of the MAO inhibitors on the endogenous peroxidase activity was discarded since the peroxidase activity was not modified by clorgyline or deprenyl (data not shown).

The characteristics of the inhibition of iodotyrosine formation by clorgyline and deprenyl indicated that MAO A is the main form of the enzyme involved in iodotyrosine formation in bovine thyroid tissue.

Tyramine, a common substrate for both forms of MAO, was used to establish the relative proportion of the forms of MAO in several tissues (8). The inhibition by clorgyline of iodotyrosine formation induced by tyramine, indicated that more than 90% of MAO involved in the iodotyrosine formation was the A form.

PEA, usually a substrate for the B form of MAO, was oxidized by MAO A in this tissue; this particular behaviour has been observed in some other tissues (11).

Only selective inhibitors of MAO A, but not those of MAO B, could be able to modify the MAO activity and probably the bovine thyroid function.

REFERENCES

1. Fischer AG, Schulz AR, and Oliner L. Life Sci 5: 995, 1966.
2. Knopp J and Torda T. Horm Metabol Res 15: 191, 1983.
3. Huang CL and Schulz AR. Life Sci 11: 975, 1972.
4. Masini-Repiso AM and Coleoni AH. Acta Endocrinol 97: 207, 1981.
5. Johnston JP. Biochem Pharmacol 17: 1285, 1968.
6. Knoll J and Magyar K. Adv Biochem Psychopharmacol 5: 393, 1972.
7. Murphy DL. Biochem Pharmacol 27: 1889, 1978.
8. Fowler CJ and Ross SB. Med Res Rev 4: 323, 1984.
9. DeGroot LJ and Davis AM. Endocrinology 70: 492, 1962.
10. Fowler CJ and Tipton KF. J Neurochem 38: 733, 1982.
11. Suzuki O, Katsumata Y, and Oya M. J Neurochem 36: 1298, 1981.

THYROID FUNCTION IN FASTING RATS

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Starvation in rats is used as an experimental model for the "low T_3 syndrome". Plasma concentrations of total and free T_4 and T_3 decrease with starvation, but T_4 more than T_3 (1,2). Plasma TSH also drops (1-3), indicating decreased thyroid secretion in starved rats.

It has been reported that thyroidal radioiodine (I*) uptake at one and four hours after I* administration in rats starved for five days is decreased, but increased at 24 hours after I*.

The aim of the present study was to further investigate thyroid function in fasted rats by measuring: thyroidal I* uptake, the distribution of I* among soluble iodoproteins and their iodoamino acid distribution and secretory activity under basal conditions after the administration of exogenous TSH or after feeding carbohydrates (CHO) which increase endogenous TSH (4).

The data obtained clearly show that food deprivation for 2-8 days in rats results in significant alterations of thyroid function.

MATERIALS AND METHODS

Male Wistar rats from our colony, weighing 300-330 g at onset, were fed a standard pelleted rat Chow (C) ad libitum, or deprived of food but with free access to tap water (F). Two (F2), three (F3), four (F4), six (F6), seven (F7), and eight (F8) days later, C and F rats received 50 µCi I* ip and were killed at five or 24 hours later. The effect of TSH (1 IU/rat, given 20 hours and immediately prior to I*) and CHO (8 g pelleted pure corn flour/rat, 20 hours prior to I*) administration was also studied in the F8 group. The rats were killed by decapitation (9-10 a.m.) and blood was collected into heparinized tubes. Plasma was separated by centrifugation and stored at -20°C. The anterior pituitaries were weighed and homogenized in 1 ml distilled water and frozen for TSH determination. Thyroids were dissected, weighed, and homogenized individually in 1 ml Tris-HCl as previously described (5). Aliquots from the homogenates were separated for ^{127}I determination and I* uptake. The remaining was centrifuged at 100,000 xg at 4°C for 60 min. A thyroglobulin (Tgb), 19 S internal marker was obtained from normal rat thyroids after ^{131}I injection and was added to aliquots of the thyroid supernates prior to application on top of 10-40% sucrose gradients,

Table 1. Effects of Starvation in Rats

Days of starvation	Group	BW g	Plasma glucose mg/dl	Thyroid wt mg/100 g	^{127}I µg/gland	Plasma PBI µg/dl	Plasma TSH µg/dl	Pituitary TSH µg/gland
0	C,5h[a]	310 ± 20	142 ± 15	15.0 ± 2.0	4.4 ± .5	3.1 ± .2	.55 ± .04	945 ± 71
0	C,24h[b]	306 ± 18	139 ± 13	14.1 ± 1.8	4.2 ± .4	2.9 ± .1	.51 ± .05	910 ± 80
2	F2,5h[a]	285 ± 17*	85 ± 5*	12.0 ± 0.7*	3.6 ± .5*	1.8 ± .1*	.30 ± .03*	796 ± 70*
3	F3,24h[b]	278 ± 19*	78 ± 7*	11.9 ± 0.7*	3.4 ± .5*	1.5 ± .1*	.26 ± .04*	616 ± 54*
3	F3,5h[a]	276 ± 15*	76 ± 9*	10.1 ± 1.0*	3.1 ± .3*	1.4 ± .1*	.25 ± .02*	610 ± 65*
4	F4,24h[b]	252 ± 13*	74 ± 8*	10.5 ± 0.8*	2.7 ± .1*	1.2 ± .2*	.20 ± .02*	509 ± 37*
6	F6,5h[a]	246 ± 11*	73 ± 4*	8.1 ± 0.7*	2.3 ± .3*	1.1 ± .1*	.15 ± .02*	450 ± 48*
7	F7,24h[b]	230 ± 10*	70 ± 6*	9.3 ± 1.0*	2.1 ± .2*	.9 ± .2*	.14 ± .02*	430 ± 20*

Data are the mean ± SD for five or six rats per group. Significance of differences is shown for comparison between data for starved groups for 2 (F2), 3 (F3), 4 (F4), 6 (F6), or 7 (F7) days vs. control group (C). *$p < 0.05$ vs. C groups. [a] and [b]: Rats sacrificed five and 24 hours after ^{125}I, respectively.

Table 2. Thyroidal ^{125}I Uptake and Plasma $PB^{125}I$ in Starved Rats

Days of starvation	0	0	2	3	3	4	6	7
Group	C,5h[a]	C,24h[b]	F2,5h[a]	F3,24h[b]	F3,5h[a]	F4,24h[b]	F6,5h[a]	F7,24h[b]
Thyroidal ^{125}I uptake (% d/gland)	5.3 ± .4	8.0 ± .5	4.8 ± .3*	9.1 ± .5*	2.4 ± .2*	5.5 ± .3*	2.0 ± .2*	2.5 ± .3*
$PB^{125}I$ (% d/ml)	.21 ± .01	.19 ± .01	.02 ± .01*	.02 ± .01*	.02 ± .01*	.02 ± .01*	.01 ± .00*	.01 ± .00*

Data are the mean ± SD for five or six rats per group. Significance of differences is shown for comparison between data for starved groups, sacrificed five or 24 hours after ^{125}I injection, and the corresponding values in C rats. *$p < 0.05$ vs. C group. [a] and [b]: Rats sacrificed five or 24 hours after ^{125}I, respectively.

which were centrifuged in a Beckman SW50.1 rotor at 23,000 r.p.m. for 16 hours, collected manually, and the sedimentation coefficient of the various peaks calculated as previously described (6,7). A portion of the soluble iodoproteins was used for I*-iodoamino acid distribution after digestion with pronase (5). Pituitary and plasma TSH contents were determined by the specific RIA developed for rat by the NIADDK of the NIH. Thyroidal iodine content and plasma PBI were determined as described by Benotti and Benotti (8). Plasma glucose concentration was determined by the glucose oxidase method (9). The results were processed by analysis of variance and Tukey's multiple range test.

RESULTS AND COMMENTS

Table 1 shows that body and thyroid weight, thyroidal iodine content, PBI, glucose, and TSH plasma concentrations and pituitary TSH content decreased rapidly as a function of time of starvation, being significant differences with control values by the second day of food deprivation. In agreement with previous results (1-3), thyroid hormone and TSH plasma concentrations decreased progressively with increasing time of food deprivation in the F group, suggesting that secondary or, more likely, tertiary hypothyroidism was induced in the starved animals (10). As shown in Table 2, thyroidal I* uptake and plasma PBI* concentrations five hours after I* were clearly reduced by the second day of food deprivation, and continued to decrease gradually thereafter. The 24 hour uptake after I* also decreased with respect to the control values but there was a transient rise of the I* uptake by the second day of food deprivation.

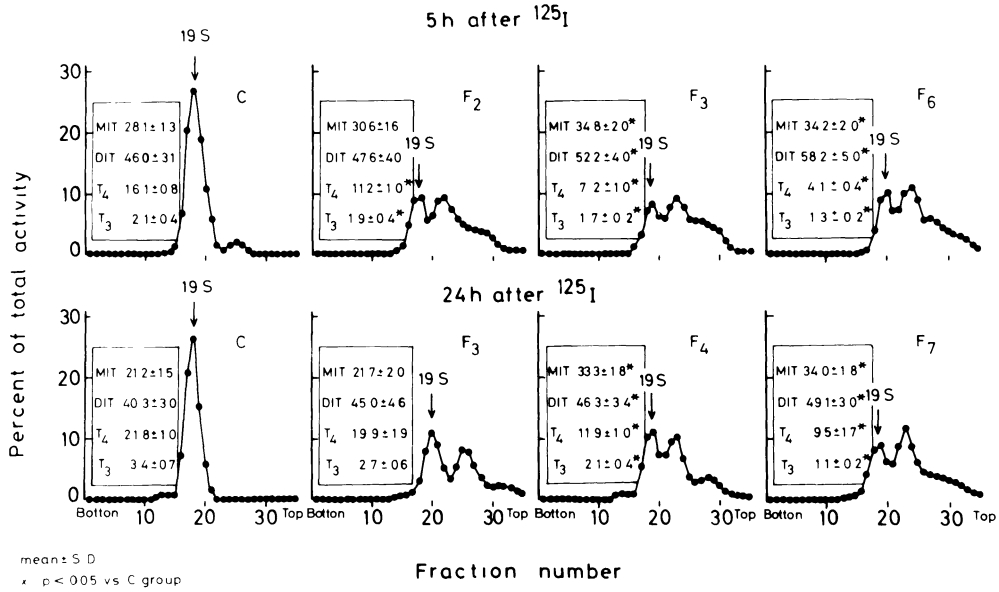

Fig. 1. Sucrose density gradients and percent iodoamino acid distribution of labeled thyroidal soluble iodoproteins, 5 and 24 h. after I* administration in fed (C) and starved rats for 2 (F2), 3 (F3), four (F4), six (F6), and seven (F7) days. The arrow indicates the location of the 19 S Tgb internal marker. The iodoamino acid distributions are given as the mean percentage of total I* in the chromatograms found as MIT, DIT, T_4, and T_3 + the SD. * indicates a value for $p < 0.05$ vs corresponding value in the C group.

Fig. 1 shows sucrose density gradients from soluble thyroidal iodoproteins and their corresponding iodoamino acid distributions both at five and 24 hours after I* in C and F rats at various times of starvation. As may be seen, both the iodoprotein profile and the iodoamino acid distribution pattern of control animals correspond to those expected in rats on an adequate iodine intake (6,7). Thus, the main peak corresponds to Tgb and is a sharp one, coinciding with that from the 19 S Tgb internal marker used, and the proportion of iodoproteins sedimenting more slowly was relatively small. On the contrary, in starved rats the Tgb peak was broader and the proportion of iodoproteins sedimenting more slowly (12-14 S) was greatly increased, suggesting a more unstable Tgb. These changes were evident by the second day of food deprivation and seem to be independent of the time of starvation or the interval between I* administration and sacrifice. It can also be seen that the proportion of iodotyrosines, both at five and 24 hours after I*, are clearly increased by the third day of food deprivation and continued to increase gradually thereafter. The proportion of iodothyronines, however, were decreased at five but not at 24 hours after I* by the second day of starvation and continued decreasing gradually until the end of the experiment, so that in F8 rats, the soluble iodoproteins contained only a very small proportion of T_4 and no detectable T_3 (Fig. 2). These results agree with those reported by Harris et al. (3) who observed a significant change in the percentual distribution of labeled iodoamino acids in rats fasted five days. The extremely high sensitivity of Tgb from starved rats to dissociation, suggests a somewhat different structure (less number of disulfide bonds per molecule?) which might impair the coupling reaction between iodotyrosines to form iodothyronines.

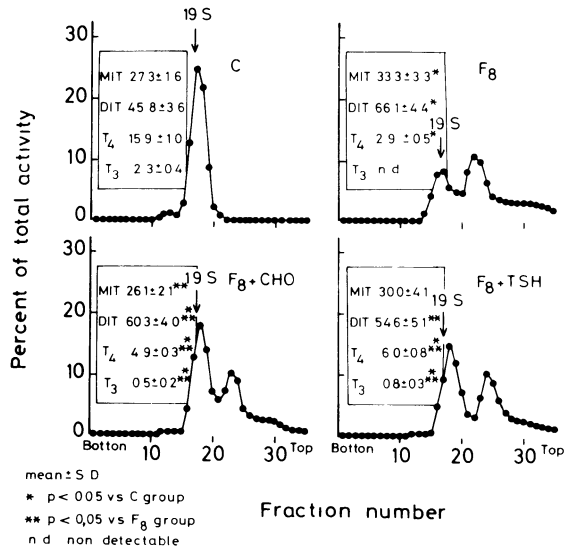

Fig. 2. Sucrose density gradients and percent iodoamino acid distribution of labeled thyroidal soluble iodoproteins, 5 h. after I* administration in fed (C), 8 days starved (F8), F8 plus TSH (1 IU, ip. 20 h. prior to I*), and F8 plus CHO rats (8 g/rat, 20 h. prior to I*). ** indicates $p < 0.05$ vs corresponding values in the F8 group.

Table 3. Effects of Carbohydrate or TSH in Starved Rats for Eight Days

Group	Thyroid wt mg/100 g	^{127}I μg/gland	Plasma PBI μg/ml	Thyroidal ^{125}I uptake (% d/gland)	Plasma $PB^{125}I$ (% d/ml)	Plasma TSH (μg/ml)	Pituitary TSH μg/gland
C	16.0 ± 1.6	4.6 ± .5	2.8 ± .3	4.8 ± .5	.230 ± .040	.52 ± .06	890 ± 110
F8	9.2 ± 2.0*	1.9 ± .3*	.9 ± .1*	1.0 ± .2*	.010 ± .001*	.12 ± .04*	309 ± 60*
F8 + CHO	12.5 ± 2.0*,**	2.4 ± .4*,**	1.4 ± .2*,**	3.0 ± .6*,**	.160 ± .030*,**	.22 ± .04*,**	260 ± 40*
F8 + TSH	11.1 ± 1.8*	2.1 ± .3*	1.3 ± .1*,**	1.9 ± .3*,**	.180 ± .015*,**	.18 ± .02*,**	240 ± 50*

Data are the mean ± SD for five or six rats per group. TSH (1 IU, ip, 20 hours and immediately prior to ^{125}I) and CHO (8 g/rat, 20 hours prior to ^{125}I) was administered to starved rats for eight days (F8). The rats were sacrificed five hours after ^{125}I. *$p < 0.05$ vs. C group; **$p < 0.05$ vs. F8 group.

Table 3 and Fig. 2 show the effect of CHO feeding and of TSH administration to F8 rats. It may be seen that the changes in the various parameters of thyroid function were practically superimposable with those seen in the previous experiment (Tables 1 and 2; Fig. 1). Both CHO feeding and TSH administration reversed, though did not completely restore, these parameters, as well as the iodoamino acid distribution pattern of thyroidal soluble iodoproteins. Surprisingly, Tgb from CHO and TSH-treated starved rats was more stable than that from the untreated starved rats (Fig. 2).

In summary, the observed alterations of the thyroid function in starved rats might be secondary to the low plasma TSH found in these animals. However, the diminished stability of the Tgb molecule found cannot be explained by the TSH changes. It is possible that some still unknown metabolic or endocrine factors, consequent to starvation, affect these parameters independently of TSH.

REFERENCES

1. Kaplan MN and Utiger RD. J Clin Invest 61: 459, 1978.
2. Kaplan MN. Endocrinology 104: 58, 1979.
3. Harris ARC, Fang SL, Azizi F, et al. Metabolism 27: 1074, 1978.
4. Hugues JO, Burges AG, Groouselle D, et al. Endocrinology 112: 715, 1983.
5. Inoue K and Taurog A. Endocrinology 81: 319, 1967.
6. Lamas L and Santisteban P. Biochim Biophys Acta 621: 265, 1980.
7. Inoue K and Taurog A. Endocrinology 83: 816, 1968.
8. Benotti J and Benotti N. Clin Chem 9: 408, 1963.
9. Hugget ASG and Nixon DA. Lancet 2: 368, 1957.
10. Campbell GA, Kurcz M, Marshall S, et al. Endocrinology 100: 580, 1977.

INSULIN DEFICIENCY IMPAIRS THYROID PEROXIDASE ACTIVITY: A STUDY IN EXPERIMENTAL DIABETES MELLITUS*

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Various abnormalities of thyroid function have been described in diabetes mellitus. Clinical, as well as subclinical, hypothyroidism have been shown to occur with increased frequency in insulin-dependent diabetes (1). Alloxan or streptozotocin (STZ)-induced diabetes is associated with a decrease in basal and TSH-stimulated thyroid function in animals (2,3).

Plasma TSH has been reported to be either normal or decreased in diabetic (DM) mice, although plasma T_4 and T_3 concentrations were markedly reduced (4-7). It has been assumed that thyroid hypofunction in diabetic rats is the result of impaired TSH secretion, since TSH response to decreased circulating thyroid hormone is lower than in control animals (7). A decrease of thyroidal response to TSH, shown by lower incorporation of iodine into hormone precursor and thyroid secretory activity, may contribute also to the decrease in serum T_4 in diabetic mice (2).

The present study was undertaken in order to evaluate the possibility of a direct impairment of thyroid hormone biosynthesis in insulin (I) deficiency.

MATERIALS AND METHODS

Eight series of young male (100 g BW) Dutch-Miranda rats were studied. In each series, 12 to 24 animals were randomly separated into three groups: 1) DM: streptozotocin-induced diabetes mellitus (60 mg STZ/Kg, IP); DM+I: STZ-induced diabetes treated with NPH insulin (2 U/day, SC) begun two days after STZ; and 3) C: controls. DM+I animals were treated with insulin for 15 days, while DM and C animals received identical volumes of 0.15 M NaCl. On the 16th day, 6 μCi $Na^{131}I$ were injected IP, and thyroid tissue and blood were obtained 24 hours later. Serum TSH, T_3, and T_4 were measured by radioimmunoassay. After ^{131}I thyroid uptake determination, thyroids were homogenized (2 glands per sample) in 1.0 ml 0.05 M Tris-HCl buffer + 1 mM KI, pH 7.2, and centrifuged at 27,000xg for two hours. Supernatants were separated for thyroglobulin hydrolysis (8) with subsequent T_3 and T_4 measurements by radioimmunoassay (9) and determination of the relative percentage of radioiodoamino acids (10). Washed pellets were resuspended in 1.0 ml of 1% digitonin for 48 hours and centrifuged at 27,000xg for 90 minutes. Thyroid

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Table 1. Serum and Thyroid T_4 and T_3 Concentrations, and TPO Activity in Experimental Diabetes Mellitus (Mean ± SD)

	DM	DM+I	C
Serum T_4 (μg/dl)	2.8 ± 1.1*	3.3 ± 0.9	3.7 ± 0.7
Serum T_3 (ng/dl)	58 ± 11*	95 ± 22	79 ± 11
Thyroid T_4 (ng/mg)	141 ± 56	166 ± 63	130 ± 56
Thyroid T_3 (ng/mg)	9.1 ± 4.1	10.1 ± 4.4	8.4 ± 2.3
TPO (U/g ptn)	782 ± 493**	1393 ± 849	1506 ± 289

DM: Streptozotocin-induced diabetes mellitus; DM+I: Insulin-treated DM; C: Control animals.
Significantly different from control mean: *$p<0.05$; **$p<0.01$.

peroxidase (TPO) activity in supernatants was assayed by a modification of the iodide oxidation method (11) described by Alexander (12), Pommier et al. (13), and Nakashima and Taurog (14). Analysis of variance and multiple range tests were used for statistical analyses. Serum TSH values were submitted to logarithmic transformation and thyroid uptake to angular transformation prior to analyses.

RESULTS AND DISCUSSION

Serum T_4 and T_3 were significantly decreased in DM but not in DM+I animals (Table 1). Serum TSH levels were slightly, but not significantly, increased in both groups (Table 2). While the low serum T_3 can be explained by a decrease in extrathyroidal deiodination (4,5), the decrease of serum T_4 cannot be attributed to a decreased serum TSH. Nevertheless, one must allow for the possibility that thyroid responsiveness to TSH might be decreased in DM animals.

Table 2. Serum TSH Concentrations and Thyroid DIT:MIT and T_4:DIT+MIT Ratios in Experimental Diabetes Mellitus (Mean and Range)

	DM	DM+I	C
Serum TSH concentration (ng/ml)	204 (151-628)	205 (104-516)	123 (71-365)
Thyroid DIT:MIT ratio	1.9 (1.5-2.1)	1.8 (1.5-2.1)	2.1 (1.7-2.6)
Thyroid T_4:MIT+DIT ratio	0.25 (0.13-0.52)	0.28 (0.12-0.50)	0.33 (0.13-0.75)

DM: Streptozotocin-induced diabetes mellitus; DM+I: insulin-treated DM; C: control animals.

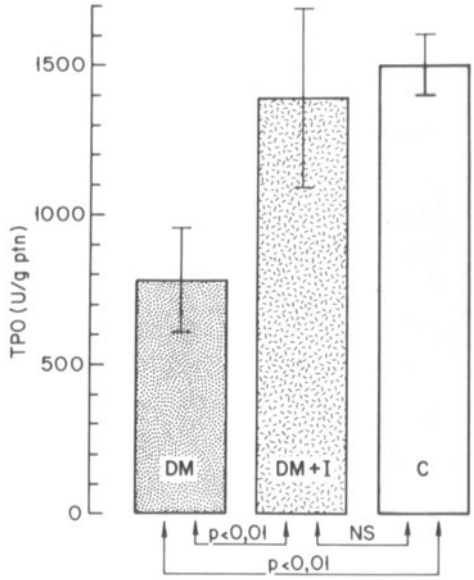

Fig. 1. Thyroid peroxidase activity in streptozotocin-induced diabetes mellitus (DM), insulin-treated DM (DM+I), and control (C) rats. (Mean ± SEM).

The TPO activity was significantly lower in DM rats than in DM+I or control animals (Table 1 and Fig. 1). Although thyroid weight and ^{131}I uptake (values not shown), as well as T_4 and T_3 thyroid levels (Table 1), did not differ among the three groups, the experimental period may have been too short to produce any appreciable change in these parameters. The slight decrease in the DIT:MIT and T_4:DIT+MIT ratios found in DM thyroids (Table 2) could indicate a subtle impairment of iodine organification and iodotyrosine coupling, but these results must be considered in light of the low sensitivity of the radiochromatographic methods.

The decreased TPO activity in STZ-induced DM was not due either to a direct effect of STZ, since in DM+I animals TPO activity was similar to that found in control animals; or to a decrease in factors essential for H_2O_2 generation, since these cofactors were provided by the *in vitro* assay system.

Our finding of a highly significant decrease of TPO activity in experimental diabetes mellitus, which cannot be related to a decrease in serum TSH, indicates that thyroid dysfunction produced by insulin lack may be due, at least in part, to an impairment of thyroid hormone biosynthesis.

ACKNOWLEDGMENT

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REFERENCES

1. Gray RS, Borsey, DQ, Irvine WJ, et al. Clin Endocrinol 19: 445, 1983.
2. Bagchi N, Brown TR, Schivers B, et al. Endocrinology 109: 1428, 1981.
3. Brown JT, Bromage NR, and Matty RJ. J Endocrinol 68: 21, 1976.
4. Chopra IJ, Wiersinga W, and Harrison F. Life Sci 28: 1765, 1981.
5. Las MS and Surks MI. Endocrinology 109: 1259, 1981.
6. Gonzalez C, Montoya E, and Jolin T. Endocrinology 107: 2099, 1980.
7. Pastor RM and Jolin T. Endocrinology 112: 1454, 1983.

8. Inoue K and Taurog A. Endocrinology 81: 319, 1967.
9. Abrams GM and Larsen PR. J Clin Invest 52: 2522, 1973.
10. Rosenthal D, Fridman J, and Lobo LCG. Arq Bras Endocrin Metab 13: 247, 1964.
11. Moura EG. Atividade Peroxidase Tireoideana: Estudo em Glandulas Humanas e no Diabetes Mellitus Experimental. Thesis, Universidade Federal do Rio de Janeiro, 1985.
12. Alexander NM. Anal Biochem 4: 341, 1962.
13. Pommier J, De Praillaune S, and Nunez J. Biochimie 54: 483, 1972.
14. Nakashima T and Taurog A. Clin Chem Acta 83: 129, 1978.

ON THE MECHANISM OF IRREVERSIBLE BRAIN DAMAGE CAUSED BY PERINATAL THYROID HORMONE DEFICIENCY

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ABSTRACT

Thyroid hormone-processing neural systems appear and become organized during the critical phase of thyroxine-dependent brain development (1). Hypothyroidism during that period causes irreversible mental impairment. Because lack of aromatic amino acids permanently damages developing neural systems which require them for neurotransmitter synthesis, we proposed permanent damage to thyroid hormone-processing systems may account for the cerebral manifestations of adult cretinism. To test this hypothesis, we studied the fate of i.v. ^{125}I-labeled T_3 and T_4 in hippocampus and cerebellum, known targets of perinatal thyroid hormone deficiency, in serial film autoradiograms (ARGs), prepared from 50 day old rats made hypothyroid from -5 to +16 days of life by PTU (neoPTU). For comparison, film ARGs were prepared after i.v. 2-deoxyglucose given to neoPTU and control (C) rats. Results: The highly resolved labeling patterns seen in selected layers of hippocampus and cerebellum of C rats given labeled thyroid hormones were blurred and distorted in neoPTU. When viewed at the same level of resolution, differences seen could not be accounted for by changes in glucose metabolism of tissue morphology. Conclusion: The prediction that thyroid hormone deficiency during the critical phase would lead to permanent abnormalities in thyroid hormone processing in adult brain was borne out by the evidence gained in these studies.

Thyroxine (T_4) and triiodothyronine (T_3) entering the brain are taken up into a morphologically identifiable system, the thyronergic system, defined as the aggregate of thyroid hormone-processing neural networks in rat brain. Within this system, both hormones are taken up by a high-affinity transport mechanism (2), differentially concentrated (3), and metabolized (4) in different regions of grey matter, axonally transported to terminal fields (5), and further concentrated and metabolized in synaptosomes and synaptic structures within these regions (6), all under strict homeostatic control (7). Recently-isolated urinary metabolites of the hormone provide new support for the possibility that T_3 is processed within these regions in a manner similar to DOPA (8).

Since the thyroid gland begins to secrete hormones at around minus four days of postnatal life, and thyronergic systems are already established in

the young (30 day old) adult brain, it seems likely that the major ontogenesis of this system occurs sometime within this interval, possibly during late fetal or early postnatal life. These epochs coincide with the so-called critical period of rat brain development. Therefore, it seemed worthwhile to study the features of brain iodocompound processing, sequentially, during this period. To carry out the investigations non-invasively, we treated dams with ^{125}I-iodide daily, from 4 days before to 20 days after birth of the offspring, knowing that thyroid secretions of the progeny would become labeled and would reach the growing brain by way of the circulation, as they are presumed to do in the course of normal development. Because this methodology brings about pervasive though not necessarily equilibrium labeling of all iodocompounds, it is possible to analyze their nature, distribution, and relative rate of accumulation in the brain from birth through weaning. Such measurements showed that the thyronergic system is established and undergoes its major ontogeny during the critical phase of thyroid hormone-dependent rat brain development (1).

Evidence assembled from these studies showed that no significant shift in the availability of T_3 relative to T_4 in brain or brain fractions could account for the uniqueness of the critical period. Labeled iodothyronine levels measured by multisystem chromatography during these experiments revealed that T_3 to T_4 ratios in brain homogenates and fractions did not change significantly between day 5 and day 19, even though serum T_3/T_4 values increased significantly during that time. Throughout, T_3 to T_4 ratios were substantially higher by a factor of 5 in brain than in liver or muscle, synaptosomal T_3 to T_4 ratios were consistently higher than those in brain homogenates by another factor of 20. However, these relationships did not change significantly over the course of the nursing period.

On the other hand, a marked and dramatic change in thyroid hormone-processing systems does occur during the critical phase. Thus, from mere rudiments found on day 1 of life, a well-established thyronergic system resembling the system previously recognized in adult brain, has become organized in all of its essential particulars in the brain of growing rats by the end of the nursing period. Accompanying this ontogenetic change is a progressive increase in specific synaptosomal T_3 binding sites, recently identified and described by Mashio and colleagues (9,10).

Other clinically important critical periods are associated with the growing brain's demand for essential molecules such as required amino acids. When aromatic aminoacidopathies occur during their critical phase, they cause irreversible changes in the differentiating systems which require them in large amounts for growth, differentiation, and particularly for neurotransmitter synthesis. Phenylketonuria is a good example of an aminoacidopathy which oversupplies phenylalanine and undersupplies tyrosine and other neural amino acids, and which, when uncorrected during its critical phase, causes irreversible brain damage (11).

In our laboratory, it is natural to think of hypo- and hyperthyroidism as aminoacidopathies because we view iodothyronines as required amino acids. Given the emergence of a neural apparatus for processing brain iodothyronines during the critical period (1), and considering the lessons learned from other perinatal aminoacidopathies, we have proposed that hypothyroidism during the critical period will not damage the entire brain, nor damage the brain randomly, but will specifically and irreversibly damage those very neural systems which require and process thyroid hormones.

Fortunately, a well-characterized animal model appropriate for testing this hypothesis was available, namely the neoPTU rat of Van Middlesworth (12). This rat model exhibits quantitative defects in auditory responses,

as well as other behaviors as a result of short-duration perinatal hyopthyroidism, induced by means of PTU administered to the dams from -4 to +15 days of life. Like iodine, the drug is transferred to the offspring and taken up by the developing thyroid gland where it acts to inhibit thyroid hormone synthesis.

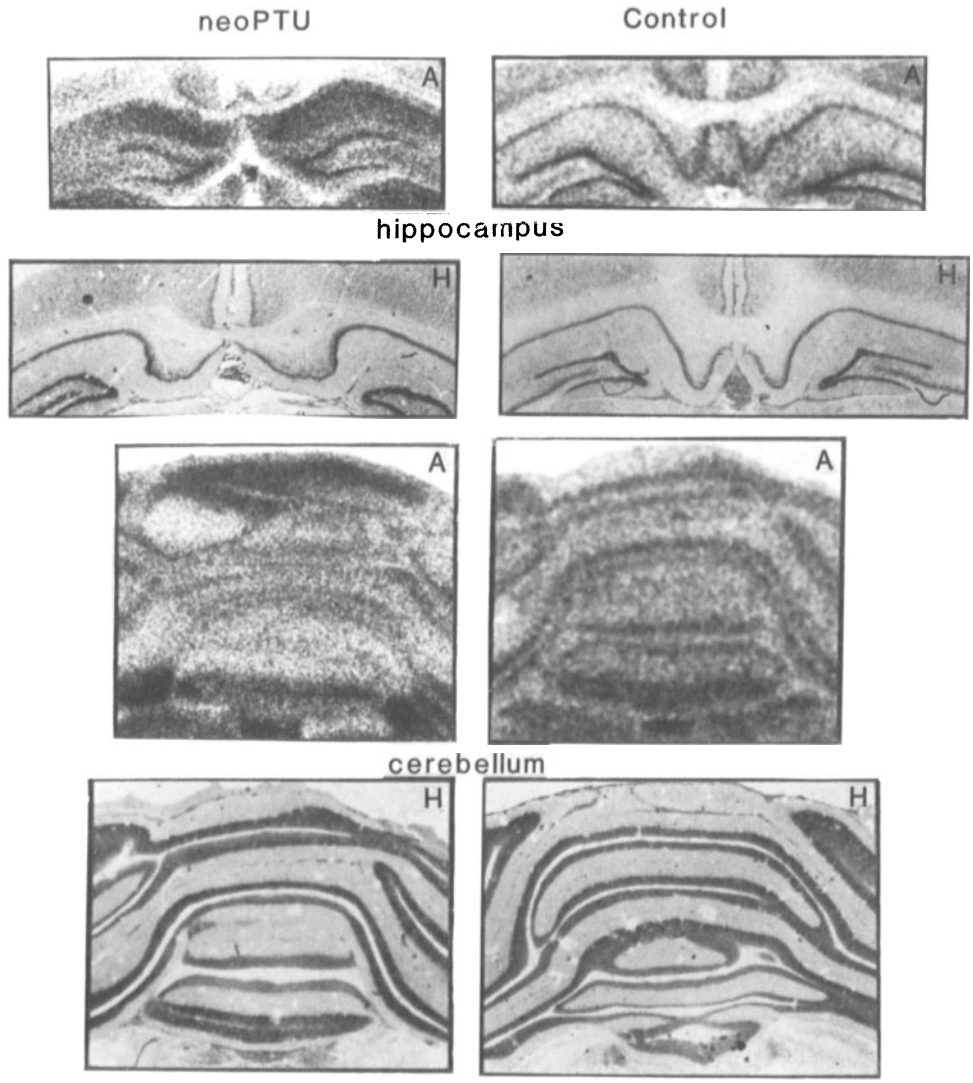

Fig. 1. Effects of perinatal hypothyroidism on thyroid hormone-processing in euthyroid adult rat brain. Pairs of adult neoPTU and Control rats received high SA ^{125}I-labeled thyroid hormones and were decapitated at regular time intervals thereafter. Serial 20 µ sections of brain were exposed to LKB ultrafilm for 10 days. ARGs (A) of hippocampus and cerebellum obtained 10 hours after i.v. ^{125}I-T_3 show blurred and poorly-resolved patterns of hormone localization in neoPTU as compared with Control. Note that histologic (H) sections of the labeled tissues used for autoradiography show no morphologic changes which account for the ARG abnormalities. Similar ARG results were observed in neoPTU after ^{125}I-T_4 (not shown).

Experience with thaw-mount film autoradiography has shown it to be capable of identifying regional defects in thyroid hormone-processing in iopdate-treated rats given radioactive T_4 (13). Therefore, we decided to use this technique to study the thyronergic system in euthyroid adult neoPTU rats as well. Hippocampus and cerebellum were selected from among the possible regions of interest on the basis of the following criteria: (a) they are known targets of perinatal thyroid dysfunction; (b) they have strongly laminated structures so their defects might be evident in film ARGs; (c) in normal adult rats, they have been shown to strongly and saturably concentrate and process thyroid hormones. Further, it was decided that any changes in thyroid hormone-processing detected would only be considered specific if the damaged regions, viewed at the same level of resolution, showed no change in general metabolic activity and no change in underlying tissue morphology. To satisfy all the required conditions, pairs of adult control and neoPTU rats were given either i.v. labeled T_4 or T_3 or intraarterial labeled 2-deoxyglucose; thaw-mount film ARGs were prepared by well-standardized methods; tissues responsible for the autoradiographic reactions were stained with toluidin blue; the ARGs were studied visually and then measured by densitometry.

RESULTS

Significant defects in hormone-processing functions of both cerebellum and hippocampus were detected in the neoPTU as compared with control animals (Fig. 1). At the same time, there was no evidence that patterns of 2-deoxyglucose uptake were altered within these regions as a result of perinatal hypothyroidism. Moreover, no morphological changes were discernible in the stained sections to account for the abnormalities in thyroid hormone processing evident in ARGs prepared after administration of labeled T_3 or T_4.

CONCLUSIONS

The proposal that brain T_3 and T_4 processing would be permanently impaired in adult euthyroid rats damaged by perinatal hypothyroidism is supported by the results obtained in these experiments. Failure to detect abnormalities of underlying histology and 2-deoxyglucose localization in cerebellum and hippocampus, viewed at the same level of resolution, also suggests that the damage inflicted by hypothyroidism during the critical period is selective for the thyronergic nervous system. It is expected that light microscopic and EM studies will reveal more details of the damage in thyronergic neural networks and their terminal fields thus far detected in hippocampus and cerebellum by means of film autoradiography. Conversely, any evidence of damage in other brain regions provided by higher-resolution methodology should give leads to other important projections of the thyronergic nervous system.

REFERENCES

1. Crutchfield FL and Dratman MB. Pediatr Res 17: 8, 1983.
2. Pardridge WM. Endocrinology 105: 605, 1979.
3. Dratman MB, Futaesaku Y, Crutchfield FL, et al. Science 215: 309, 1982.
4. Dratman MB and Crutchfield FL. Am J Physiol 4: 639, 1978.
5. Dratman MB, Crutchfield, FL, Futaesaku Y, et al. Submitted.
6. Dratman MB, Crutchfield FL, Axelrod J, et al. Proc Nat Acad Sci 73: 941, 1976.

7. Dratman MB, Crutchfield FL, Gordon JT, et al. Am J Physiol 245: 185, 1983.
8. Gordon JT, Dratman MB, and Wassel MS. 67th Annual Meeting of The Endocrine Society, 1985, Abstract #847.
9. Mashio Y, Inada M, Tanaka K, et al. Endocrinology 110: 1257, 1982.
10. Mashio Y, Inada M, Tanaka K, et al. Acta Endocrinol 104: 134, 1983.
11. Andersen AE, Rowe V, and Guroff G. Proc Nat Acad Sci 71: 21, 1974.
12. Van Middlesworth L and Norris CH. Endocrinology 106: 1686, 1980.
13. Crutchfield FL, Dratman MB, and McNamara MB. 67th Annual Meeting of The Endocrine Society, 1985, Abstract #1100.

REGULATION OF 19,000 Mr SOLUBLE PROTEIN AND HIGH MOBILITY GROUP PROTEIN (HMG 14) PHOSPHORYLATION BY TRIIODOTHYRONINE (T_3) IN PRIMARY ASTROCYTIC CULTURES*

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While most of the information available concerns the numerous effects of thyroid hormones on the maturation of neurons, it has been noted that glial cells also are affected by thyroid status (1). In order to further investigate the mechanism of action of thyroid hormones on the brain, we developed a system of cultured cells from cerebral hemispheres of postnatal rat, which are predominantly composed of astrocytes (2). It is now well established that protein phosphorylation-dephosphorylation is a major general mechanism by which external stimuli controls the intracellular activity. Therefore, in this study, we have examined the effects of triiodothyronine (T_3) on protein phosphorylation in different subcellular fractions of cultured astrocytes.

MATERIAL AND METHODS

Cerebral hemispheres from 2 day-old rats were dissociated mechanically and cultured for 17 days in Dulbecco's modified Eagle's medium containing horse serum (5%) and newborn calf serum (5%) depleted of thyroid hormones (3). The cultures were incubated with T_3 (10^{-8} M) for 0 to 72 hours. All phosphorylation experiments were performed 17 days after seeding (100 µC of [^{32}P]-phosphate/dish) for the last 4 hours of incubation.

Soluble and nuclear fractions were prepared by centrifugation at 105,000 x g and 800 x g, respectively. High mobility group (HMG) proteins were extracted from the nuclear preparation with 0.75 M perchloric acid. Phosphorylated proteins were usually analyzed by polyacrylamide gel electrophoresis in one dimension (4) and/or two dimensions (5). In addition, HMG extracts and samples of bovine thymus HMG proteins were dissolved in 0.9 M acetic acid and aliquots were then subjected to two-dimensional gel electrophoresis (4,6). The phosphorylated proteins were revealed by autoradiography and [^{32}P]-labeled protein spots were cut of the gels for counting.

*Supported by MRC grant MT-5730.

RESULTS

Action of T_3 on the Soluble 19 kDa Protein

Figure 1 shows a typical pattern of protein phosphorylation of cells grown in the presence of T_3 for 0 to 72 hours. This figure indicates that T_3 induces a marked decrease in the phosphorylation of a protein (Mr = 19,000). As illustrated in the graph, a small decrease (-5.6%) is already detectable after 2 hours and is significantly different (-26.4%, $p<0.05$) after 4 hours in the presence of T_3. The maximal effect is attained after 7 hours (-42%) and remains unchanged up until 72 hours. Subsequent analysis by two-dimensional polyacrylamide gel electrophoresis (Fig. 1A and 1B) indicates that the 19 kDa protein is composed of two spots with isoelectric points of 6.6 and 6.8 (Fig. 1B). It also confirms the effect of T_3 on the phosphorylation of this protein.

Action of T_3 on the Nuclear 17 kDa HMG Protein

The HMG proteins were separated by electrophoresis and revealed by autoradiography (Fig. 2). A marked increase in the phosphorylation of a 17 kDa protein is observed after incubation with T_3. As shown in the graph, the maximal effect is already observable by 7 hours after T_3 addition (+

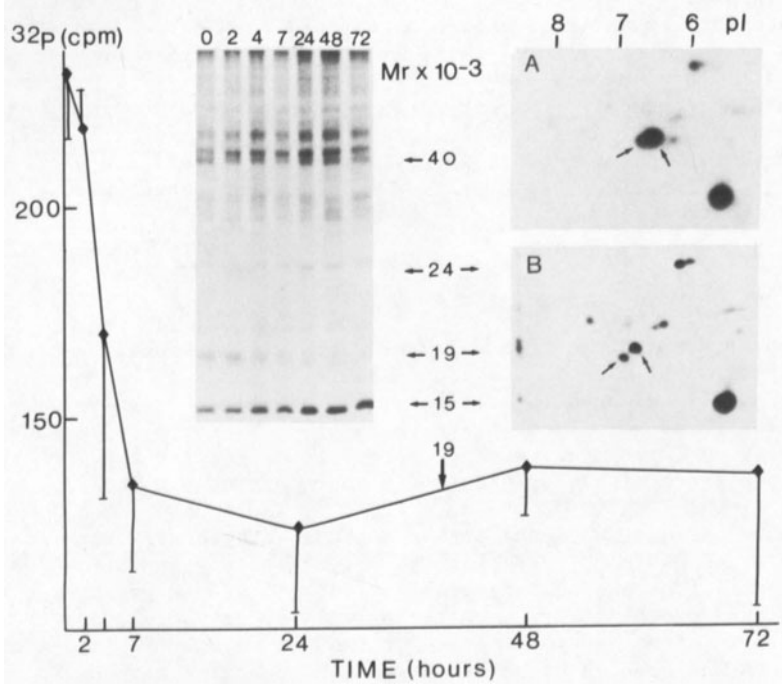

Fig. 1. Time course of 19 kDa protein phosphorylation. Cells were incubated with T_3 for 0 to 72 hours and labeled with ^{32}P (4 hours). The soluble phosphoproteins were separated by SDS-PAGE (autoradiograms: left panel). The graph represents the time course of the 19 kDa protein phosphorylation. Panels A (control) and B (T_3 for 71 hours): two-dimensional gel electrophoresis. The 19 kDa spots are indicated by the arrows.

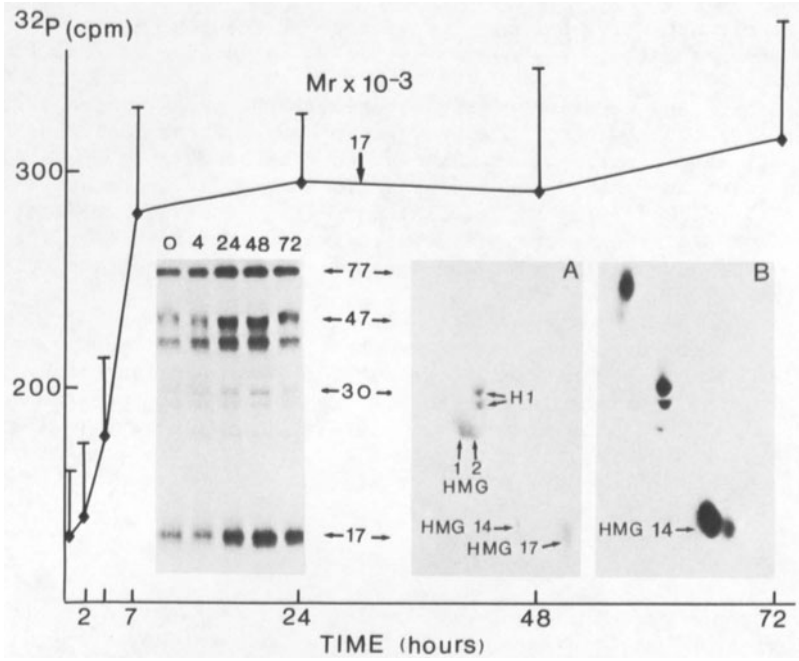

Fig. 2. Time course of 17 kDa HMG protein phosphorylation. Cells were treated as described in Fig. 1. HMG proteins with histone H1 complex were extracted and separated by polyacrylamide gel electrophoresis (autoradiograms: left panel). The graph represents the time course of the 17 kDa protein phosphorylation. In panels A and B the astrocytic HMG phosphoproteins were mixed with bovine thymus HMG proteins and submitted to acid-urea and SDS-PAGE gel electrophoresis. Panel A: Coomassie blue stained gel. Panel B: corresponding autoradiogram.

115%, $p<0.05$) and remains unchanged for up to 72 hours. The phosphorylation of the other fractions, not illustrated, does not change significantly. These phosphorylated proteins were analyzed in acid-urea and SDS by two-dimensional gel electrophoresis and compared with partially purified bovine thymus HMG proteins (Fig. 2A and 2B). The phosphorylated astrocytic 17 kDa protein comigrates with HMG 14.

DISCUSSION

The results presented here show that triiodothyronine produces very selective effects on protein phosphorylation in primary glial cell cultures. In the soluble fraction, a protein with a molecular mass of 19,000 daltons incorporates less [^{32}P]-phosphate after incubation with T_3. In contrast, an increased phosphorylation of a 17 kDa protein in the HMG fraction is observed following T_3 stimulation. This protein behaves as HMG 14 with regard to its electrophoretic activity in two-dimensional gel electrophoresis.

The action of hormones on the phosphorylation of nuclear HMG proteins is now well documented (7). It is thus believed that these proteins can play an important role in gene transcription and that phosphorylation may

modulate their activity (8-10). Therefore, our results may indicate that thyroid hormones influence gene expression in cultured glial cells.

The nature and function of the soluble 19 kDa protein need further characterization. However, this protein has some biochemical analogy with the actin-binding protein of 19 kDa described by Bamburg et al. (11). In accordance with this, the amount of β actin is rapidly increased at the cytoskeleton level following T_3 stimulation. If it is true, the fact that the phosphorylation of this protein is controlled by thyroid hormones could open a new field of investigation on the mechanism involved in actin polymerization at the cellular level.

While Kolodny et al. (12) reported that high affinity nuclear T_3 receptors are absent in glial cells, recent work from our laboratory (13) demonstrates that nuclear T_3 receptor is present in astrocytes cultured for 14 days. Our results clearly demonstrate that T_3 produces direct effects on cultured astrocytes and suggest that these cells are a target for thyroid hormones in the developing brain.

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REFERENCES

1. Legrand J. In C DiBenedetta, R Balazs, G Combos, et al. (eds), Multidisciplinary Approach to Brain Development, Elsevier/North-Holland Biomedical Press, Amsterdam, 1980, p 279.
2. Schousboe A. Cell Mol Biol 26: 505, 1980.
3. Samuels HH, Stanley F, and Casanova J. Endocrinology 105: 80, 1979.
4. Laemmli UK. Nature (Lond.) 227: 680, 1970.
5. O'Farrell PH. J Biol Chem 250: 4007, 1975.
6. Panyim S and Chalkley R. Arch Biochem Biophys 130: 337, 1969.
7. Cooper E and Spaulding SW. Mol Cell Endocrinol 39: 1, 1985.
8. Gazit B, Panet A, and Cedar H. Proc Natl Acad Sci (USA) 77: 1787, 1980.
9. Weisbrod S, Groudine M, and Weintraub H. Cell 19: 289, 1980.
10. Levy-Wilson B. Proc Natl Acad Sci (USA) 78: 2189, 1981.
11. Bamburg JR, Harris HE, and Weeds AG. FEBS Lett 121: 178, 1980.
12. Kolodny JM, Leonard JL, and Larsen PR. 60th Meeting of the American Thyroid Association, New York, Abstract T-2, 1984.
13. Luo M, Faure R, and Dussault JH. 9th International Thyroid Congress, Sao Paulo, 1985.

REGULATION BY n-BUTYRATE OF THYROID HORMONE RECEPTOR LEVELS IN CULTURED CELLS OF GLIAL ORIGIN

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Sodium butyrate has been reported to affect the differentiation, proliferation, and biochemical properties of different types of mammalian cells in culture, including neural cells (1).

The acetylation of core histones (mainly H3 and H4) and other nuclear proteins is the best studied effect of butyrate and is secondary to the inhibition of the nuclear deacetylases (2). Other important effects of butyrate in nuclei include modifications in the extent of phosphorylation, methylation, and ADP-ribosylation of chromatin-associated proteins (3,4) or an alteration in DNA methylation (5).

The thyroid hormone receptor is a chromatin-associated protein which appears to mediate the actions of thyroid hormones in mammalian cells. Although little is known regarding wich components or aspects of chromatic structure might modify the nuclear concentrations of receptor, it is expected that post-synthetic modifications of nuclear proteins may alter the thyroid hormone receptor. Using GH1 cells, Samuels et al. have demonstrated that butyrate elicits a reduction in the nuclear receptor levels which is inversely related to the extent of histone acetylation (6). Using a photoaffinity label probe, it has been shown that in GH1 cells the receptor has two different molecular weight forms, an abundant 47,000 Mr and a less abundant 57,000 Mr form (7) and that butyrate decreases receptor levels primarily by shortening the half-life of the 47,000 Mr form (8). In addition, at low concentrations of butyrate (0.5 mM or less), a small increase in receptor number is observed (9), which is due to an increase of the 57,000 Mr form (8).

In this study, we have examined the characterization and regulation by butyrate of the nuclear thyroid hormone receptors in C6 cells, a rat glioma cell line. C6 cells are a very valuable model for normal glia in a number of respects (10), and their responsiveness to several hormones closely resembles that of non-transformed glial cells in culture (11).

MATERIALS AND METHODS

C6 cells were grown in monolayer cultures in RPMI medium containing 10% horse serum-2.5% fetal calf serum. Cells were depleted of thyroid hormone

before each experiment by culturing them for 24 h in medium supplemented with AG 1-X8 resin-charcoal-treated newborn calf serum (10%, v/v) which has been shown to be depleted of thyroid hormone (12). For the determination determination of T_3 binding to intact cel monolayers (13), the medium was replaced with serum-free RPMI medium which was supplemented with 0.8 nM ^{125}I-T_3, which saturates more than 85% of receptor. T_3 binding to isolated nuclei was determined as previously described (13). DNA was determined by the method of Burton (14). After extraction (6,15), the histones were electrophoresed in slab acid-urea polyacrylamide gels (16).

RESULTS

The characteristics of the thyroid hormone nuclear receptor in C6 cells were very similar to those described in other cell types: the Kd 0.1-0.3 nM both in intact cells and isolated nuclei, the sedimentation coefficient was approximately 4S, and in serum-free medium the affinity for T_3 was higher than for T_4 or Tetrac and lower that for Triac (data not illustrated).

After 24 hours, 2 mM butyrate caused an almost twofold increase of both nuclear and extranuclear hormone (Table 1). This increase was more accentuated at 48 hours (almost threefold), but longer incubation times did not elicit a further elevation.

The increase in nuclear binding may merely be a consequence of the higher cellular hormone content. Figure 1 illustrates the influence of a 48 hr incubation with 2 mM butyrate on the Scatchard plot of T_3 nuclear binding assessed in isolated nuclei and, therefore, without any interference of extranuclear T_3 levels. The estimated Kd values are similar (0.27 and 0.19 nM) in untreated and in butyrate-treated cells, indicating that the affinity is not altered and that there is an almost threefold increase in the maximal binding capacity.

These data indicate that butyrate affects the entry (or permanence time) of T_3 in the cell, and this is accompanied by an actual increase in nuclear receptor number.

Figure 2 illustrates the disappearance of receptor in the presence of 5 μM cycloheximide which inhibited protein synthesis by more than 95%. The

Table 1. Effect of Butyrate on Nuclear and Extranuclear T_3 Levels

Time of butyrate treatment (h)	Nuclear (fmol T_3/100 μg DNA)	Extranuclear (fmol T_3/100 μg DNA)
0	23	175
24	40	344
48	54	517

C6 cells were incubated with 2 mM butyrate and nuclear and extranuclear bound T_3 determined as described under "Materials and Methods".

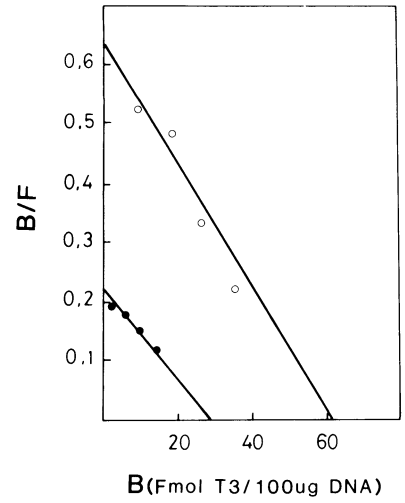

Fig. 1. Scatchard plot of T_3 binding to isolated nuclei. C6 cells were incubated with butyrate 2 mM for 48 h. The nuclei were then isolated and incubated with different concentrations of ^{125}I-T_3. ●:control; ○:butyrate.

estimated t 1/2 of disappearance was 11-12 hr in control cells, whereas receptor t 1/2 increased by almost threefold in cells incubated with 2 mM butyrate for 48 hr. Therefore, this compound probably raises receptor number by decreasing receptor degradation.

Other short-chain fatty acids have effects qualitatively similar to butyrate on both nuclear and extranuclear T_3. Table 2 shows the results obtained after an incubation of 48 hr with a 5 mM concentration of different carboxylic acids. All of them increased nuclear receptor and extranuclear hormone with the following potencies: butyrate > valerate > propionate > acetate.

This rank order is similar to that obtained for the inhibition of histone deacetylation (17), which suggests that the effect of butyrate could be explained by a modification of chromatic structure secondary to acetylation. However, as can be observed in Fig. 3, butyrate and other analogs did not detectably increase the level of multiacetylated forms of histone H3 and H4 in C6 cells.

The lack of effect of butyrate on histone acetylation in C6 cells might only be apparent. It is possible that the percentage of histones which can be modified in C6 cells is much lower than in other cell types (1,6,17) and

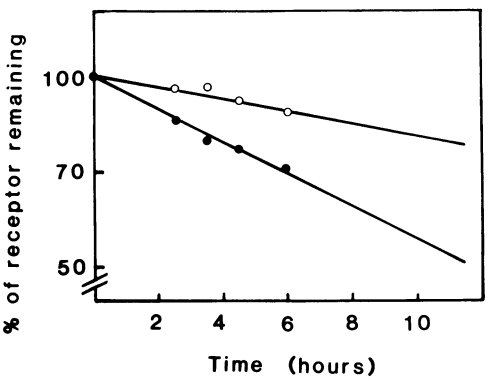

Fig. 2. Receptor disappearance after inhibition of protein synthesis. C6 cells were incubated in the presence or absence of 2 mM butyrate for 48 h. The cells were then incubted with 5 µM cycloheximide for the times indicated and nuclear receptor determined. At time 0, the levels of receptor were 25 and 61 fmol/100 µg DNA in control and butyrate-treated cells, respectively. The data are expressed as % of these values. ●:control; ○:butyrate.

Table 2. Influence of Short-Chain Fatty Acids on Nuclear Receptor and Extranuclear T_3 Levels

Fatty Acid	Nuclear (fmol T_3/100 µg DNA)	Extranuclear (fmol T_3/100 µg DNA)
None	23	121
Acetate	42	173
Propionate	85	430
Valerate	100	614
Butyrate	114	1262

C6 cells were incubated for 48 h with a 5 mM concentration of the compounds indicated.

that, therefore, an inhibition of the nuclear deacetylases cannot be observed when merely studied by gel electrophoresis. In agreement with this explanation, Kumar et al. (11) have shown that the kinetics of turnover of ^{3}H-acetate incorporation into histones is markedly slowed down by aliphatic carboxylic acids in C6 cells.

The different degree of histone modification caused by butyrate in C6 and GH1 cells might explain the different regulation of the thyroid hormone receptor by butyrate in both types of cells. In this respect, it must be pointed out that in GH1 cells, butyrate also elicits an increase in receptor levels (although not as marked as that observed in C6 cells) at low concentrations (8,9). On the other hand, the different regulation of the thyroid hormone receptor might also reflect a different organization of the receptor in the chromatin, or even to be due to differences in the receptor itself; for example, in the percentage of the 57,000 and 47,000 Mr forms (7) between pituitary and glial cell lines.

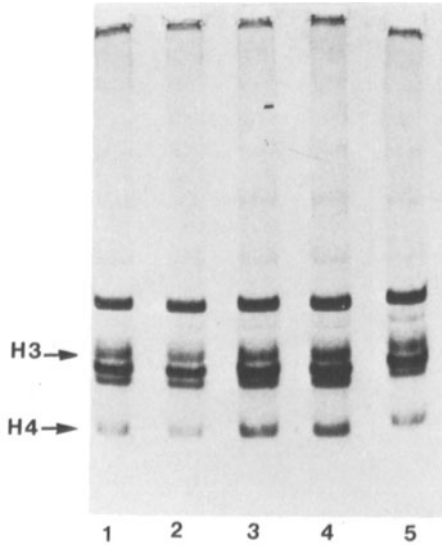

Fig. 3. Influence of carboxylic acids on histone pattern. C6 cells were incubated for 48 h with different compounds, each at 5 mM. The histones were extracted and electrophoresed in slab acid-urea gels. Lane 1:control; 2:acetate; 3:propionate; 4:valerate; 5:butyrate.

REFERENCES

1. Prasad KN and Sinha PK. In Vitro 12: 125, 1976.
2. Riggs MG, Whittaker RG, Neumann JR, et al. Nature 268: 462, 1977.
3. Boffa LC, Gruss RJ, and Allfrey VG. J Biol Chem 256: 9612, 1981.
4. Rastl E and Swetly P. J Biol Chem 253: 4333, 1978.
5. Christman JK, Weich N, Schoenbrun B, et al. J Cell Biol 86: 366, 1980.
6. Samuels HH, Stanley F, Casanova J, et al. J Biol Chem 255: 2499, 1980.
7. Pascual A, Casanova J, and Samuels HH. J Biol Chem 257: 9640, 1982.
8. Stanley F and Samuels HH. J Biol Chem 259: 9768, 1984.
9. Casanova J, Horowitz ZD, Copp RP, et al. J Biol Chem 259: 12084, 1984.
10. Weingarten DP, Kumar S, Bressler J, et al. In W. Norton (ed), Advances in Neurochemistry, Plenum Press, New York, 1984, p 299.
11. Kumar S, Weingarten DP, Callahan JW, et al. J Neurochem 43: 1455, 1984.
12. Samuels HH, Stanley F, and Casanova J. Endocrinology 105: 80, 1979.
13. Samuels HH and Tsai JR. J Clin Invest 53: 656, 1974.
14. Burton K. Biochem J 62: 315, 1956.
15. Vidali C, Boffa LC, Bradbury EM, et al. Proc Natl Acad Sci USA 75: 2239, 1978.
16. Panyim S and Chalkley R. Arch Biochem Biophys 130: 337, 1969.
17. Sealy L and Chalkley R. Cell 14: 115, 1978.

INHIBITORY EFFECT OF THYROID HORMONE ON PROTEIN SYNTHESIS BY FETAL RAT NEURONS IN PRIMARY CULTURE

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ABSTRACT

The effect of thyroid hormones on protein synthesis by fetal cortical cells in primary culture was studied by assessing (^{3}H)-phenylalanine incorporation into trichloroacetic acid precipitable proteins. 48 h incubation with thyroid hormones, concentrations between 10^{-12} M and 10^{-7} M, produced a dose-dependent inhibition of protein synthesis. The time course study showed that T_4 effect needed a longer period than T_3 to achieve a significant inhibition of labeled amino acid incorporation into proteins. 48 h incubation of the cells with T_3 (10^{-7} M) plus Actinomycin-D (5 µg/ml) resulted in a significant potentiation of T_3 inhibitory effect. These results indicate that thyroid hormones inhibit total protein synthesis in fetal rat neurons.

INTRODUCTION

Thyroid hormone (TH) deficiency at birth is known to cause marked impairment of brain differentiation (1,2). These observations are supported by changes in DNA and protein synthesis (3,4). TH effect on different tissues at different doses can be dramatically different as concluded from rat liver and amphibian tadpole studies (5,6). Since there is no well-defined data on TH regulation of protein synthesis in rat fetal cortical cells, our aim was to clarify TH role in these tissues.

MATERIAL AND METHODS

Timed pregnant Wistar rats were raised in our laboratory. On day 17 of fetal life, the embryos were removed. The cerebral cortices were taken and collected in Hank's Balanced Salt Solution. The cortical cells were dispersed by mechanoenzymatic techniques and cultured as described (7). Final cell suspensions were plated at 10^7 cells/25cm^2 per tissue culture flask. Media were changed twice weekly.

At the beginning of each experiment, the plates were rinsed with Minimum Essential Medium (MEM), without serum and phenylalanine, and fresh medium containing (^{3}H)-phenylalanine (50-100 µCi/dish). At the termination

of the experiment, culture dishes were chilled on ice, media were removed, cells were rinsed with 0.1 N HCl, extracted in the same, and frozen. Total proteins were precipitated by the addition of trichloroacetic acid. T_4 and T_3 were dissolved in 0.1 N NaOH and neutralized with 0.1 N HCl. The stock solution was diluted with MEM to get the desired concentrations. Total proteins were measured by the Lowry method (8), and DNA was estimated by the Burton method (9). Mean and standard errors of samples were calculated. Student's t test was sued to evaluate the differences between the control and experimental groups.

RESULTS

Cell cultures were exposed to T_3 or T_4 for various time periods. A significant decrease in counts incorporated into proteins (cells + media) was evident as early as 2 h after exposure to T_3 (10^{-8} M). This decrease was of the same magnitude after 3 and 6 h and was more evident at 24 h (Fig. 1) and 48 h (data not shown). However, when cells were incubated with T_4 (10^{-7} M), the inhibitory effect was not detectable until 24 h of pretreatment, indicating that T_4 effect needed a longer lag period than T_3 to achieve a significant inhibition of protein synthesis. Since the inhibitory action of both T_3 and T_4 was more effective at 48 h, this time was selected from then on. A dose response study (Fig. 2) showed that T_4 concentrations between 10^{-12} to 10^{-8} and T_3 between 10^{-11} to 10^{-7} M significantly inhibited counts incorporated into proteins. This effect was already evident with 10^{-12} M of T_4 and 10^{-10} M of T_3.

In order to investigate whether this inhibitory action of TH could be mediated by a newly synthesized protein, cells were simultaneously exposed

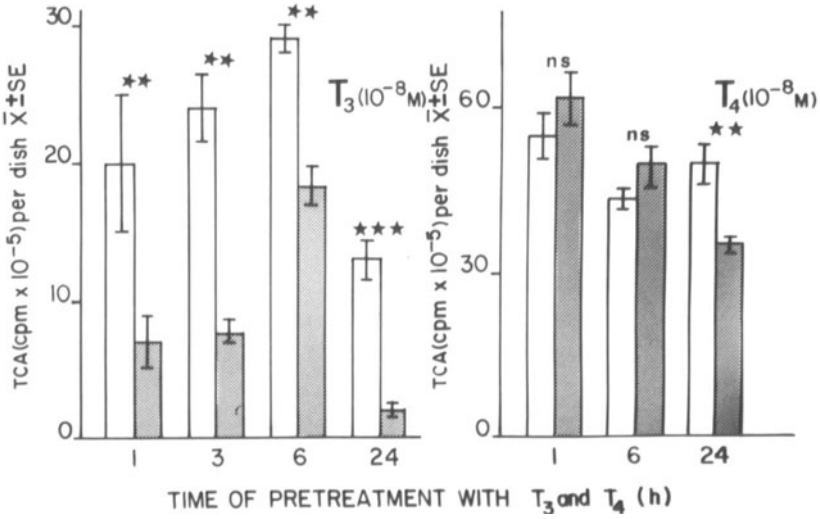

Fig. 1. Time course of [^{3}H]-phenylalanine incorporation into proteins by fetal cortical cells in culture. TH were added to culture at 1, 3, 6, and 24 h before the addition of [^{3}H]-Phe. The cultures were then incubated for an additional 3 h. Incorporation was reduced by 1 h pretreatment with T_3 and 24 h with T_4. Open columns: control. Dotted columns: T_3 and T_4-treated. ns: not significant. **$p<0.01$; ***$p<0.001$.

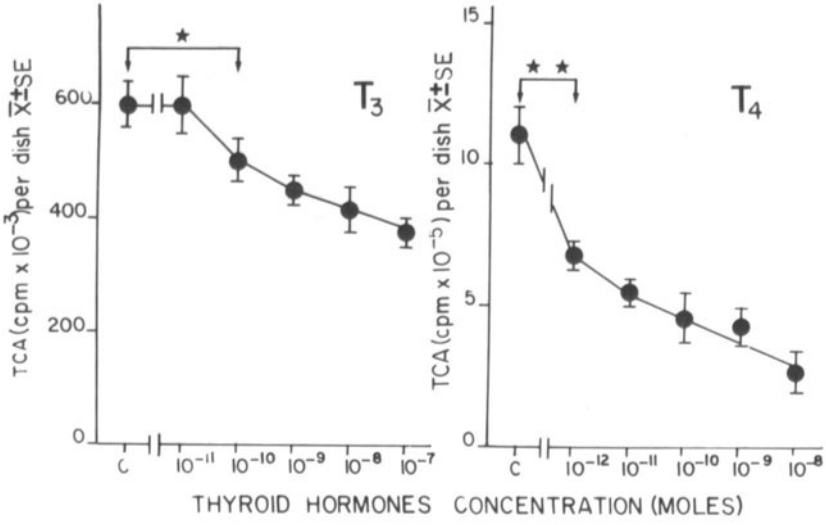

Fig. 2. Dose response of thyroid hormone effect on [^{3}H]-Phe incorporation into TCA precipitable counts. TH was added to cultures 48 h before the addition of labeled amino acid. The cultures were then incubated for an additional 3 h. Incorporation was reduced by 10^{-10} M T_3 and 10^{-12} M T_4. *$p<0.05$; **$p<0.01$.

to Actinomycin-D (Act-D) plus T_3. As is shown in Fig. 3, Act-D significantly reduces the synthesis of proteins, just as T_3 does. The concomitant addition of both magnifies this inhibition. Total proteins measured by the Lowry method were also decreased by T_3 treatment in a dose-response manner (data not shown). Preliminary data on DNA and RNA has shown no evidence of change under TH treatment.

DISCUSSION

The present data indicate that protein synthesis in fetal rat cortical cells is decreased by thyroid hormone. The effect is observed in a wide range of doses of T_4 and T_3. The lag time for T_4 effect is 24 h, being only 1 h for T_3. This is the first demonstration of the inhibitory action of TH on protein synthesis in the brain. In other tissues, such as tadpole tail muscle, it has been shown that the rate of protein synthesis is decreased in spontaneous metamorphosis after T_3 administration (10). The specificity of the inhibition is strengthened by the fact that total proteins were also decreased by TH treatment, as measured by the Lowry method. Evidence of this effect at low and physiological TH concentrations does not support increased degradation, although our data do not completely exclude this possibility. Under the same experimental conditions, physiological doses of T_3 (10^{-9} M) increased the synthesis of somatostatim (11), indicating that this neuropeptide could be under positive TH control at this state of brain development. The inhibitory mechanism was not elucidated in this study. Since Act-D potentiates T_3 inhibitory effect, it seems that the inhibition is not mediated by the synthesis of a new protein, as described in tadpole metamorphosis.

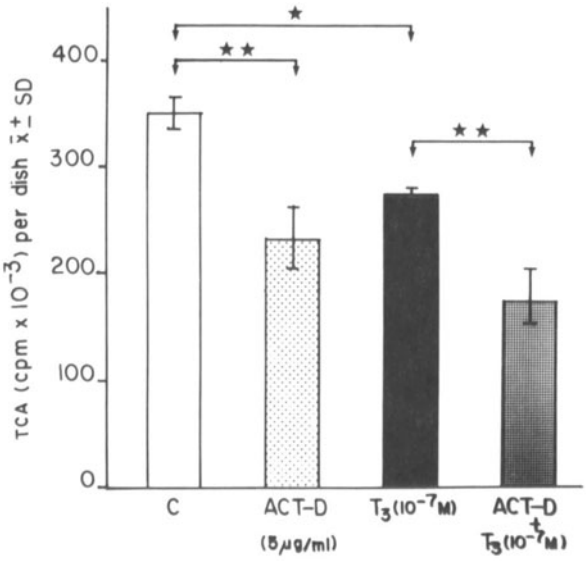

Fig. 3. Effect of Act-D treatment on T_3 inhibition of protein synthesis. Act-D and T_3 were added to culture 48 h before the addition of [^{3}H]-phenylalanine. The cultures were then incubated for an additional 3 h. Simultaneous incubation with Act-D and T_3 further reduces protein synthesis. *$p<0.05$; **$p<0.01$.

REFERENCES

1. Eayrs JF and Taylor SM. J Anat (Lond) 85: 350, 1951.
2. Balazs R, Kovacs S, Cocks WA, et al. Brain Res 25: 555, 1971.
3. Patel A and Balazs R. Biochem J 121: 469, 1977.
4. Clark BR and Weichsel Jr ME. J Neurochem 29: 91, 1977.
5. Widnell CC and Tata JR. Biochem Biophys Acta 72: 506, 1963.
6. Atkinson B, Just J, Atkinson K, et al. Dev Biol 29: 162, 1972.
7. Vaccaru D and Messer A. Tissue Culture Assoc Manual 3: 561, 1977.
8. Lowry OH, Rosebrough NJ, and Farr AL. J Biol Chem 293: 265, 1951.
9. Burton K. Biochem J 62: 315, 1955.
10. Tonoue T and Frieden E. Biochem Biophys Res Commun 37: 81, 1969.
11. Cacicedo L and Sanchez-Franco F. 7th International Congress of Endocrinology, Quebec, Canada, Abstract 410, 1984.
12. Salcem M and Atkinson B. J Biol Chem 253: 1378, 1978.

EFFECTS OF T_3 AND DEXAMETHASONE ON PROLIFERATION AND ENZYME ACTIVITIES OF GLIAL CELLS IN CULTURE

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Thyroid hormones affect myelination in vivo (1), as well as in brain explants (2) and dissociated cultures (3), which indicates that these hormones are important for a normal maturation and differentiation of glial cells.

The glucocorticoid induction of glycerol phosphate dehydrogenase (EC 1.1.1.8; GPDH) is a very well-characterized hormonal effect in oligodendrocytes and C6 cells, a rat glioma cell line (4,5). Other hormones, such as catecholamines, potentiate the glucocorticoid induction of GPDH (6) and specifically regulate the synthesis of lactate dehydrogenase (EC 1.1.1.27; LDH) (7) in C6 cells. Malic Enzyme (EC 1.1.1.27; ME) activity is under thyroid hormone control in several tissues (8), although it fails to respond to thyroid hormones in the brain (9). We have examined the effect of L-triiodothyronine (T_3) and dexamethasone (Dex) on cell proliferation and on the activity of these enzymes in C6 cells and in primary cultures of dissociated embryonic mouse brain cells.

MATERIAL AND METHODS

C6 cells were grown in monolayer cultures, and 24 hours before the beginning of the experiments the growth medium (RPMI-10% horse serum-2.5% fetal calf serum) was replaced by a medium containing 10% newborn calf serum treated with resin AG 1-x10 and charcoal, as previously described (10), to eliminate thyroid hormones and glucocorticoids.

Cerebral hemispheres of 14- and 15-day-old mouse embryos were dissociated mechanically into single cells and cultured as described (11). The medium was replaced by serum-free medium (DMEM) 24 h before the addition of the hormones.

Preparation of cytosols and enzyme assays was carried out as previously described (12,13). One unit of enzyme is defined as that amount which causes the oxidation of 1 nmol of NADH/min.

The incorporation of ^{3}H-Leucine (5 μCi/ml for 2 h at 37°C) into trichloroacetic acid-insoluble material was used as a measure of total protein synthesis in C6 cells. Protein and DNA concentrations were determined by the method of Lowry (14) and Burton (15), respectively.

Table 1. Effect of T_3 and Dexamethasone on the Growth and Protein Synthesis of C6 Cell Cultures

	Protein (µg)	DNA (µg)	^{3}H-Leucine incorporation (dpm/100 µg protein)
Control	188	19	74461
T_3	190	19	77607
Dex	90	10	86702
T_3 + Dex	94	9	83673

C6 cells were incubated with 5 nM T_3, 50 nM dexamethasone (Dex) or the combination of both for 72 h. Total protein and DNA/culture, as well as the incorporation of ^{3}H-Leucine into total protein were determined as described under Material and Methods.

All data shown are the mean of 3-5 cultures which did not vary among them more than 5-20%.

RESULTS AND DISCUSSION

Incubation of C6 cells with 5 nM T_3 does not change total protein or DNA content/culture (Table 1), whereas 50 nM Dex decreased both by approximately 50% without altering the protein/DNA ratio. T_3 did not modify the effect of Dex on cell proliferation, since T_3 + Dex had the same effect as Dex alone. Exposure to T_3, Dex, or the combination of both had no effect upon ^{3}H-Leucine incorporation into proteins (Table 1), which supports the idea that cytotoxicity is not responsible for the reduction of cell numbers in the glucocorticoid-treated cultures. T_3 did not alter total protein in the primary cultures (2.7 and 3.4 mg protein/flask at 14 and 60 days in control cultures vs 2.3 and 3.5 mg protein in T_3-treated cultures).

Table 2. Effect of T_3 and Dexamethasone (Dex) on GPDH Levels in C6 Cells and in Primary Cultures

	GPDH Activity			
	Control	T_3	Dex	T_3 + Dex
C6 Cells	53	20	273	223
Primary Cultures				
14 DIC*	2.6	1.3	6.5	5.9
60 DIC	0.8	0.3	-	-

*Days in culture. C6 cells or cells dissociated from brain embryonic mouse were incubated with 5 nM T_3 for 72 h, or with 50 nM dexamethasone (Dex) for 48 h. Data represents GPDH specific activity expressed as units/mg cytosolic protein.

Table 2 shows the effect of T_3 and Dex on GPDH activity in C6 cells and in cultures of fetal brain mouse cells. Basal activity was higher in C6 cells than in the primary cultures at 14 days in culture (mostly composed of astrocytes and oligodendrocytes) or at 60 days in culture (mainly microglia although oligodendrocytes are still present). As expected (4,5), Dex treatment increased GPDH activity in both C6 cells and brain cultures. By contrast, T_3 decreased basal GPDH by 50-70% and also caused a slight decrease of the glucocorticoid-induced levels. This finding indicates that the enzyme inhibition observed in C6 cells is not merely a reflection of malignant transformation, and it opens the possibility that thyroid hormones could modulate this activity in the brain in vivo.

It has been described that noradrenaline potentiates the glucocorticoid-induced specific activity of GPDH (6). Fig. 1A shows that in C6 cells, noradrenaline (3 µM for 18 h) did not alter the uninduced level, but it potentiated the response to Dex. T_3 reduced basal activity and the response to Dex or to the combined effects of Dex and noradrenaline. Fig. 1B illustrates LDH specific activity obtained in the same cells. As expected (7), noradrenaline increased LDH levels by nearly twofold, and T_3 did not alter the uninduced or the catecholamine-induced LDH activity.

The effects of noradrenaline appear to be mediated by a transient rise in the intracellular cyclic-AMP levels. Fig. 2 shows the influence of T_3 on the response to Dex and dibutyryl cyclic-AMP (1 mM for 48 h). Dibutyryl cyclic-AMP greatly potentiated Dex induction of GPDH, and T_3 reduced the activity in basal conditions and even in conditions of very high stimulation. By contrast and in agreement with the results desribed in Fig. 1B, T_3 did not change LDH activity (data not shown). These results show that the effect of T_3 on GPDH response does not represent a generalized effect on enzyme activities. This specificity is also demonstrated by the finding that T_3 did not change ME activity (a good parameter of thyroid hormone action in other cell types) either alone or in combination with Dex or Dex plus insulin (not illustrated).

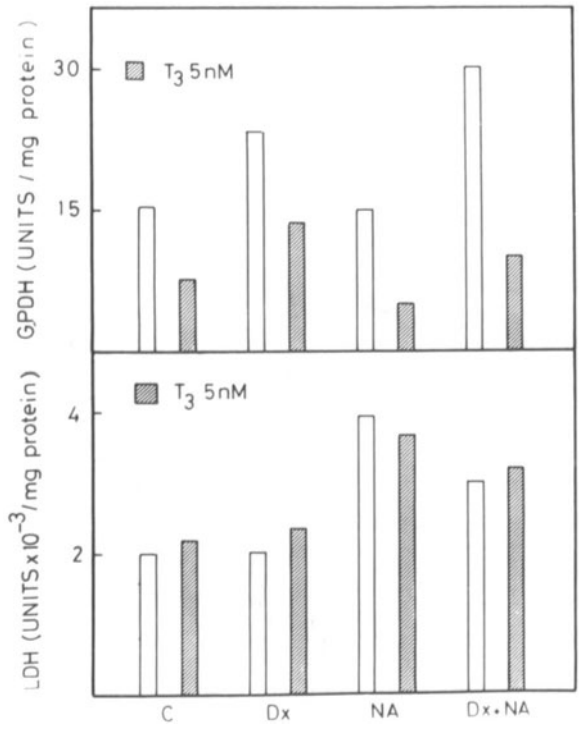

Fig. 1. Differential effect of T_3 on GPDH (panel A) and LDH panel B) levels in C6 cells. C represents uninduced controls. Dx represents cells incubated with 50 nM dexamethasone for 48 h, NA are cells treated with 3 µM noradrenaline for the last 18 h, and Dx+NA are cells incubated with dexamethasone and noradrenaline. In all groups, hatched bars represent cells treated with 5 nM T_3 for 72 h (i.e., the incubation with T_3 was started 24 h before the addition of Dx), and open bars represent cells which were not exposed to T_3.

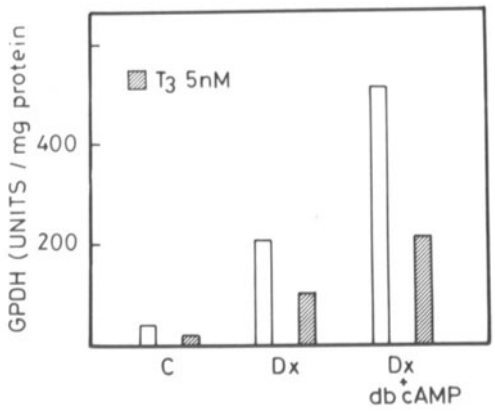

Fig. 2. Effect of T_3 on GPDH induction with dexamethasone and dibutyryl cyclic-AMP. C6 cells were incubated with or without 5 nM T_3 for 24 h. The cells were then treated for an additional 48 h period in the presence (hatched bars) or absence (open bars) of T_3 with 50 nM dexamethasone (Dx), dexamethasone + 1 mM dibutyryl cyclic-AMP (Dx+dbcAMP), or medium (C).

The inhibitory effect of T_3 on GPDH is rather surprising, since most of the effects of thyroid hormones on enzymes and other proteins are stimulatory. However, we have yet to establish whether the hormone is altering the synthesis or shortening the half-life of the enzyme. Interestingly, sodium butyrate, which has a profound influence on the chromatin and greatly increases T_3 receptor levels in C6 cells (16), has an effect similar to T_3, blocking the GPDH induction by glucocorticoids (5). It will be of interest to determine whether or not T_3 and butyrate modulate GPDH activity through similar mechanism(s).

REFERENCES

1. Dalal KB, Valcana T, Timiras PS, et al. Neurobiology 1: 211, 1971.
2. Hamburgh M. In A Moscona and A Mouroy (eds), Current Topics in Developmental Biology, Academic Press, New York, 1969, p 109.
3. Bhat NR, Rao GS, and Pieringer RA. J Biol Chem 256: 1167, 1981.
4. De Vellis J, McGinnis JF, Breen GAM, et al. In S Fedoroff and L Hertz (eds), Cell, Tissue and Organ Cultures in Neurobiology, Academic Press, New York, 1977, p 485.
5. Weingarten DP, Kumar S, Bressler J, et al. In WT Norton (ed), Advances in Neurochemistry, Plenum Press, New York, Vol. 5, 1984, p 299.
6. Breen GAM, McGinnis JF, and de Vellis J. J Biol Chem 253: 2554, 1978.
7. Jungmann RA, Kelley DC, Miles MF, et al. J Biol Chem 258: 5312, 1983.
8. Tarentino AL, Richert DA, and Westerfeld WW. Biochim Biophys Acta 124: 295, 1966.
9. Hemon P. Biochim Biophys Acta 151: 681, 1968.
10. Samuels HH, Stanley F, and Casanova J. Endocrinology 105: 80, 1979.
11. Sarlieve LL, Delaunoy JP, Dierich A, et al. J Neurosci Res 6: 659, 1981.
12. McGinnis JF and de Vellis J. Biochim Biophys Acta 364: 17, 1974.
13. Hsu RY and Lardy HA. Methods of Enzymology 13: 230, 1969.
14. Lowry OH, Rosebrough NJ, Farr AL, et al. J Biol Chem 193: 265, 1951.
15. Burton K. Biochem J 62: 315, 1956.
16. Ortiz-Caro J, Montiel F, Pascual A, et al. Proceedings of the 9th International Thyroid Congress, Plenum Press, New York, 1985.

HEALING EFFECTS OF IODINE AND THYROID HORMONE ON EXPERIMENTAL RICKETS

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INTRODUCTION

The Steenbock 2965 diet has been regarded as a standard rachitogenic diet, but in 1961 Shimazaki and Yagi pointed out that this diet is also deficient in iodine, and the rachitic findings produced on this diet could be ameliorated with potassium iodide (KI). Further, in 1984 Shimazaki et al. (1) found that triiodothyronine (T_3) is more effective in this respect than iodine and even vitamin D (v.D). In this study, to determine the physiological basis of this beneficial effect, the actions of T_3 on mineral metabolism have been investigated and compared with those of v.D.

METHODS

Animals and Materials

Weanling male Wistar rats (SPF) were obtained from Shizuoka Laboratory Center. Two kinds of diets, deficient in v.D and iodine, were basically the AIN - 76™ diet (2) in which v.D and KIO_3 were omitted and Ca and P contents changed. Diet A contained high Ca and low P (1.13% Ca and 0.135% P), and diet B contained adequate Ca and P (0.409% Ca and 0.454% P). The animals had free access to distilled water.

Experimental Design

Several protocols of study were followed. Group 1 rats were raised on diet A without further treatment (rachitic group). Group 2 rats were raised on diet A and treated with 3 μg T_3 in 0.1 ml of 5 mM NaOH intraperitoneally (i.p.). Group 3 rats were on diet A and treated i.p. with 0.25 μg v.D_3 in 0.1 ml of 0.1% ethanol in propylene glycol mixture. Group 4 rats were on diet A and treated i.p. with 0.25 μg v.D_3 and 3 μg KI in 0.1 ml of saline. Group 5 rats were on diet B without further treatment. Group 6 rats were on diet B and treated i.p. with 0.25 μg v.D_3 and 3 μg KI, and served as normal controls. All the animals were kept in stainless steel cages for 30 days.

Table 1. Effects of T_3, v.D_3 and KI on Intestinal Pi and Ca Transport, Plasma Pi and Ca Levels, Plasma T_3 and T_4 Levels, and Renal Pi Excretion

Diet	D (-) I (-) P depleted				D (-) I (-) P normal	
Treatment	-	T3	D	D+KI	-	D+KI
Ca transport (S/M)	12.22 ± 0.74	5.47 ± 0.69***	12.97 ± 2.71	14.74 ± 1.41	7.39 ± 0.53***	9.02 ± 0.55***
Pi transport (S/M)	0.89 ± 0.05	1.16 ± 0.03***	1.13 ± 0.12	1.12 ± 0.17	1.14 ± 0.05***	1.95 ± 0.41
Plasma Ca mg/dl	8.5 ± 0.2	8.3 ± 0.2	11.7 ± 0.6***	11.7 ± 0.6***	11.3 ± 0.1***	11.0 ± 0.2***
Plasma Pi mg/dl	5.2 ± 0.2	7.1 ± 0.2***	7.4 ± 0.3***	7.3 ± 0.7*	8.2 ± 0.1***	8.1 ± 0.4***
Plasma T_3 ng/ml	0.94 ± 0.05	6.79 ± 0.45***	0.70 ± 0.09*	0.66 ± 0.08	1.13 ± 0.10	1.05 ± 0.06
Plasma T_4 µg/dl	4.53 ± 0.83	1.09 ± 0.18***	3.06 ± 0.48	2.50 ± 0.33	4.35 ± 0.83	4.63 ± 1.19
U_{Pi} X V µg/day	3.0 ± 0.4	6.9 ± 2.1	0.9 ± 0.6**	3.3 ± 1.0		

Mean ± standard error; compared with rachitic control (t test). *$p<0.05$; **$p<0.02$; ***$p<0.01$.

Table 2. Effects of T_3 and v.D_3 on Plasma Ca and Pi Levels in Nephrectomized Rachitic Rats

Nephrectomy	-	+	+	+	+
Treatment	-	-	T_3	D	T_3+D
Plasma Ca mg/dl	15.0 ± 0.4*	20.5 ± 1.8	19.7 ± 1.1	13.1 ± 1.4	18.2 ± 1.2
Plasma Pi mg/dl	5.8 ± 0.6	8.1 ± 0.8	8.5 ± 0.7	10.8 ± 1.7	10.2 ± 0.8

*Mean ± standard error.

Chemical Determination

Plasma Ca and inorganic phosphate (Pi) levels were determined by the OCPC method and by molybdenum blue direct method, respectively. Total plasma T_3 and T_4 levels were measured by RIA.

Histological Examination

Bilateral tibias were fixed in buffered formalin solution, decalcified, sectioned longitudinally and stained with hematoxylin and eosin (H.E.). The kidneys were routinely processed and stained with H.E.

Clearance Study

To examine renal excretion of Pi, each rat raised on diet A was housed in a metabolic cage and urine was collected for 24 hours. The rats were allowed to drink and feed ad lib during this period.

Nephrectomy Study

Rachitic rats raised on diet A were bilaterally nephrectomized and immediately thereafter injected with either 6 μg T_3, 0.4 μg 1α(OH)D_3, or 6 μg T_3 + 0.4 μg 1α(OH)D_3 and examined for the biochemical and histological changes for 16 hours.

Transport of Ca and Pi by Everted Sacs of Intestine

Ca and Pi transports of intestine were studied by a modification of the method of Martin et al. (3) and that of Chen et al. (4), respectively. Rats were fasted for 18 hours and killed by decapitation. For measuring Ca transport the upper duodenum was used and a ratio of serosal Ca/mucosal Ca (S/M) was calculated, and for Pi transport the jejunum was used.

Determination of 25(OH)D_3-1α- and 24-hydroxylase Activities of Kidney Homogenate In Vitro

Rats were anesthetized with ether, exsanguinated and perfused with 0.01 M PBS through the kidney. The activities of 25(OH)D_3-1α- and 24-hydroxylase were measured, using the kidney homogenate, by a modification of the method of Horiuchi et al. (5).

Table 3. Effects of T_3, v.D_3 and KI on Renal 1α,25(OH)D_3 and 24-Hydroxylase Activities

Diet	D (-) I (-) P depleted				D (-) I (-) P normal	
Treatment	-	T3	D	D+KI	-	D+KI
1α,25$(OH)_2D_3$ produced	14.3 ± 3.9	4.3 ± 1.6	1.8 ± 1.1*	<1.0***	3.0 ± 1.2*	<1.0**
24-25$(OH)_2D_3$ produced pmol/300 mg tissue/20 min	<1.0	<1.0	21.8 ± 13.3	28.3 ± 6.6**	1.4 ± 0.7	20.6 ± 6.9*

Mean ± standard error; compared with rachitic control (t test). *$p<0.05$; **$p<0.02$; ***$p<0.01$.

RESULTS

The rachitic group showed a low plasma Pi level and characteristic bony changes in the proximal end of tibia and also a remarkable atrophy in renal tubules. On the other hand, the T_3 group revealed a nearly normal level of plasma Pi, and histologically no sign of rachitic bony changes and no atrophic changes in renal tubules (Table 1).

In the nephrectomy study, all groups had high plasma levels of Ca and Pi. Groups receiving T_3 revealed a sign of healing (Table 2).

The clearance study showed that the T_3 group excreted more Pi (Table 1). It has been reported that, in the hyperthyroid state, the urinary excretion of Pi increases in spite of an enhanced reabsorption of Pi because of a significant increase in filtered load of Pi.

In the intestinal transport experiment, only the rachitic group actually secreted Pi into intestinal lumen, and the T_3 group showed an accelerated pI absorption, but a depressed Ca transport (Table 1).

Renal 1α- and 24-hydroxylase activities were as follows. The rachitic group had a high renal 1α-hydroxylase activity and undetectable 24-hydroxylase activity. On the contrary, normal rats showed a high 24-hydroxylase activity and undetectable 1α-hydroxylase activity. The T_3 group had a suppressed renal 1α-hydroxylase activity and no increase of 24-hydroxylase activity (Table 3).

DISCUSSION AND CONCLUSION

It was recently reported that thyroid hormone could be a factor in maintaining the increased level of renal 25(OH)v.D-1α-hydroxylase activity observed in phosphate deprivation (6). This postulated potency for thyroid hormone in accelerating the v.D activating pathway could explain the antirachitic ability of T_3. In fact, however, this explanation is unsatisfactory because in this study T_3 was effective even in nephrectomized rats and T_3 suppressed the renal 1α-hydroxylase activity. Consequently, there remain two possibilities to be considered: first, T_3 may activate the target cells for active metabolites of v.D, which cells, in turn, hypertrophy and respond more vigorously, and secondly, T_3, independent of v.D, stimulates its target cells, which presumably include those cells engaged in mineral metabolism, e.g., intestinal, renal and osteo-cartilagenous cells. Here again, the first possibility must be ruled out because T_3, compared with v.D, had a contrary, suppressing effect on the intestinal transport of Ca. After all, it seems rational that T_3 activates its own target cells, leading to an increase of transmembranous Pi uptake, possibly by accelerating de novo synthesis of Na K ATPase and/or modulating plasma membrane lipid composition, and in consequence brings about healing of rickets. It follows from this that T_3 may have some beneficial effects on certain types of hypophosphatemic rickets.

REFERENCES

1. Shimazaki M, Masuda H, Mitsuhashi T, et al. Osaka City Med J 30: 113, 1984.
2. Report of the American Institute of Nutrition Ad Hoc Committee on Standards for Nutritional Studies. J Nutr 103: 1340, 1977.
3. Martin DL and DeLuca HF. Am J Physiol 216: 1351, 1969.
4. Chen TC, Castillo L, and Korycka-dahl M. J Nutr 104: 1056, 1974.

5. Horiuchi N, Shinki T, Suda S, et al. Biochem Biophys Res Commun 121: 174, 1984.
6. Gray RW. Garthwaire TL, and Phillips LS. Calcif Tissue Int 35: 100, 1983.

THYROID HORMONE STIMULATES RED BLOOD CELL Ca^{++}-DEPENDENT ATPase ACTIVITY THROUGH A "NON-HORMONAL" EFFECT*

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INTRODUCTION

Thyroid hormones (TH) elicit a wide variety of metabolic and physiological responses in several tissues of higher organisms. The majority of these effects require the interaction of TH with protein receptors located at the cell nucleus. However, exranuclear actions of TH have elicited growing interest during the last decade. Red blood cells have been shown to possess plasma membrane-binding sites for both T_3 and T_4. Using this as a cellular model, a direct stimulation of erythrocyte membrane Ca^{++}-dependent ATPase (Ca^{++}-ATPase) by physiological concentrations of TH has recently been demonstrated (1,2). Among TH analogues, T_4 exerts the highest stimulatory effect on basal enzyme activity which is not dependent upon prior conversion to T_3 (3). This effect of T_4 requires the mediation of calmodulin (CaM), an ubiquitous cytosolic protein which plays a major role in the regulation of Ca^{++}-ATPase (4).

Present studies were undertaken to characterize more fully the effects of TH on this enzyme system, using rabbit and human erythrocyte membranes.

MATERIAL AND METHODS

Erythrocyte membranes were prepared, as depicted in Fig. 1, using two procedures (1,5). The effect of TH was investigated by preincubating membranes with T_4 or T_3 (10^{-14} M to 10^{-8} M) for 60 min at 37°C. Enzyme activity was measured using the procedure of Strittmater (6), as modified by Davis et al. (1), and was expressed as specific inorganic phosphate release (Pi) from ATP (1 mM).

RESULTS

Basal Ca^{++}-ATPase activity was higher in rabbit than in human red blood cell membranes. The effect of TH was investigated with rabbit membranes preincubated with T_4 or T_3 (Table 1). With 10^{-14} M T_4, no effect was found.

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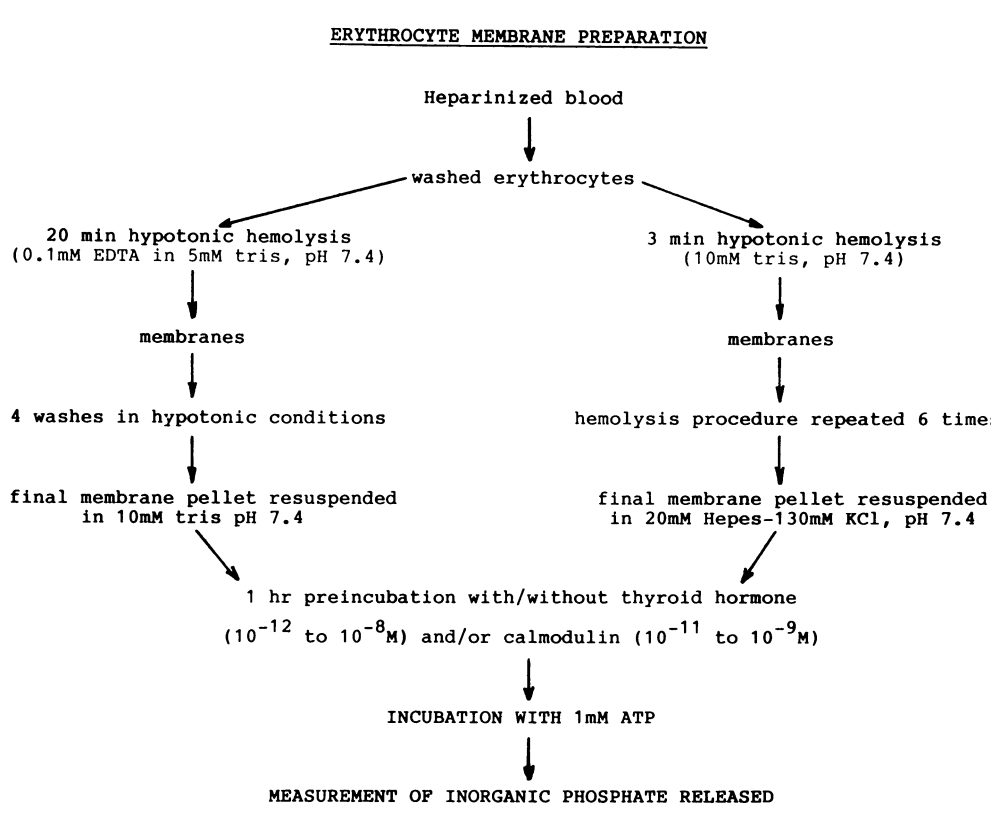

Fig. 1. Enzyme activity (mean ± SEM) was expressed as μmoles Pi/mg protein/90 min (membranes prepared in Tris and EDTA-washed) or 30 min (membranes prepared in Hepes). Statistical significance was calculated by paired t test.

Table 1. Ca^{++}-dependent ATPase Activity*

	Membranes	Basal	$+T_4$	ΔT_4	
Rabbit	Tris (a)	1.32 ± 0.04	1.44 ± 0.05	0.12 ± 0.04	$p<0.05$**
	Hepes (b)	0.20 ± 0.01	0.21 ± 0.03	0.01 ± 0.01	N.S.
Human	Standard (c)	0.74 ± 0.02	0.75 ± 0.02	0.01 ± 0.01	N.S.
	EDTA-washed (d)	0.39 ± 0.02	0.38 ± 0.02	-0.01 ± 0.01	N.S.

*Enzyme activity is expressed as μmol Pi/mg protein/90 minutes (a,c,d) or 30 minutes (b). Results represent mean ± SEM of 7 (a), 3 (b), 6 (c) and 4 (d) experiments.

**Statistical significance was calculated by paired t test.

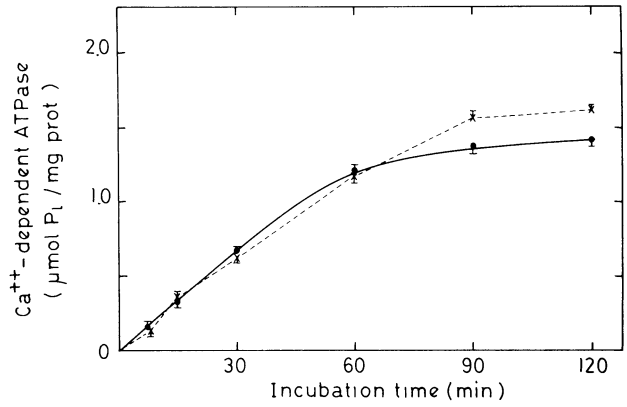

Fig. 2. Rabbit membrane suspensions (0.25 mg protein/tube) were preincubated with or without T_4 for 60 min at 37°C. Experimental points are the mean ± SEM of quadruplicates. Basal activity without T_4 (●); in the presence of 10^{-10} M T_4 (x).

With 10^{-10} M T_4, a moderate, but significant, stimulation of enzyme activity was observed and was not further changed by increasing T_4. Reduction of the ATP concentration in the incubation medium did not modify the stimulatory effect of T_4. The effect of T_3 was also investigated. Although a positive effect on enzyme activity was suggested, it was not statistically significant. In humans, the addition of 10^{-8} M CaM during preincubation of membrane suspensions yielded a 30-40% increase of basal enzyme activity (similar to that obtained with rabbit erythrocytes), but preincubation with 10^{-10} M T_4 did not modify basal enzyme activity.

The time course of Pi release is illustrated in Figure 2. In the absence of T_4, enzyme kinetics were linear up to 60 min, and Pi release reached a plateau between 90 and 120 min of incubation. In the presence of 10^{-10} M T_4, enzyme kinetics were identical to those during the first 60 min of incubation. At 90 and 120 min of incubation, however, T_4 induced a relative increase in the Pi release by 15%. These results provided the first evidence of a membrane mechanism of action of thyroid hormones on erythrocyte Ca^{++}-dependent ATPase.

The response of Ca^{++}-ATPase activity to exogenous CaM is illustrated in Figure 3. Human erythrocyte membranes were incubated with increasing concentrations of CaM and enzyme activity was increased above 5×10^{-10} M CaM ($p<0.005$). In addition, in the presence of T_4, enzyme activity was further increased by approximately 10% at CaM concentrations ranging from 5.10^{-12} M to 10^{-9} M ($p<0.05$). Membranes were washed extensively with EDTA to remove residual endogenous CaM. "EDTA-washed" membranes gave a 42-49% reduction in basal activity. Addition of exogenous CaM ($>5 \times 10^{-11}$ M) was accompanied by a striking increase in enzyme activity. With 10^{-9} M CaM, enzyme activity was increased by 250%, and was nearly identical in both "standard" and "EDTA-washed" membranes. These results suggested that the effect of TH could be observed in specific states of enzyme activation by CaM, providing additional evidence for an indirect mechanism of action of erythrocyte Ca^{++}-ATPase.

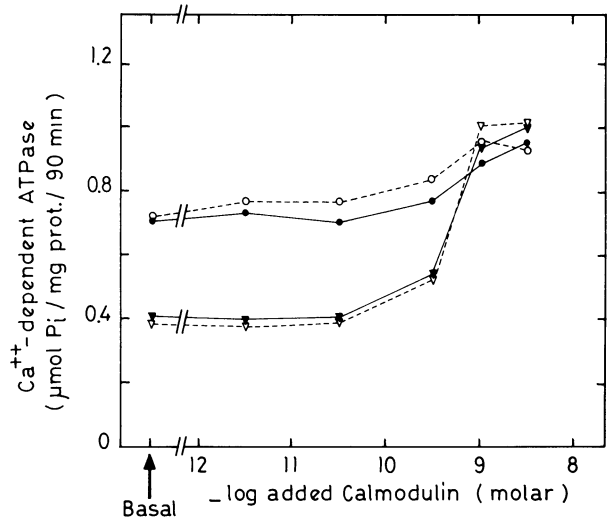

Fig. 3. Standard (○ , ●) or EDTA-washed (▽ , ▼) membranes (0.3 mg protein/tube) were preincubated for 60 min at 37°C with or without T_4. Enzyme activity in the presence of 10^{-10} M T_4 is represented by ○----○ ("standard" membranes) and by ▽-----▽ (EDTA-washed membranes).

To test this hypothesis further, incubation of erythrocyte membranes was carried out in media with alternative free calcium concentrations. Using these conditions, a widespread range of Ca^{++}-dependent ATPase responses to exogenous CaM was achieved, up to 320% of basal enzyme activity. Figure 4 indicates that in the low range of enzyme stimulation by CaM (Δ CaM : 40% or less), preincubation with T_4 induced an additional stimulation of enzyme activity of 8-10% ($t = 2.94$; $p<0.05$). At higher enzyme activity stimulation by CaM (between 50 and 320%), T_4 had no effect.

COMMENTS

Davis et al. were the first to report an in vitro stimulatory effect of physiological concentrations of TH on Ca^{++}-ATPase from human and other mammalian erythrocytes (1,2). Stimulation by T_4 of Ca^{++}-ATPase was shown to require the presence of CaM. The effect of T_4 could be amplified by the addition of exogenous CaM and was blocked by antibodies against CaM or by trifluoperazine, a CaM action inhibitor (4).

Results of present studies confirm an increase in Ca^{++}-ATPase activity with both T_4 and T_3 administration. Thyroid hormone effects were small (15% or less), and the effect of T_3 was not significant. Moreover, the time course of Pi release did not reflect any stimulatory effect of T_4 during the first hour of incubation. The effect of T_4 became apparent 1 hr after the addition of ATP (ΔT_4 : 15%). With human membranes, T_4 induced a relative stimulation of the Ca^{++}-dependent ATPase of 8-10% ($p<0.05$) in experimental conditions where the enzyme was not maximally stimulated by CaM (Δ CaM over basal activity : 5-40%). In conditions of high CaM stimulation (Δ CaM : 50-320%), T_4 had no effect.

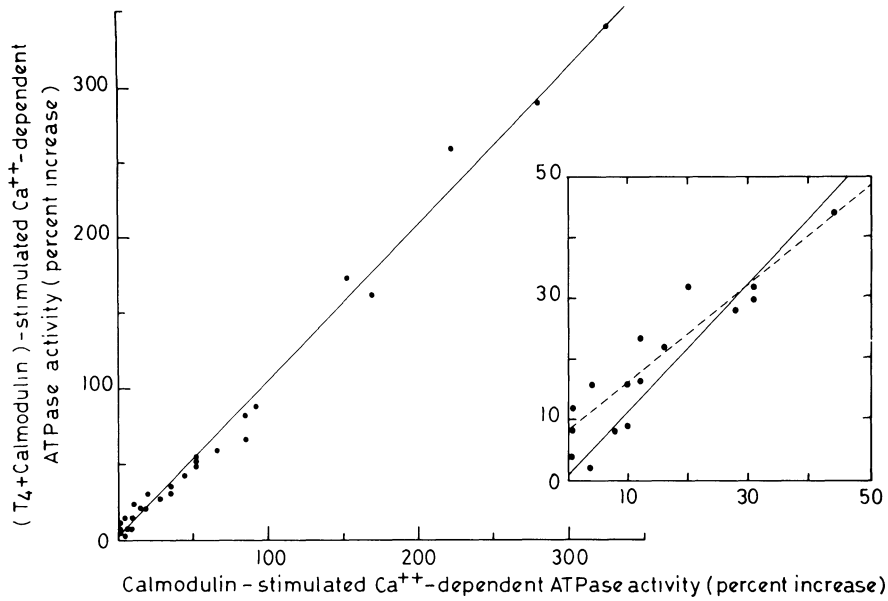

Fig. 4. Results are expressed as % increase over basal enzyme activity induced by CaM alone (abscissa) and by CaM + 10^{-10} M T_4 (ordinate). Exogenous CaM added ranged from 5 x 10^{-11} M to 5 x 10^{-9} M. Basal and CaM-stimulated enzyme activity were measured in the presence of 20, 50, or 100 µM Ca^{++} and results were pooled. Regression lines were calculated by the least square method for the entire set of data (y = 0.57 + 1.06 x) (solid line) and for ΔCaM lower than 50% (y = 7.55 + 0.82 x) (dotted line) (see insert).

CONCLUSION

These results do not provide evidence for a direct stimulation of the Ca^{++}-ATPase by T_4 in vitro, but suggest an indirect effect on the red blood cell membrane. This "non-hormonal" action of T_4 could be accounted for by a membrane stabilization effect, preventing either enzyme degradation or breakdown of other membrane constituents which could influence Ca^{++}-ATPase activity. It is possible that interaction with membrane phospholipids could mediate T_4 action (7). In erythrocytes, the fatty acid composition of membrane phospholipids can influence kinetic properties of the Ca^{++}-ATPase through changes in membrane fluidity (8). It has also been shown in rats that thyroid hormone either increased or decreased erythrocyte Ca^{++}-ATPase activity, depending on the fatty acids added to the diet (9). Finally, thyroid hormones could act on the "environment" of the Ca^{++}-dependent ATPase, thereby facilitating the binding of CaM to the enzyme. A similar mechanism has been reported for insulin, which increases binding of CaM to rat adipocyte plasma membranes (10). Recently, a mechanism of action of thyroid hormone has been proposed by Davis et al. The authors have presented evidence for the "non-specific" stimulation of rabbit myocardial Ca^{++}-dependent ATPase by thyroid hormones and non-iodothyronine homologues in vitro (11). The physiological relevance of the effect of thyroid hormones on erythrocyte Ca^{++}-ATPase remains to be elucidated.

REFERENCES

1. Davis PJ and Blas SD. Biochem Biophys Res Comm 99: 1073, 1981.
2. Davis FB, Kite JH, Davis PJ, et al. Endocrinology 110: 297, 1982.
3. Davis FB, Cody V, Davis PJ, et al. J Biol Chem 258: 12373, 1983.
4. Davis FB, Davis PJ, and Blas SD. J Clin Invest 71: 579, 1983.
5. Ronner P, Gazzotti P, and Carafoli E. Arch Biochem Biophys 179: 578, 1977.
6. Strittmater WJ, Hirata F, and Axelrod J. Biochem Biophys Res Comm 88: 147, 1979.
7. Hillier AP. J Physiol 211: 585, 1970.
8. Galo MG, Bloj B, and Farias RN. J Biol Chem 250: 6204, 1975.
9. Galo MG, Unates LE, and Farias RN. J Biol Chem 256: 7113, 1981.
10. Goewert RR, Klave NB, and McDonald JM. J Biol Chem 258: 9995, 1983.
11. Davis FB, Mylotte KM, Davis PJ, et al. Proc. 60th Meeting of the American Thyroid Association. Endocrinology 115: T-18, 1984.

INDUCTION OF METAMORPHOSIS AND THYROID FUNCTION IN THE LARVAL LAMPREY

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A larval lamprey undergoes metamorphosis into the adult form. This metamorphosis is remarkable and is accompanied by external changes in oral apertures, branchiopores, eyes, and other parts of the body. Simultaneously, thyroid follicles are formed from the endostyle located underneath the pharyngeal region. Up to the present, it has not been clear which hormonal or neuronal factors are involved in this metamorphosis. Recently, we observed that potassium perchlorate ($KClO_4$) induces metamorphosis completely within three months. In this paper, we report the results of those investigations and the plasma thyroxine (T_4) level in the larvae during and after induction of metamorphosis to clarify whether or not thyroid function is involved in metamorphosis.

MATERIALS AND METHODS

The larval lampreys, Lampetra reissneri, were collected from streams and were reared with sand at 22 ± 2°C in well water. These larvae were treated with 0.05% and 0.01% $KClO_4$, respectively. Other larvae were reared as a control under the same conditions. After the experiment, the animals were anesthetized with MS222 and blood samples were collected into heparinized capillaries. Plasma T_4 was measured by an appropriate radioimmunoassay (RIA) method, as previously reported (1,2). For histological observation, these larvae were fixed in Bouin's solution and their tissues were processed by the routine method for making serial paraffin section (5 μm). The slides were stained with hematoxylin and eosin. Metamorphic stages were determined by the criteria of Youson and Potter (3).

RESULTS

Induction of Metamorphosis

In some larvae treated with $KClO_4$, a slight sign of metamorphosis in oral apertures, branchiopores, and nostrils was observed after about four weeks. Some of these larvae finished metamorphosis within three months and stuck to the wall of the aquarium. Metamorphic stages in the larval lampreys treated with 0.05% $KClO_4$ for 3 months are shown in Table 1. Complete metamorphosis (stage 7) was induced in 6 of 14 larvae. In three metamorphosed animals, an indent was observed in the pharyngeal region. In five

Table 1. Metamorphic Stage and Plasma T_4 Level in the Larval Lamprey After Treatment with 0.05% Potassium Perchlorate for Three Months

Animal no.	Sex	Total length (cm)	Metamorphic stage	Plasma T_4 (ng/ml)
1	F[a]	17.3	Stage 7[b]	0.6
2	F	15.1	Stage 7	ND[c]
3	F	14.5	Stage 7	0.6
4		13.6	Stage 7	ND
5		13.1	Stage 7	0.2
6	F	12.5	Stage 7	0.4
7		14.9	Stage 7, abnormal pharynx	ND
8		14.7	Stage 7, abnormal pharynx	ND
9		12.8	Stage 7, abnormal pharynx	ND
10	F	14.1	Stage 7, eyes (stage 3)	ND
11		12.4	Stage 7, eyes (stage 4)	ND
12		12.5	Oral apertures (stage 5), branchiopores (< stage 2), eyes (stage 1)	ND
13		12.5	Oral apertures (stage 3), branchiopores (< stage 2), eyes (stage 1)	1.7
14		11.7	Oral apertures (stage 4), branchiopores (< stage 2), eyes (stage 1)	1.4

[a]F, female; [b]Stage 7, completely metamorphosed lamprey; [c]ND, not detectable, less than 0.2 ng/ml.

animals, partial metamorphosis was induced; oral apertures, branchiopores, and eyes where at different metamorphic states and the dorsal fins were slightly enlarged. Even after prolonged treatment, these animals were at different stages, failed to complete metamorphosis, and became malformed. Such incomplete metamorphosis was induced in the smaller larvae. Similar results were also obtained by treatment with 0.01% $KClO_4$. It was shown that the completely metamorphosed animals treated with $KClO_4$ (total length, more than 12.5 cm) were smaller than the spontaneously metamorphosed ones (total length, more than 14.1 cm).

Plasma T_4 Levels in the Larval Lampreys Treated with $KClO_4$

Plasma T_4 values in the larval lampreys treated with 0.05% $KClO_4$ for 3 months were very low (<0.2 to 1.7 ng/ml), as shown in Table 1. However, in the larvae treated with 0.01% $KClO_4$ for 3 months, the plasma T_4 values fluctuated considerably and were in the range 8.5 to 60.2 ng/ml. There was no significant difference among the plasma T_4 values in completely or incompletely metamorphosed animals. The plasma T_4 values in these animals were higher than those in the untreated larvae. Moreover, large larvae with a total length of 14.4 - 16.3 cm were treated with 0.01% $KClO_4$ for only 3 weeks. In these large larvae, no external changes were observed and the plasma T_4 values were at an undetectable level (<0.2 ng/ml).

Histological Observation

In completely metamorphosed animals (stage 7) treated with $KClO_4$, thyroid follicles were formed from endostyle, as seen in spontaneous metamorphosis. In some animals, abnormality in the vascular system was observed near the thyroid region. In incompletely metamorphosed animals, marked changes in the endostyle were observed; cell type I of the endostylar cells degenerated and a few thyroid follicles were formed as formation of the vascular system progressed. Such changes in the endostyle were similar to those found at the early stage of spontaneous metamorphosis. Treatment with $KClO_4$ hastened the growth of eggs (oocytes). The diameter of the eggs in completely metamorphosed animals was almost the same as in spontaneously metamorphosed animals.

DISCUSSION

It has been reported that the endostyle of the larval lamprey is a forerunner of the thyroid and it synthesizes thyroglobulin and secretes thyroid hormones (4-6). Plasma thyroid hormones increase gradually with the growth of the larvae and reach the highest level in the largest larvae just before metamorphosis. During metamorphosis, the hormone levels drop and remain at the lowest level of adult lamprey. Such a change in plasma thyroid hormones from the larval to metamorphic stages is similar to that in amphibian metamorphosis. However, it has been shown that thyroid hormones do not have any effect on lamprey metamorphosis (7-11). Thus, it seems that the involvement of thyroid hormones in lamprey metamorphosis is different from that in amphibian metamorphosis. Hoheisel and Sterba (12) first found that $KClO_4$ induces some external signs of metamorphosis after 8-12 months in the larval lamprey. However, they could not get completely metamorphosed animals. The results of the present study indicated that complete metamorphosis is induced by treatment with $KClO_4$ within 3 months only in the larger larvae. In the smaller larvae treated, the external structures of the body in part were at different metamorphic stages. $KClO_4$ has been shown to induce goiters in mammals (13,14) and in lower vertebrates (15,16). In the present study, we showed that plasma T_4 values are extremely low 3 weeks after treatment with $KClO_4$, before the treatment results in external signs of metamorphosis. It is probable that a lowering of plasma T_4 has a close relationship to the initiation of lamprey metamorphosis, being mediated by the pituitary gland or neuronal factors such as the pineal gland (17,18). It was reported that goitrogens such as thiourea, thiouracil, and sodium thiocyanate induce marked changes in the endostyle of the larval lamprey rll,19,20). The results of these experiments should be reconsidered in view of the involvement of thyroid function in lamprey metamorphosis.

REFERENCES

1. Larsen PR, Dockalova J, Sipula D, et al. J Clin Endocrinol Metab 37: 177, 1973.
2. Suzuki S and Suzuki M. Gen Comp Endocrinol 45: 74, 1981.
3. Youson JH and Potter IC. Can J Zool 57: 1808, 1979.
4. Suzuki S and Kondo Y. Gen Comp Endocrinol 21: 451, 1973.
5. Wright GM and Youson JH. J Exp Zool 89: 391, 1977.
6. Suzuki S. Develop Growth and Differ 24: 416, 1982.
7. Marine D. J Exp Med 17: 379, 1913.
8. Remy P. Compt Rend Soc Biol 86: 129, 1922.
9. Horton FM. J Exp Biol 11: 257, 1934.
10. Stokes M. Proc Soc Exp Biol Med 42: 810, 1939.
11. Klenner JJ and Schipper AL. Anat Rec 120: 790, 1954.
12. Hoheisel G and Sterba G. Z Mik Anat Forsch 70: 490, 1963.
13. Julin T, Morreale de Escobar G, and Escobar del Rey F. Endocrinology 83: 620, 1968.
14. Ortiz-Caro J, Pastor RM, and Julin T. Acta Endocrinologica 103: 81, 1983.
15. Pflugfelder O. Roux' Archiv fur Entwicklungsmechanik 151: 78, 1959.
16. Pflugfelder O. Roux' Archiv fur Entwicklungsmechanik 153: 236, 1961.
17. Eddy JMP. J Endocr 44: 451, 1969.
18. Cole WC and Youson JH. J Exp Zool 218: 397, 1981.
19. Jones RP. Nature 160: 638, 1947.
20. Barrington EJW and Sage M. Gen Comp Endocrinol 3: 153, 1963.

DIFFERENTIAL EFFECT OF HYPOTHYROIDISM ON THE DENDRITIC DENSITY OF PYRAMIDAL NEURONS OF THE CEREBRAL CORTEX*

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It is known that, given the extreme complexity of the central nervous system, the effect that some pathological conditions have on its development is far from being evident by a simple inspection of a tissue preparation at the microscope.

To look through the microscope at a section of the cerebral cortex (C.C.), stained according to the rapid Golgi procedure (1), is, in some way, similar to looking at one screen where many pictures are projected at the same time. If we do not have the proper filter which allows us to see each picture separately, we could reach a paradox; because of the excess of information, we do not get the message contained on the screen. Mathematical models and some special mathematical algorithms are, in a sense, one type of "filter" which allows us to study the properties and structure of the C.C., and how this structure is affected by pathological or abnormal conditions (2).

Eayrs (3) was one of the first to apply quantitative techniques to study the effect that hypothyroidism has on the development of the C.C. and, although his results were not conclusive, he mentioned that this pathological condition affects the density of the dendritic arborization of the neurons.

In 1970, Ruiz-Marcos and Valverde (4) developed a special matricial algorithm which makes it possible to find the mean dendritic density distribution of a group of neurons with respect to their soma, and to compare the distributions of two homogeneous groups of neurons, one belonging to control animals and the other to animals affected by a certain pathological condition. Using this method, one can determine how a pathological condition affects the development of the dendritic arborization of the group of neurons studied.

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This algorithm, which was later perfected (5), has been used in the present work to study the effect that neonatal hypothyroidism has on the morphological development of the pyramidal neurons of the C.C. in order to elucidate whether this pathological condition affects the whole dendritic field of the neurons homogeneously, or if it affects some parts of their morphology with preference to others.

MATERIAL AND METHODS

A total of 20 pyramidal neurons, stained according to the rapid Golgi procedure (1) and chosen at random from layer III of the visual area of the C.C. of six control (C) rats 80 days old, and an equal number of neurons from a group of five rats of the same age which were surgically thyroidectomized ($\bar{T}$) when they were 10 days old, were drawn using a camera lucida. The three spatial coordinates (X,Y,Z) of the more important points of the dendritic aborization of the neurons were transferred and stored in the permanent magnetic disk memory of a PDP 11/40 computer by means of a special program. Following the instructions of another program, the computer solves, as has been described in detail elsewhere (5), the already mentioned matricial algorithm, finding the mean dendritic density distribution corresponding to the two groups (C and $\bar{T}$) of neurons studied, storing them in the form of numerical matrices (series of numbers arranged in rows and columns), named ACRONS (from Averaged Computed neuRONS) (4), in its permanent memory for further analysis. The overall difference between these two matrices was assessed by means of a two-way analysis of variance to the logarithmic transformed original data. This transformation was necessary in order to obtain the homogeneity of the variances which were tested using the Bartlett test (6). The computer further found the differences ($C-\bar{T}$) between each pair of homologous elements (those with the same position inside the matrices) of the two previously calculated ACRONS by dividing each of these differences by its corresponding C value, in order to obtain the relative decrease (($C-\bar{T}$) positive) or increase of the mean dendritic density on each place of the dendritic field with respect to the C value on this same place. The results obtained from these computations are given in the form of an alphanumerical pattern (Fig. 1), where it is indicated with the letter E (from "Empty") those positions on the dendritic field where the machine has found dendrites of C neurons and not of $\bar{T}$ neurons, with the letter C where it has found the opposite situation, indicating with numbers ranging from 9 to -9 the percentage of increment in the mean dendritic density found in each place of the dendritic field (number 9 indicating 90% of C value). On this and the next graph, the letter X represents the position of the soma of the pyramidal neurons studied, with their apical shafts going upwards, perpendicular to the pia surface which is located parallel and just below the upper IIIII line. As the unitary square chosen in this particular study to calculate the dendritic densities was 40 μm, the actual distance between two consecutive symbols is 40 μm.

Finally, the whole series of differences obtained were studied by means of a one-way analysis of variance, followed by a Duncan test (7), in order to detect the site, inside the dendritic field, where the maximum difference occurs and those places where the differences encountered do not differ from the maximum by more than the 0.05 level. As a result of this procedure, the machine prints a graphical pattern (Fig. 2), on which there appears a "+" sign on those places where these differences ($C-\bar{T}$) are positive (density decrease), a "-" sign where these differences are negative, and nothing where the value of the differences differ from the maximum beyond the 0.05 level of significance.

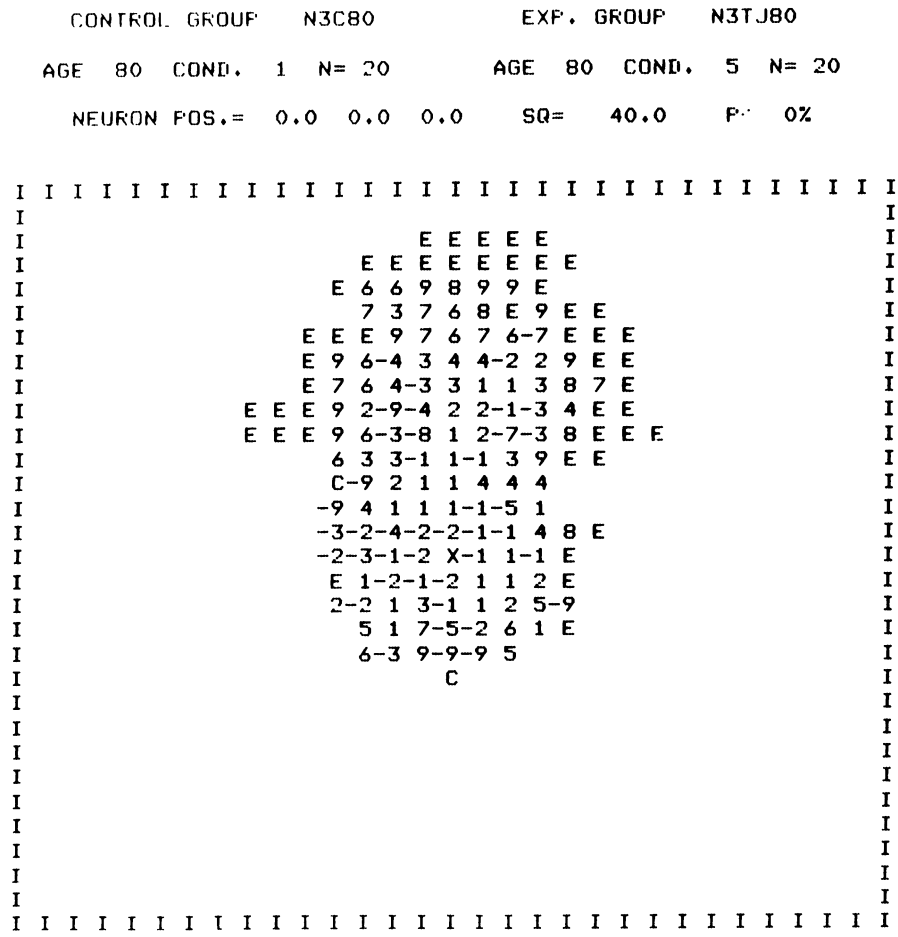

Fig. 1. Percentage of differences with respect to control. Alphanumerical pattern produced by the computer as part of the comparison made between the mean dendritic density distributions of the two groups of pyramidal neurons of control and hypothyroid animals studied at 80 days old. For meaning of symbols, see Material and Methods.

RESULTS

The value of F obtained as the result of overall comparison of the two numerical matrices, representing the mean dendritic density distributions of the group of C versus $\bar{T}$ neurons, was 18.15 with 1 and 173 degrees of freedom, meaning that the difference between these two distributions is highly significant.

The results obtained from the detailed comparison between these two distributions are summarized on Figs. 1 and 2. (For full explanation of the symbols of these graphics, see the Material and Methods section).

Fig. 1 shows the extension of the whole dendritic field of the group of C and $\bar{T}$ neurons studied. The position of the letter E on this graph indicates that $\bar{T}$ has more deeply affected the development of the apical shafts of these neurons than their basal dendrites. This conclusion is reinforced

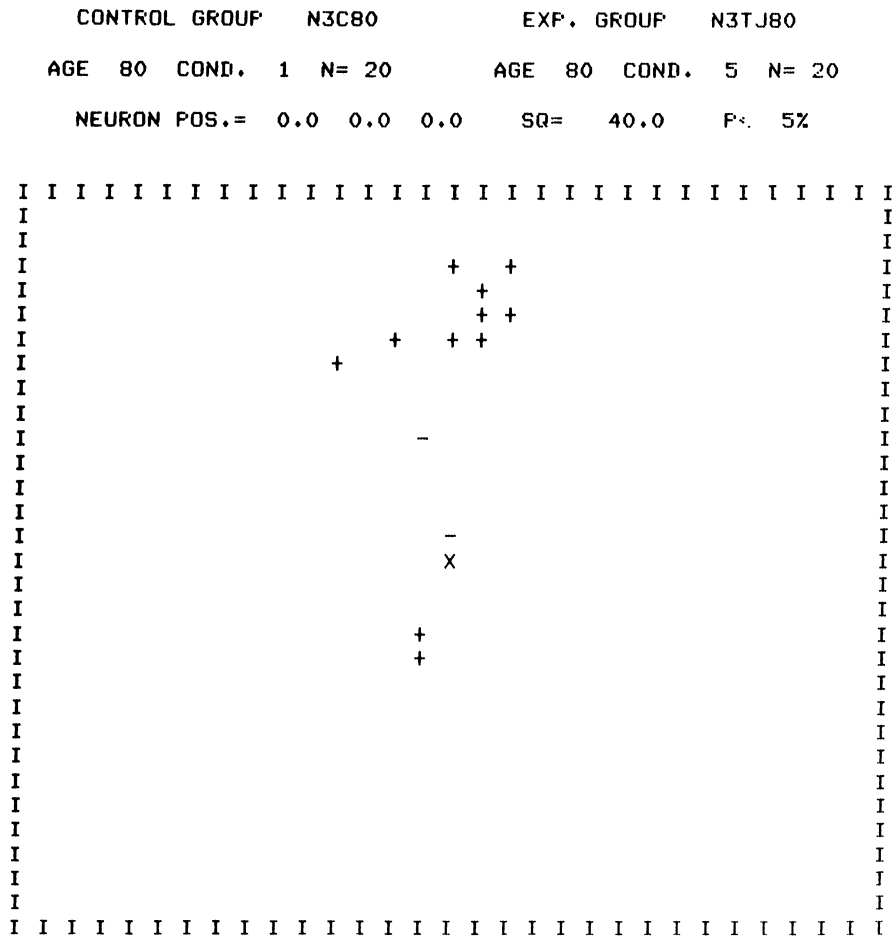

Fig. 2. Comparative acron. Graphical pattern produced by the computer as a result of the comparison made between the mean dendritic density distributions of the same groups of neurons mentioned on Fig. 1. For meaning of symbols, see Material and Methods.

by the fact that the highest numbers are localized on the upper part of the graph, meaning that the stronger relative reduction of the mean dendritic density with respect to the original control value is localized on the upper part of the apical shafts of the group of pyramidal neurons studied, reaching 80 to 90% (numbers 8 and 9) on the more distal part of them.

The observation of this graph led us to believe that $\bar{T}$ does not affect very deeply the development of the basal arborization of the pyramidal neurons and, surprisingly, on some parts of this field, an increase (negative numbers) has been observed in the mean dendritic density of the neurons of hypothyroid animals when compared to those of control animals.

Furthermore, the position of signs "+" on Fig. 2, representing the places on the dendritic field where the differences between C and $\bar{T}$ are higher, are again an indication that the influence of hypothyroidism on the development of the structure of the dendritic tree arborization of the pyramidal neurons is stronger on the more distal part of the apical shafts of these neurons.

DISCUSSION

Using a mathematical model (2) which coherently describes the properties of the distribution of spines along the dendrites of cortical neurons, it has been found (8) that $\overline{T}$ performed on rats at 10 days of age has a selective effect on the development of these spines, counted along the apical shafts of pyramidal neurons, affecting more deeply this development on the superficial layers of the C.C. It has also been reported recently (9) that $\overline{T}$ also more deeply affects the development of myelinated profiles on the superficial layers of the C.C. than on the rest.

The results obtained in the present study are in agreement with all previous results mentioned above and indicate that $\overline{T}$ also affects the morphology of the dendritic arborization of the pyramidal neurons on its more superficial region inside the C.C.

Previous results obtained from the study of the effect that $\overline{T}$ has on the microtubules inside the apical shafts of pyramidal neurons (10) seemed to indicate that the more profound effect, reported here, of $\overline{T}$ on the development of the more distal parts of these apical shafts could be due, at least in part, to damage of the general metabolism of the neuron which provokes a deficit of transport to the more distal parts of its morphology. If that would be the case, however, a more profound derangement of the distal parts of the basal dendrites of the pyramidal neurons should have been observed. In this respect, the results obtained seem to indicate that the more profound effect of hypothyroidism on the more distal part of the apical shaft arborization of the pyramidal neurons is due to an overall derangement on the most superficial layers of the C.C.

Still, the question arises whether this general derangement, produced by neonatal hypothyroidism on the more superficial layers of the C.C., is because these layers are the first to mature (11), or if it is also due to preference of this pathological condition for this region of the brain. In order to elucidate this last question, it would be necessary to study the effect that hypothyroidism, induced at an adult age, has on the different parts of the morphology of the neuron, a type of study that could be done with application of the mathematical techniques used for the present and previous studies.

SUMMARY

In order to study the influence that neonatal hypothyroidism has on the development of the density of the dendritic arborization of the pyramidal cells of the cerebral cortex (C.C.), a total of 20 neurons belonging to control rats at 80 days old, and an equal number of neurons belonging to animals of the same age which were surgically thyroidectomized ($\overline{T}$) when they were 10 days old, were studied using the matricial algorithm developed by Ruiz-Marcos and Valverde (4), and other statistical algorithms developed for this study. The results obtained show that $\overline{T}$ significantly affects the development of the more distal upper part of the dendritic field of the neurons studied, while affecting very little the development of the dendritic arborization of the basal dendrites of these neurons.

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REFERENCES

1. Cajal SR and De Castro F. Elementos de Tecnicas Micrograficas, Edit. Salvat., Barcelona, Spain, 1972, p 65.
2. Ruiz-Marcos A and Valverde F. Exp Brain Res 8: 284, 1969.
3. Eayrs JT. In The Scientific Basis of Medicine. Ann Rev 317, 1966.
4. Ruiz-Marcos A and Valverde F. Brain Res 19: 25, 1970.
5. Ruiz-Marcos A. In Proceedings of the Intern. Symp. Workshop on Iodine Nutrition, Thyroxine and Brain Development, New Delhi, India, 1985, in press.
6. Dixon WJ and Massey FJ. Introduction to Statistical Analysis, McGraw-Hill Co., New York, 1969, p 308.
7. Snedecor GW. Statistical Methods, Iowa Univ. Press, Iowa USA, 1967, p 244.
8. Ruiz-Marcos A, Sanchez-Toscano F, Obregon MJ, et al. Brain Res 239: 559, 1982.
9. Berbel P, Escobar del Rey F, Morreale de Escobar G, et al. Trabajos del Instituto Cajal, Vol. LXXV, 1984, abstr. 37.
10. Berbel P, Escobar del Rey F, Morreale de Escobar G, et al. Neurosciences Lett Suppl 14: S-26, 1983.
11. Rakic P. In FO Schmitt, FG Worden, G Adelman, et al. (eds), The Organization of the Cerebral Cortex, The MIT Press, Cambridge, MA, 1981, p 15.

PROSTACYCLIN (PGI_2) PRODUCTION IN HYPERTHYROID RATS AND PATIENTS WITH GRAVES' DISEASE

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Prostaglandins can stimulate thyroid hormone secretion from the thyroid gland (1). Conversely, T_3 can stimulate prostacyclin synthesis by cultured vascular smooth muscle cells (2). These findings seem very interesting for understanding the mechanism of the anti-atherosclerotic action of thyroid hormone. Therefore, we have examined the effect of thyroid hormone administration *in vivo* on prostacyclin synthesis in rats, and the mechanisms of thyroid hormone action were also investigated.

MATERIALS AND METHODS

Male Wistar rats (weighing about 250 g) were treated with T_4 (200 μg/100 g body wt. ip every 24 h for either 3, 7, or 14 days; groups 1, 2, and 3, respectively) or methimazole (0.01% in drinking water for 14 days; group 7). For controls, rats were given corresponding amounts of saline ip every 24 h for 3, 7, and 14 days (groups 4, 5, and 6, respectively). Serum T_3 and T_4 levels were determined by RIA. Plasma PGI_2 levels and PGI_2 release by aortic rings incubated *in vitro*, were measured at 6-keto-$PGF_{1\alpha}$ by RIA after extraction and separation by silicic acid column chromatography as reported previously (3). Phospholipase A_2 activity and lipid analysis of rat aorta were carried out by established methods (4,5). Plasma 6-keto-$PGF_{1\alpha}$ levels in hyperthyroid patients with Graves' disease were measured before and after treatment with antithyroid drugs. All data are shown as mean ± SEM.

RESULTS AND DISCUSSION

Serum T_4 levels in hyperthyroid rats (12.4 ± 0.9, 8.3 ± 0.9, and 9.2 ± 1.2 μg/dl for groups 1, 2, and 3, respectively) were significantly higher than those in control rats (4.5 ± 0.3, 4.2 ± 0.2, and 4.9 ± 0.5 μg/dl for groups 4, 5, and 6, respectively). The methimazole-treated group showed a significantly decreased serum T_4 level (0.9 ± 0.1 μg/dl). Serum T_3 levels in all T_4-treated groups were also significantly higher and in the methimazole-treated group significantly lower than in controls.

Plasma 6-keto-$PGF_{1\alpha}$ levels in groups 1, 2, and 3 were significantly higher than those in corresponding controls, as shown in Table 1. However, plasma 6-keto-$PGF_{1\alpha}$ levels in group 7 were not different from that

Table 1. Effects of Thyroxine and Methimazole Treatments on Plasma 6-keto-$PGF_{1\alpha}$ and on *In Vitro* Production of 6-keto-$PGF_{1\alpha}$ by Aortic Rings

Groups	Treatment	Plasma 6-keto-$PGF_{1\alpha}$ (pg/ml)	6-keto-$PGF_{1\alpha}$ produced *in vitro* (ng/mg aortic ring/15 min)
1	T_4, 3 days	129.8 ± 14.6**	20.5 ± 1.0
2	7 days	137.5 ± 17.3**	30.4 ± 2.0***
3	14 days	114.6 ± 14.9*	34.2 ± 2.0***
4	Saline, 3 days	65.9 ± 11.2	20.7 ± 1.0
5	7 days	53.3 ± 10.2	18.0 ± 2.2
6	14 days	58.2 ± 6.8	20.4 ± 1.2
7	Methimazole, 14 days	60.3 ± 7.1	14.8 ± 0.7***

Aortic rings obtained from abdominal aorta were incubated in 0.5 ml Krebs-Ringer bicarbonate buffer (pH 7.4), and 50 mg % glucose for 15 min at 37°C. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs their corresponding controls.

in controls despite their decreased serum thyroid hormone levels. Since it is difficult to conclude that the enhanced PGI_2 production by vascular tissues in T_4-treated rats resulted in the increased plasma 6-keto-$PGF_{1\alpha}$ levels, we determined the PGI_2 production by aorta *in vitro* directly.

The *in vitro* production of PGI_2 by aortic rings obtained from normal rats is shown in Fig. 1. The addition of arachidonic acid (20 μg/ml) into the incubation medium caused a significant increment in PGI_2 production, and production was significantly inhibited by the addition of 1×10^{-5} M indomethacin. Direct addition of T_4 into the incubation medium did not cause any significant changes in PGI_2 production (data not shown). Under these experimental conditions, *in vitro* production of PGI_2 by aortic rings obtained from experimental rats was determined as shown in Table 1. The PGI_2 production by aortic rings in groups 2 and 3 was significantly higher

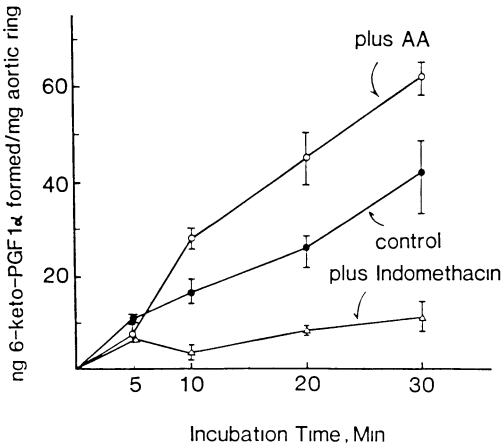

Fig. 1. Effects of A.A. and indomethacin on PGI_2 production *in vitro* by aortic rings obtained from normal rats.

Table 2. Effects of Thyroxine and Methimazole Treatments on Phospholipid Content and Composition of Rat Aorta

	Phospholipid μg/100 mg wet wt.	% of total PL			
		PC	PE	PI-PS	SM
Normal	497 ± 52	37.6 ± 3.3	28.7 ± 3.9	16.5 ± 3.1	11.3 ± 2.6
T_4 (7 days)	578 ± 49	36.5 ± 0.1	32.2 ± 1.2	15.6 ± 2.2	10.2 ± 3.3
T_4 (14 days)	515 ± 25	38.2 ± 1.3	31.0 ± 1.6	16.4 ± 5.9	8.7 ± 4.3
Methimazole	546 ± 81	37.0 ± 0.1	32.3 ± 1.8	17.0 ± 5.9	10.4 ± 4.0

than in corresponding controls, but no significant difference in PGI_2 production was observed between groups 1 and 4. In contrast, methimazole treatment caused a significant suppression.

These results show the stimulatory effect of thyroid hormone on PGI_2 production by vascular tissues, and also that T_4 treatment for more than three days was necessary to stimulate PGI_2 synthesis by vascular tissues.

The rate-limiting step in prostaglandin synthesis is believed to be the release of arachidonic acid from membrane phospholipid. We determined the effect of thyroid hormone on phospholipid content, fatty acid composition of phospholipid, and phospholipase A_2 activity in aortas (Table 2). Phospholipid content of aorta in T_4- and methimazole-treated groups was not different from those of controls. Also, fatty acid composition of phospholipids in the aorta was not different between controls and either T_4- or methimazole-treated rats (data not shown). Furthermore, no significant differences in phospholipase A_2 activity were found between controls and either T_4- or methimazole-treated rats (Table 3).

These results suggest that thyroid hormone may act on (stimulate) some steps after the release of arachidonic acid from phospholipid, though it is not clear whether T_3 and/or T_4 can only stimulate PGI_2 production from these experiments.

Table 3. Phospholipase A_2 Activities in Aortic Homogenates Obtained from Control, T_4-treated, and Methimazole-treated Rats

	p moles AA released/mg protein/2 hr
Control groups 4, 5, 6	55.9 ± 7.6
T_4-treated rats	
group 1	41.6 ± 2.3
group 2	62.8 ± 6.8
group 3	55.1 ± 9.2
Methimazole-treated rats	
group 7	57.5 ± 6.8

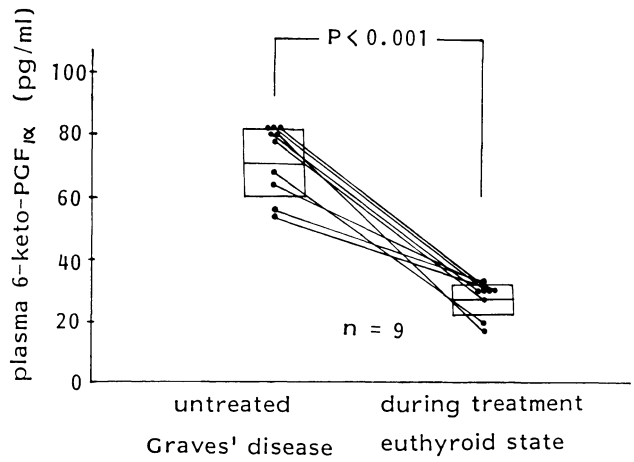

Fig. 2. Plasma-6-keto-PGF$_{1\alpha}$ in patients with Graves' disease before and after the treatment with antithyroid drugs.

Since it is interesting to examine these effects of thyroid hormones in humans, we measured plasma 6-keto-PGF$_{1\alpha}$ levels in hyperthyroid patients with Graves' disease before and after treatment with antithyroid drugs. As shown in Fig. 2, plasma 6-keto-PGF$_{1\alpha}$ levels in patients with Graves' disease decreased significantly after treatment.

Finally, although further studies have to be done to clarify the mechanisms, the present data further confirmed the stimulatory effects of thyroid hormone on PGI_2 synthesis by vascular tissues in vivo.

SUMMARY

The effects of thyroid hormone administration in vivo on PGI_2 production by vascular tissues were investigated in rats, and plasma PGI_2 levels in hyperthyroid patients with Graves' disease were determined. PGI_2 was measured as 6-keto-PGF$_{1\alpha}$ by RIA. Plasma PGI_2 levels in T_4-treated rats were significantly higher than those of control rats. Aortic rings obtained from rats given T_4 for 7 and 14 days showed significantly increased release of PGI_2 into the incubation medium. In contrast, rats given methimazole for 14 days showed a significant decrease in PGI_2 production by aortic rings.

No significant changes were observed in phospholipid content, fatty acid composition of phospholipids, or phospholipase A_2 activity between controls and experimental rat aortas.

These results suggest a stimulatory effect of thyroid hormone on PGI_2 synthesis in vivo, though the mechanism of thyroid hormone action is not yet clear.

Furthermore, the stimulatory effect was confirmed by finding plasma 6-keto-PGF$_{1\alpha}$ levels significantly decreased in patients with Graves' disease after treatment with antithyroid drugs.

REFERENCES

1. Onaya T and Solomon DH. Endocrinology 86: 423, 1970.
2. Nakao J, Chang WC, Murota S, et al. Atherosclerosis 39: 439, 1981.
3. Noguchi T, Kazama Y, Kanemaru Y, et al. Prostaglandins (in press).
4. Folch J, Lees M, and Sloane-Stanley GH. J Biol Chem 226: 497, 1957.
5. Eisenberg S, Stein Y, and Stein O. Biochim Biophys Acta 164: 205, 1968.

REGULATION OF RAT LIVER GLYCEROPHOSPHOLIPID FATTY ACID COMPOSITION IN HYPO- AND HYPERTHYROIDISM*

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INTRODUCTION

The fatty acid composition of microsomal phospholipids isolated from rat liver has been shown to be altered in hyperthyroidism and hypothyroidism (1-4). In hypothyroidism, the proportions of linoleic (C18:2ω6), eicosatrienoic (C20:3ω6), eicosapentaenoic (C20:5ω3), and docosahexaenoic (C22:6ω3) acids were increased, while the proportion of arachidonic (C20:4ω6) acid was decreased (2). In contrast, chronic hyperthyroidism increased the proportions of stearic (C18:0) acid and C20:4ω6 and concomitantly decreased the proportions of palmitic (C16:0) acid, palmitoleic (C16:1ω9) acid, C18:2ω6 and C20:3ω6 (1). The increased C18:2ω6 and decreased C20:4ω6 in hypothyroidism may be due to diminished fatty acid desaturation (Δ6 desaturase). However, in hyperthyroidism, changes in C18:2ω6 and C20:4ω6 are reversed without evidence of increased Δ6 desaturase activity. Furthermore, the other changes in fatty acid composition cannot be readily explained by the observed alterations in fatty acid desaturation and in many cases are opposite to that predicted by the altered desaturation (1,2).

Therefore, we have studied the effect of hypo- and hyperthyroidism on several enzymes involved in phospholipid synthesis and degradation, alterations of which might result in changes in phospholipid fatty acid composition. The enzymes studied and reported here are: acyl-CoA: glycerol 3-phosphate acyltransferase (GPAT), acyl-CoA: 1-acylglycerol 3-phosphorylcholine acyltransferase (GPCAT), phospholipase A_2, and lysophospholipase. The first enzyme (GPAT) is known to catalyze a rate-limiting step for the synthesis of phosphatidic acid (incorporating saturated and monounsaturated fatty acids) in rat liver microsomes and mitochondria (5,6). The GPCAT and phospholipase A_2 form an acylation-deacylation cycle that is known to play an important role in introducing polyunsaturated fatty acids into phosphoglycerides. Finally, lysophospholipase catalyzes the degradation of phosphoglycerides.

METHODS

The experiments were carried out using age-matched normal and thyroidectomized white male Sprague-Dawley rats. Hyperthyroidism was produced in

*A more complete version of this manuscript has been accepted for publication in the journal, Lipids.

normal rats by daily intraperitoneal injection of 25 μg of triiodothyronine (T_3) per 100 g body weight for 14 days. Hypothyroidism was verified in the thyroideceomized rats by declining growth rate and low serum thyroxine (T_4) and T_3 values. In the T_3-treated hypothyroid rats, 25 μg T_3 per 100 g body weight was injected intraperitoneally daily for 4 days. Mitochondria, microsomes and 100,000 g supernatants (cytosol) of the homogenized liver were isolated, protein determinations done, and samples stored at -70°C as described previously (7). Microsomal and mitochondrial GPAT, microsomal GPCAT, mitochondrial phospholipase A_2, and cytosol lysophospholipase assays were carried out as described previously (7). The results of replicate experiments were pooled for statistical analysis. Significant differences between groups were determined by using Student's t test.

RESULTS

Table 1 indicates that hyperthyroidism significantly decreased the activities of microsomal GPAT 34% and GPCAT 33%. Hyperthyroidism, however, did not affect mitochondrial GPAT, phospholipase A_2, or cytosol lysophospholipase activity. These data demonstrate that hyperthyroidism depresses activities of the phospholipid synthetic enzymes (GPAT and GPCAT) in microsomes but has no effect on activities of the phospholipid degradative enzymes (phospholipase A_2 and lysophospholipase).

Hypothyroidism had no effect on microsomal GPAT activity, but is significantly increased mitochondrial GPAT activity 38% and microsomal GPCAT activity 19%. Hypothyroidism also resulted in a 36% decrease in phospholipase A_2 activity and a 56% decrease in lysophospholipase activity. Thus, in contrast to the decreased enzyme activities of phospholipid synthesis seen in hyperthyroidism, hypothyroid rat liver increases phospholipid synthesis and simultaneously decreases enzyme activities of phospholipid degradation. T_3 treatment of hypothyroid animals corrected the activities of phospholipase A_2 and lysophospholipase to the level of the control rats. The increased mitochondrial GPAT activity was not corrected, and the microsomal GPAT (unaffected by hypothyroidism) and increased GPCAT activities were decreased to the level of the hyperthyroid rat.

DISCUSSION

The decreased activities of the microsomal acyltransferases GPAT and GPCAT is consistent with the reported decrease in microsomal phospholipid content in the hyperthyroid rat liver (4). Other factors such as the decrease in the availability of glycerol 3-phosphate (8) and diacylglycerol (9) and a greater proportion of diacylglycerol being diverted to the synthesis of triacylglycerol (10) could also contribute to diminished synthesis of liver microsomal phospholipids. The diminished liver GPCAT activity in hyperthyroidism is in contrast to that found in other tissues such as lung in which T_3 has been shown to stimulate activites of microsomal GPCAT and phospholipase A_2 (11).

The acyltransferases GPAT and GPCAT are known to play an important role in determining the fatty acid composition in phosphoglycerides, with the microsomal GPAT preferentially catalyzing the incorporation of saturated and monounsaturated fatty acids into phospholipids, and the microsomal GPCAT preferentially catalyzing the incorporation of polyunsaturated fatty acids into phospholipids. Therefore, the decrease in microsomal GPAT and GPCAT activities would contribute to the decreased proportions of certain unsaturated fatty acids (C16:1ω9, C18:2ω6, and C20:3ω6) found in microsomal phospholipids of hyperthyroid rat liver (1). However, neither these enzyme changes nor the diminished Δ^6 desaturase activity (1) can explain the increased C20:4ω6 levels seen.

Table 1. Effects of Hyperthyroidism and Hypothyroidism on Phospholipid Metabolism

Enzymes	Euthyroid (control)	Hyperthyroid	% Change	Hypothyroid	% Change	Treated Hypothyroid	% Change
GPAT (microsomal)	2.62 ± 0.12 (13)	*1.72 ± 0.04 (11)	-34	2.82 ± 0.14 (12)		*1.80 ± 0.09 (7)	-31
(mitochondrial)	0.29 ± 0.02 (6)	0.28 ± 0.02 (7)		*0.40 ± 0.03 (7)	+38	*0.42 ± 0.03 (7)	+45
GPCAT							
-spectrophoto-metric assays	48.8 ± 1.4 (15)	*32.8 ± 1.4 (11)	-33	*58.2 ± 2.4 (19)	+19	*34.6 ± 2.7 (6)	-29
-radioactive tracer assays	32.1 ± 2.1 (15)	*23.0 ± 2.5 (11)	-28	36.5 ± 1.5 (19)	+14	*21.9 ± 2.2 (7)	-32
Phospholipase A_2	0.46 ± 0.03 (15)	0.44 ± 0.04 (11)		*0.29 ± 0.02 (17)	-36	0.40 ± 0.03 (7)	
Lysophospholipase	2.56 ± 0.34 (9)	2.39 ± 0.31 (7)		*1.20 ± 0.04 (12)	-56	2.60 ± 0.19 (7)	

Numbers of rats studied in each group are shown in (). Enzyme activities are expressed as nmoles/mg protein/min, except for phospholipase A_2 which is expressed as nmoles/mg protein/hr. Values are shown as mean ± SEM. Significant differences between hyperthyroid rats, hypothyroid rats, or T_3-treated hypothyroid rats versus control rats are shown by: *$p<0.01$. Percent (%) increase (+) or decrease (-) in enzyme activities with respect to control rats are shown.

Our results also indicate that hypothyroidism decreases the enzyme activities of phospholipid degradation: mitochondrial phospholipase A_2 and cytosol lysophospholipase, and concomitantly increases enzyme activities of phospholipid synthesis: mitochondrial GPAT and microsomal GPCAT. The increase in GPCAT activity in hypothyroidism in contrast to the decreased activity in hyperthyroidism may explain the opposite effects of hyperthyroidism and hypothyroidism on the observed alterations in phospholipid fatty acid composition. Specifically, the increased GPCAT in hypothyroidism would increase proportions of the polyunsaturated fatty acids such as C18:2ω6, C20:3ω6, C20:5ω3, and C22:6ω3 in microsomal phospholipids of hypothyroid rats (2). Of course, the diminished Δ^6 desaturase activity in hypothyroidism would best account for the decreased C20:4ω6 and increased C18:2ω6 levels (2).

Phospholipid fatty acid composition in hypothyroidism and hyperthyroidism is undoubtedly multifactorial, being influenced by altered fatty acid desaturase activity, altered fatty acyl-CoA incorporation into and removal from phospholipids, and additional yet unstudied factors such as altered utilization of various fatty acids.

REFERENCES

1. Faas FH and Carter WJ. Biochem J 193: 845, 1981.
2. Faas FH and Carter WJ. Biochem J 207: 29, 1982.
3. Hoch FL, Subramanian C, Dhopeshwarker GA, et al. Lipids 16: 328, 1981.
4. Ruggiero FM, Landriscina C, Gnoni GV, et al. Lipids 19: 171, 1984.
5. Lloyd-Davies KA and Brindley DN. Biochem J 152: 39, 1975.
6. Sanchez M, Nicholls DG, and Brindley DN. Biochem J 132: 697, 1973.
7. Dang AQ, Faas FH, and Carter WJ. Lipids 19: 738, 1984.
8. Olubadewo J, Wilcox HG, and Heimberg M. J Biol Chem 259: 8857, 1984.
9. Glenny HP and Brindley DN. Biochem J 176: 777, 1978.
10. Young DL and Lynen F. J Biol Chem 244: 377, 1969.
11. Das DK. Arch Biochem Biophys 224: 1, 1983.

EFFECT OF THYROXINE REPLACEMENT THERAPY ON SERUM THYROTROPIN*

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INTRODUCTION

Several studies have shown that the replacement doses of oral thyroxine are associated with supranormal concentrations of serum T_4, although normal concentrations of triiodothyronine (T_3) (1-4) and the underlying mechanisms remain debatable. The present study confirms the high serum T_4 and normal T_3 with the conventional doses of oral T_4, but also identifies a greater prevalence of unmeasurable TSH concentration in these patients.

PATIENTS AND METHODS

The study group comprised 86 patients (72 women and 14 men), all treated for hypothyroidism or euthyroid goiter. The mean age in this group was 50 years (range, 16-70). At the time of the study, all patients were clinically euthyroid and had been taking various doses of thyroxine (Synthyroid, Flint Div., Baxter-Travenol, Deerfield, IL) as indicated in Table 1. Blood samples were obtained several hours after the ingestion of T_4 for measurement of serum T_4, T_3, TSH, free T_4, T_3U, and FTI.

RESULTS

Table 1 shows the results for patients on three different doses of T_4. The mean total T_4 and free T_4 (FT_4) were significantly higher than in the control group regardless of the administered dose of T_4. The concentration of T_4 between the three groups of patients was not significantly different. Similarly, FTI in the group of patients taking T_4 was significantly higher than in controls. However, serum T_3 and T_3U values in individual groups of patients were not significantly different as compared with the control group. Forty-one percent of the patients had T_4 above normal (11 μg/dl) (Fig. 1), while only 3.7% of the patients had an elevated T_3 (Fig. 2). Analysis of linear regression of serum T_4 and the dose of T_4 showed statistically non-significant coefficient correlation (r=0.151). FT_4 in patients was elevated in 63% of subjects; there was a significant correlation between T_4 and FT_4 in the patients taking the dose of 0.15 ($p<0.001$) and 0.2 mg T_4 daily ($p<0.02$). Serum TSH was determined in 56 T_4-treated patients (Table 2).

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Table 1. Thyroid Function Tests in Groups of Patients on Thyroxine Replacement

Dose (mg/day)	N	T_4 (μg/dl)	T_3U %	FTI	T_3 (ng/dl)	FT_4 (ng/ml)
0.1	20	10.9 ± 2.9*†	29.4 ± 4.5	10.0 ± 2.3†	158 ± 41 (15)**	2.4 ± 0.5 (8)†
0.15	44	11.4 ± 2.6†	31.1 ± 4.3	11.5 ± 2.8†	158 ± 34 (31)	3.0 ± 0.8 (31)†
0.2	16	11.5 ± 3.5†	33.3 ± 4.3¶	13.0 ± 4.3¶†	163 ± 37 (11)	2.6 ± 0.5 (13)†
Total	86	11.4 ± 2.9†	30.7 ± 4.3	11.4 ± 3.1†	157 ± 37 (75)	2.8 ± 0.7 (53)†
Control	246	7.1 ± 1.1	30.6 ± 2.9	7.2 ± 1.1	149 ± 24	1.9 ± 0.3 (144)

*Mean ± SD. **Not all patients had measurements of T_3 and FT_4; figures in ( ) indicate the numbers of such determinations. †Significantly ($p<0.001$) greater than control. ¶$p<0.05$ vs 0.1 mg group.

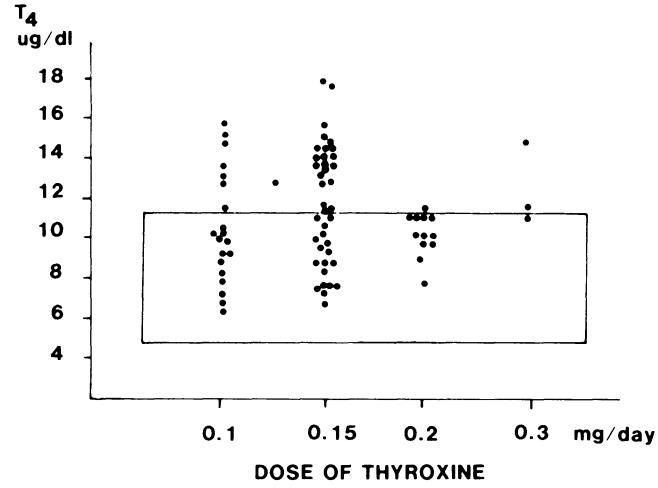

Fig. 1. Serum T_4 in 86 patients on thyroxine replacement. Box represents normal range for serum T_4 concentration.

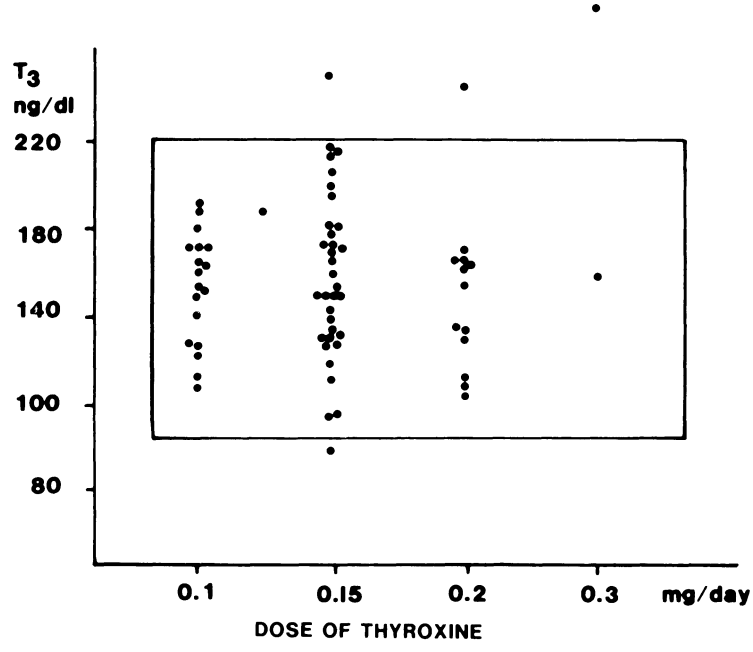

Fig. 2. Serum T_3 in 75 patients on thyroxine replacement. Box represents normal range for serum T_3 concentration.

In 77% of the patients, serum TSH was < 0.5 μU/ml, that is the lowest measurable concentration for our assay, compared to 22% of control subjects (Table 2). Of the patients on T_4 with serum TSH < 0.5 μU/ml, 33% had a normal T_4 and 20% had a normal FT_4 (Table 3); serum T_3 level in these patients was in the normal range. A similar prevalence of serum TSH < 0.5 μU/ml, was found in those patients taking 0.1, 0.15, and 0.2 mg of T_4 daily (71-77%); all patients on a T_4 dose more than 0.2 mg daily had an unmeasurable serum TSH.

Table 2. Serum TSH Concentration in Patients on Thyroxine Replacement

Group	N	TSH (μU/ml) <0.5	0.5-3.5	3.6-5.6
Thyroxine treated	56	43	13	0
Control	40	9 (22%)	29 (76%)	2 (5%)

DISCUSSION

In the present study, we have shown that both T_4 and FT_4 were above the normal range in many patients on T_4 replacement. Although the FT_4, metabolically active fraction of T_4, was in the hyperthyroid range in these patients, none had symptoms of hypermetabolism, similar to the findings in some previous studies (2). The mechanism for T_4 elevation in this group of patients remains unexplained, although it is very likely that a higher serum T_4 in the patients on replacement is required to replace the lack of thyroid production of T_3 (1,5). In our group of T_4-treated patients, 77% had undetectable serum TSH, in contrast to 22% of controls. Since our T_4-treated patients typically had a supranormal concentration of T_4 and/or FT_4, while that of T_3 was normal, the frequently suppressed TSH may indicate that pituitary secretion of TSH responds to the concentration of T_4 available for conversion to T_3 by pituitary gland, rather than the serum concentration of T_3, as previously published by others (6,7).

Table 3. Total and Free T_4 in Patients With Unmeasurable Serum TSH

	Normal	Elevated
T_4 (n=43)	33%	67%
FT_4 (n=33)	20%	80%

REFERENCES

1. Salmon D, Rendall M, Williams J, et al. Arch Int Med 143: 571, 1982.
2. Ingbar JC, Borges M, Iflah S, et al. J Endocrinol Invest 5: 77, 1982.
3. Deam DR, Cambell DG, and Ratnaike S. Med J Austral 2: 374, 1983.
4. Pearce CJ and Himsworth RL. Brit Med J 288: 693, 1984.
5. Stock JM, Surks MI, and Oppenheimer JH. N Engl J Med 290: 529, 1974.
6. Silva JE and Larsen PR. Science 198: 617, 1977.
7. Silva JE and Larsen PR. J Clin Invest 61: 1247, 1978.

THYROXINE-INDUCED MOLTING AND GONADAL FUNCTIONS OF LAYING HENS

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INTRODUCTION

In most avian species, molting and feather regeneration are related to breeding or seasonal changes. In addition to spontaneous molting, forced molting of laying domestic fowls is induced by a combination of food-water restriction and short photoperiod or excess dose of thyroxine (T_4), followed by a reduction in egg production rate. The forced molting technique is used to avoid replacing pullets every year, although during the molting the rate of egg production is nearly zero. It has been proposed that changes in various endocrine functions are involved in both spontaneous and forced molting (1-9).

This paper aims to clarify the mechanism of forced molting caused by excessive T_4 in relation to gonadal functions.

MATERIALS AND METHODS

Twenty white Leghorn hens of 238 days of age were reared in individual cages in a windowless house under artificial light (light on 06:00; light off 20:00 h). They were divided into 4 groups receiving daily injections, into the breast muscle, of 0, 20, 100, and 500 μg/kg of T_4 (L-thyroxine, Sigma Co.), dissolved in slightly alkaline saline solution, for 4 weeks. The control group (0 μg T_4 received the same volume of vehicle. Blood samples were obtained from the brachial vein before T_4 injection once a week. Serum levels of T_4 (10), T_3 (10), rT_3, LH (11), estradiol (E2) (12), and progesterone (12) were measured by appropriate RIA systems. Body weights were measured once a week and egg production was recorded daily. Statistical significance was evaluated by the one way analysis of variance (13).

RESULTS

Changes in Body Weight and Egg Production Rate after T_4 Injection

The mean body weights of the groups treated with 20 and 100 μg T_4 did not differ from that of the control group, whereas that of the 500 μg T_4

group decreased significantly, from 1788 ± 119 to 1494 ± 67 g (mean ± SEM) after one week. Also, the egg production rates of the 20 and 100 μg T_4 groups did not differ from that of the control group, but the rate of the 500 μg T_4 group decreased markedly after one week, and stopped completely thereafter. In addition, molting in the latter group was induced 10 days after T_4 injection.

Changes in Circulating Iodothyronine Levels After T_4 Injection

Serum T_4, T_3, and rT_3 levels of each group during the experiment are shown in Fig. 1(A). Serum T_4 levels rose as the T_4 dose increased. In the

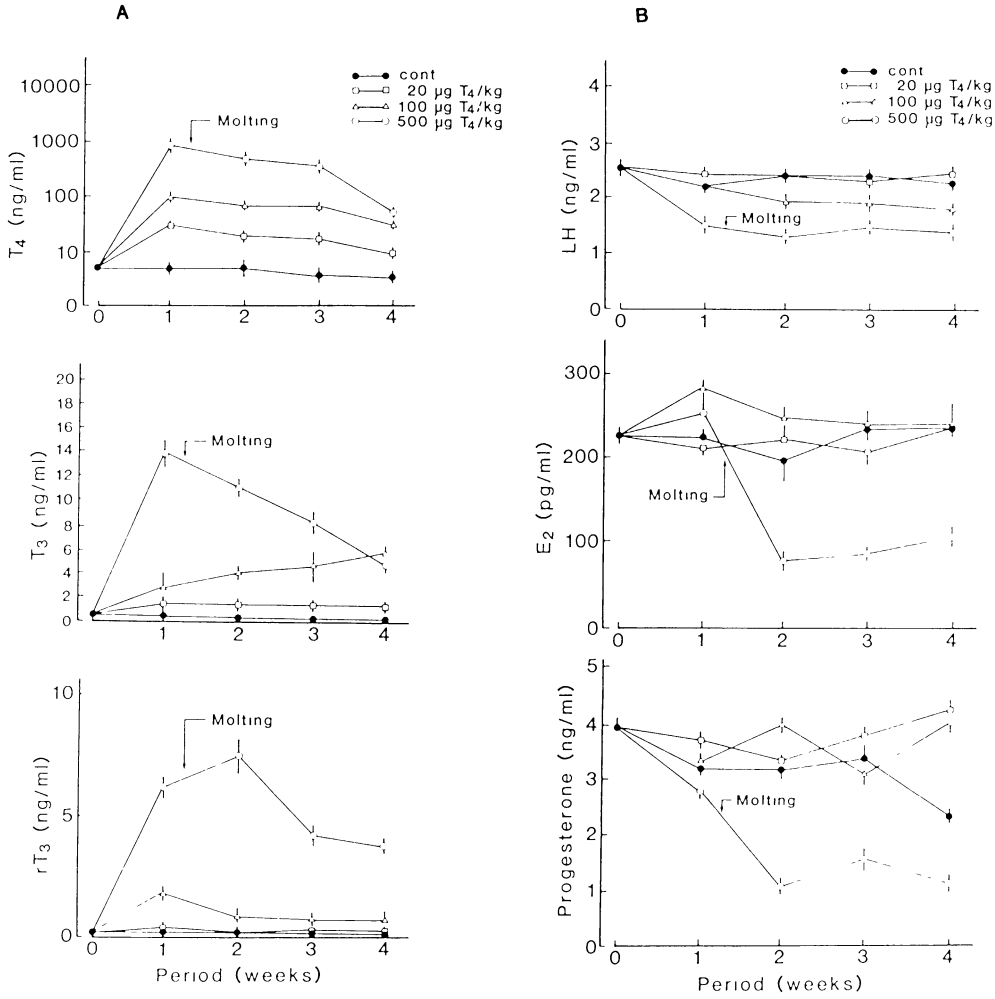

Fig. 1. Changes in circulating iodothyronine levels (A) and gonadal hormone levels (B) after graded doses of L-T_4 injection (0, 20, 100, and 500 μg/kg) for four weeks. Each point represents the mean ± SEM.

500 μg T_4 group, the serum T_4 increased markedly after one week and reached a peak of 931 ± 43 ng/ml, which was 150-fold as high as the control level, but was followed by a gradual decrease. T_3 and rT_3 levels showed similar changes. The peak values were 16.3 ± 1.2 ng T_3/ml after one week and 7.5 ± 0.9 ng rT_3/ml after two weeks. These values were respectively about 30-fold and 50-fold higher than those of controls.

Changes in Circulating Gonadal Hormone Levels after T_4 Injection

Serum LH, E_2, and progesterone levels of each group during the experiment are shown in Fig. 1(B). The LH level of the 20 μg T_4 group did not change; that of the 100 μg T_4 group decreased slightly after 2 weeks; and that of the 500 μg T_4 group decreased markedly, from 2.5 ± 0.1 to 1.5 ± 0.1 ng/ml after 1 week. The E_2 and progesterone levels of the 20 and 100 μg groups did not differ from those of control. There were no changes in the E_2 and progesterone levels of the 500 μg T_4 group after 1 week, but after 2 weeks those levels decreased markedly, from 226 ± 12 to 80 ± 9.4 pg/ml (E_2) and from 3.9 ± 0.2 to 1.1 ± 0.1 ng/ml (progesterone). A significant decrease in LH level after 1 week preceded a decrease in egg production rate, which was evident after 2 weeks.

DISCUSSION

The present study was undertaken to observe how excessive doses of T_4 act on laying hens to induce molting and to relate these events to changes in circulating iodothyronines and gonadal hormone levels.

In 1925, Zavadovsky (14) reported molting 7 or 10 days after desiccated thyroid administration and concluded that thyroid hormone plays a specific role in the regulation of growth and molting. In the present experiment, we found molting after a similar latent period. Some workers here reported that thyroid hormone is not directly involved in molting of laying hens because the administration of thiouracil or progesterone could also induce molting (1,5). However, as shown in Fig. 1(B), serum progesterone level decreased during T_4-induced molting. We found an increase in serum thyroid hormone levels, while gonadal hormone levels decreased during the forced molting due to a deprivation of food and water (unpublished data). Therefore, it is probable that an increase in circulating thyroid hormone levels is one of the events preceding molting in laying hens.

SUMMARY

Twenty laying hens were divided into 4 groups which received daily intramuscular injections of graded doses of L-T_4 (0, 20, 100, and 500 μg T_4 per kg) for 4 weeks. There were no changes in body weight and egg production rate of the control (0 μg T_4), 20 μg and 100 μg T_4 groups, but marked decreases in the 500 μg T_4 group, and molting started in the latter group 10 days after T_4 injection. Circulating levels of T_4, T_3, and rT_3 in the 500 μg T_4 group were 30- to 150-fold higher than those of controls, while gonadal hormone levels (LH, E_2, and progesterone) decreased inversely. From these results, we conclude that an excess dose of T_4 (500 μg T_4 per kg per kg per day) induces an increase in circulating iodothyronine levels, which may directly stimulate the growth of feather germ, inducing molting.

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REFERENCES

1. Sulman F, Perek M. Endocrinology 41: 515, 1947.
2. Shaffner CS. Poultry Science 34: 840, 1955.
3. Adams JL. Poultry Science 34: 702, 1955.
4. Perek M and Ekstein B. Poultry Science 38: 996, 1959.
5. Himeno K and Tanabe Y. Poultry Science 36: 835, 1957.
6. Juhn M and Harris P. Proc Soc Exp Biol Med 92: 709, 1956.
7. Shibata S. Shin Niwatori no Hanshoku. Youkei no Nihonsha, Nagoya, 1956, p 182.
8. Brake J and Thaxton P. Poultry Science 58: 699, 1979.
9. Brake J, Thaxton P, and Benton EH. Poultry Science 58: 1345, 1979.
10. Nishikawa K, Hirashima T, Suzuki S, et al. Endocrinol Japan 26: 731, 1979.
11. Hattori M and Wakabayashi K. Gen Comp Endocrinol 39: 215, 1979.
12. Watanabe G, Taya K, and Sasamoto S. J Endocrinol 106: 31, 1985.
13. Snedecor GW and Cochran WG. Analysis of Variance, In Statistical Methods (Japanese, ed), Iowa State Univ. Press, Ames, Iowa, 1976, p 246.
14. Zavadovsky B. Endocrinology 9: 125, 1925.
15. Ringer RK. In PD Sturkie (ed), Avian Physiology, Third ed., Springer Verlag, New York, 1976, p 356.

GLUCAGON CAUSES DISAPPEARANCE OF A T_3 AND CARBOHYDRATE-INDUCIBLE RAT HEPATIC mRNA ($mRNA_{S14}$): UNEXPECTED CIRCADIAN DEPENDENCY OF RESPONSE*

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We have employed a rat hepatic mRNA sequence ($mRNA_{S14}$) as a model for studies of hormonal and nutrient effects on the regulation of gene expression in vivo. Attention was drawn to this sequence, which encodes a cytosolic protein of M_r 17,010, pl 4.9, because of its rapid induction to 15X the basal hypothyroid level within 4 h after T_3 administration (1). Subsequent studies showed that hepatic $mRNA_{S14}$ expression is regulated by other factors as well. Starvation or experimental diabetes mellitus, for example, markedly attenuate $mRNA_{S14}$ levels, whereas carbohydrate feeding augments $mRNA_{S14}$ (2,3). Moreover, Mariash et al. in our laboratory have shown that induction of this mRNA by T_3 and dietary carbohydrate exhibits the synergistic action which typifies their induction of several lipogenic enzymes in rat liver (2,4,5). The hypothesis has been advanced that a product of carbohydrate metabolism interacts in a multiplicative fashion with a signal generated by the association of T_3 with its receptor to induce specific mRNA sequences encoding lipogenic enzymes. Participation of the S_{14} protein in lipogenesis is further suggested by its abundancy only in lipogenic tisses--liver, fat, and lactating mammary gland (5).

Studies reported here arose from two lines of investigation. First, we wished to characterize the determinants of the circadian variation in hepatic $mRNA_{S14}$ expression observed by Jump et al. in our laboratory (5). Secondly, we undertook studies to determine whether glucagon could cause disappearance of $mRNA_{S14}$, presumably by inhibiting glycolysis and thus diminishing the carbohydrate signal at the nuclear level. Our data indicate that glucagon can cause $mRNA_{S14}$ disappearance, and that this effect is rapidly reversed by T_3. Moreover, we observed an unexpected circadian dependency of the potency of both T_3 and glucagon in this model.

METHODS

Male Sprague-Dawley rats weighing approximately 200 g were fed ad lib on a standard chow and kept on a 12 h photoperiod (lights on 0700 h). Awake animals received the indicated doses of glucagon (Eli Lilly), T_3, or the

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appropriate vehicle, and were killed by aortic exsanguination under light ether anesthesia.

Livers were frozen (-80°C) for extraction of RNA by the guanidine HCl method (5). Quantitation of relative $mRNA_{S14}$ expression was by dot hybridization of total RNA on nitrocellulose paper (50% formamide, 60°C) employing ^{32}P-dCTP-labeled $cDNA_{S14}$ - E7 (6). The E7 probe corresponds to 620 base pairs of the 3' coding region of $mRNA_{S14}$. Autoradiographs prepared from the blots were quantitated by the computer-assisted videodensitometric technique of Mariash et al. (7).

RESULTS

Circadian Variation in Hepatic $mRNA_{S14}$

Initial studies were designed to investigate the circadian regulation of the S_{14} gene. The circadian rhythm of hepatic $mRNA_{S14}$ peaks in the evening (2200 h) at a level 3 times above the nadir (1000 h) in euthyroid rats (5). Rats killed at 4 h intervals starting 24 h after administration of a dose of T_3 sufficient to fully occupy hepatic receptors for the duration of study continued to exhibit circadian periodicity in $mRNA_{S14}$ expression, although the mean level was increased 4-fold. This indicated to us that T_3 did not cause the rhythm and that the circadian factor could periodically antagonize a maximal T_3 effect.

Since regulation of $mRNA_{S14}$ expression by dietary constituents has been documented (2), we sought to determine whether $mRNA_{S14}$ periodicity was primarily entrained to the photoperiod or to the mostly nocturnal food intake of the rat. Accordingly, animals were adapted for 15 days to feeding only during the light (0700-1900 h) or dark portions of the cycle. Surprisingly, this had no major effect on the phase of the rhythm. Adaptation to photoreversal for the same period, however, resulted in the expected 180° phase shift in $mRNA_{S14}$ periodicity. This suggested to us the existence of a modulator, probably neurohumeral, of S_{14} gene expression which is primarily entrained to the photoperiod.

Opposite Effects of Glucagon and T_3 on $mRNA_{S14}$ Expression

In other studies, we wished to determine whether glucagon could influence the level of $mRNA_{S14}$. We reasoned that inhibition of glycolysis by this hormone could produce a rapid attenuation of the carbohydrate-induced regulatory factor at the level of the S_{14} gene.

Accordingly, glucagon (25 μg/100 g bw ip at 15 min intervals x 3 doses) was administered to euthyroid rats starting at 1700 h, when levels of $mRNA_{S14}$ are high. This resulted in a decline to 20% of the initial value over a 4 h period. Time course studies showed a monoexponential decline after glucagon (t1/2, 90 min) suggesting cessation of $mRNA_{S14}$ synthesis. This inference was supported by nuclear run-on assays which indicated >90% reduction in $mRNA_{S14}$ transcription following glucagon. Repetition of this experiment in hyperthyroid rats (T_3, 200 μg/100 g bw ip 18 h before glucagon) resulted in a marked diminution of the glucagon-induced disappearance (t1/2, 440 min). This indicated that T_3 antagonized the effect of glucagon on S_{14} gene regulation. Nuclear run-on assays in this group showed a lesser diminution after glucagon, suggesting that the antagonism of glucagon by T_3 occurred, at least in part, at the transcriptional level.

Circadian Dependency of Glucagon and T_3 Effects on $mRNA_{S14}$ Expression

We wished to determine whether the T_3-mediated resistance to the glucagon effect on $mRNA_{S14}$ was a rapid direct effect of thyroid hormone

or secondary to an increase in glucagon turnover in the hyperthyroid state. To this end, euthyroid animals received glucagon (25 μg/100 g bw ip x 3 at 15 min intervals) followed 15 min later by T_3 (200 μg/100 g bw ip) or vehicle starting at 1700 h. Glucagon-treated rats exhibited the expected decline in $mRNA_{S14}$, whereas a dose of T_3 administered after glucagon completely reversed the decline. Surprisingly, however, rats treated with T_3 alone at this hour failed to exhibit an increase in hybridizable $mRNA_{S14}$. These findings indicated the rapidity with which T_3 antagonizes the effect of glucagon, as well as the unexpected circadian sensitivity of the liver to respond to T_3 with an increase in $mRNA_{S14}$.

The circadian sensitivity to glucagon and T_3 was further demonstrated when these experiments were repeated during the morning (0800 h) when $mRNA_{S14}$ levels are low. T_3 alone affected the expected rise in $mRNA_{S14}$; however, glucagon at this hour had no effect. The lack of glucagon effect on $mRNA_{S14}$ during the day was further emphasized by failure of glucagon to interfere with the T_3-induced rise in $mRNA_{S14}$.

DISCUSSION

These studies demonstrate that glucagon can cause immediate disappearance of a T_3 and carbohydrate mRNA sequence in rat liver. Moreover, this glucagon effect may be rapidly reversed by administration of a receptor-saturating dose of T_3. The monoexponential decline of $mRNA_{S14}$ after glucagon, as well as the nuclear run-on assay, suggest that these effects occur, at least in part, at the transcriptional level. However, an additional effect of glucagon on $mRNA_{S14}$ turnover cannot be excluded by these studies.

These results are consistent with a multiplicative interaction between T_3 and a carbohydrate-induced regulatory factor which declines after inhibition of glycolysis by glucagon. Antagonism of the glucagon effect by T_3 may result from multiplication of the residual carbohydrate-induced signal at the level of the S_{14} gene.

Surprisingly, hepatic responsiveness to each of these opposing hormones is dependent on one or more circadian factors. During the day, when $mRNA_{S14}$ levels are low, there is no demonstrable effect of glucagon on $mRNA_{S14}$, whereas T_3 affects a rapid induction. Conversely, sensitivity to glucagon is maximal at night, and the glucose-initiated decrease is rapidly reversible by T_3. It is, therefore, possible that circadian switching of hepatic sensitivity to glucagon-like effects could underlie the periodicity of $mRNA_{S14}$ expression. Several models could be advanced to explain this periodicity of response. One possability is that these changes are produced by circadian variation of an endogenous, glucagon-like suppressor. Maximal action of such a repressor in the early morning hours could explain both the falling levels of $mRNA_{S14}$ at that time and the failure of exogenous glucagon to exert additional effects.

Regardless of the molecular mechanism of the T_3-glucagon interaction, it is apparent that it is markedly influenced *in vivo* by circadian factors. The rapid diminution of hybridizable $mRNA_{S14}$ after glucagon administration may also provide a useful model for study of the well-established interaction of thyroid hormone and dietary carbohydrate. The rapidity of the action and interaction of these hormones on $mRNA_{S14}$ expression strongly suggests that they are mediated at the pretranslational level.

REFERENCES

1. Seelig S, Jump DB, Oppenheimer JH, et al. Endocrinology 110: 671, 1982.

2. Liaw C, Seelig S, Towle HC, et al. Biochem 22: 213, 1983.
3. Carr FE, Bingham C, Oppenheimer JH, et al. Proc Natl Acad Sci USA 81: 974, 1984.
4. Mariash CN, Kaiser FE, Schwartz HL, et al. J Clin Invest 65: 1126, 1980.
5. Jump DB and Oppenheimer JH. Endocrinology, in press.
6. Liaw CW and Towle HC. J Cell Biochem Suppl 6: 299, 1982.
7. Mariash CN, Seelig S, and Oppenheimer JH. Anal Biochem 121: 388, 1982.

SUPPRESSION OF TRIIODOTHYRONINE-INDUCED GROWTH HORMONE GENE EXPRESSION BY INSULIN-LIKE GROWTH FACTOR-I IN THYROIDECTOMIZED RAT PITUITARY CELLS*

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The secretion of GH from the anterior pituitary is under hypothalamic control and also under complex multihormonal regulation. Thyroid hormone plays a major role in stimulating GH gene expression. Somatomedin C has been shown to inhibit GH secretion in vivo and in vitro. We have previously shown that physiologic levels of insulin inhibit basal and T_3-stimulated GH secretion and mRNA levels by GH_3 pituitary tumor cells (1,2) and by normal rat pituitary cells (3,4). The recent availability of a recombinant human IGF-I analogue (5) has allowed us to examine the effects of the peptide on T_3-induced GH in vitro secretion by thyroidectomized rat pituitary cells. The aim of this study was to characterize the effect of IGF-I on GH gene expression induced by physiologic levels of T_3.

METHODS

Two month old male Sprague-Dawley rats were surgically thyroidectomized (Charles River Labs) and maintained for 4-7 weeks. Anterior pituitary glands were enzymatically dispersed (3,6) and cells incubated in Ham's F-10 medium and fetal calf serum (10%) which had been stripped of T_3 and T_4 by ion exchange resin (7). After 24 hour preincubation with this medium, the cells were treated with the indicated amounts of T_3 and/or IGF-I (Thr 59, Amgen, Thousand Oaks, CA) for up to 72 hours. Rat GH cDNA (8) was nick translated using ^{32}p-dCTP and ^{32}p-dATP as described (2) to yield a specific activity of 10^8 cpm/μg DNA. Denatured RNA (5 x 10^5 cells) was immobilized on nitrocellulose paper and hybridized with modifications as described (2,9). The hybridization signal was quantified by counting the radioactivity of the individual spots after subtraction of background counts.

RESULTS

Effect of IGF-I on GH Secretion

Pituitary cells were incubated for 72 hours in the presence of IGF-I, 0.65 to 13 nM. Control cells secreted low levels of GH which were stimulated by T_3 in a time- and dose-dependent manner. IGF-I did not alter the low basal GH secretion. When T_3 (0.25 nM) was added to the hypothyroid

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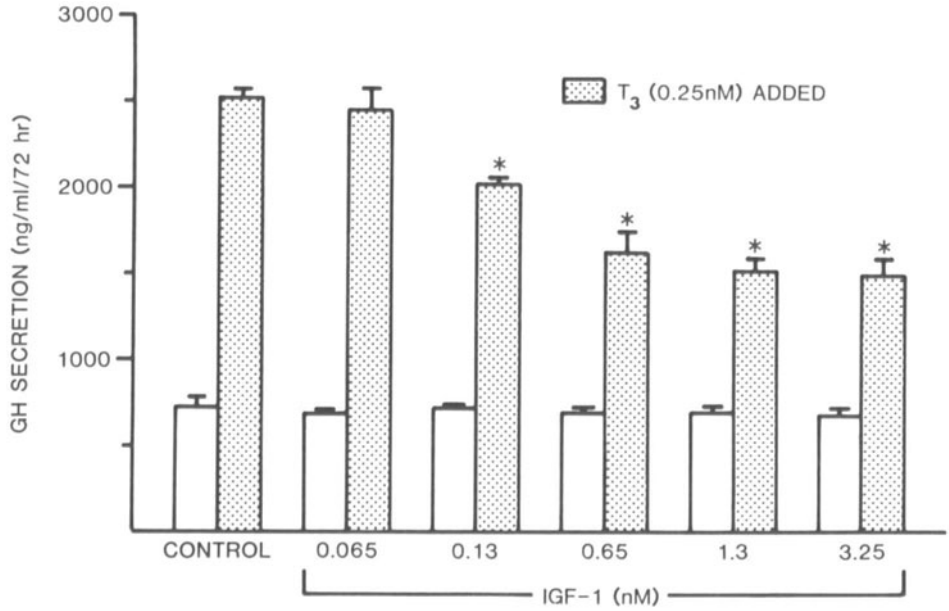

Fig. 1. Dose-response of suppressive effects of IGF-I on T_3-induced GH secretion. Each bar represents mean ± SEM of three triplicate wells. *$p<0.001$ vs control (T_3 alone).

medium, GH secretion was stimulated by 337% at 72 hours as compared to basal secretion (Fig. 1). IGF-I (0.13 nM) suppressed the T_3-induced GH secretion by 20% and there was a dose-related suppression of T_3-induced GH secretion by increasing doses of IGF-I. The inhibitory effect of IGF-I on T_3-induced GH secretion also occurred in the absence of glucose, and IGF-I did not alter the PRL levels. The T_3-stimulated GH secretion was not altered by other growth factor peptides, including EGF and FGF.

Effect of IGF-I on GH mRNA Levels

The effects of T_3 on the levels of pituitary GH mRNA sequences were tested by incubating the cells for 72 hours with varying doses of T_3 (0.01 to 0.05 nM). T_3 stimulated the hybridization signal in a dose-dependent manner (Fig. 2A). Treatment of the cells with T_3 (0.25 nM) caused a 3-fold increase in the levels of GH mRNA sequences. Cells were treated with a constant dose of T_3 (0.25 nM) and varying doses of IGF-I (0.65 to 13 nM). Significant suppression of T_3-induced GH mRNA levels was seen with 3.25 nM IGF-I ($p<0.005$) (Fig. 2B). Low basal levels of GH mRNA were not further suppressed by IGF-I.

DISCUSSION

The human IGF-I analog used in these experiments clearly suppressed the T_3-induction of GH secretion and GH mRNA levels in thyroidectomized rat pituitary cells. The inhibition of T_3-induced GH secretion by IGF-I occurred in a dose- and time-dependent manner and in the absence of glucose. We have previously shown that IGF-I inhibits basal and stimulates GH secretion by euthyroid rat pituitary cells (10). In this study, however, low basal GH secretion by thyroidectomized rat pituitary cells appeared to be unaffected by IGF-I. This may be due to the already low levels of GH secretion being unable to be further suppressed by IGF-I or it would appear that the

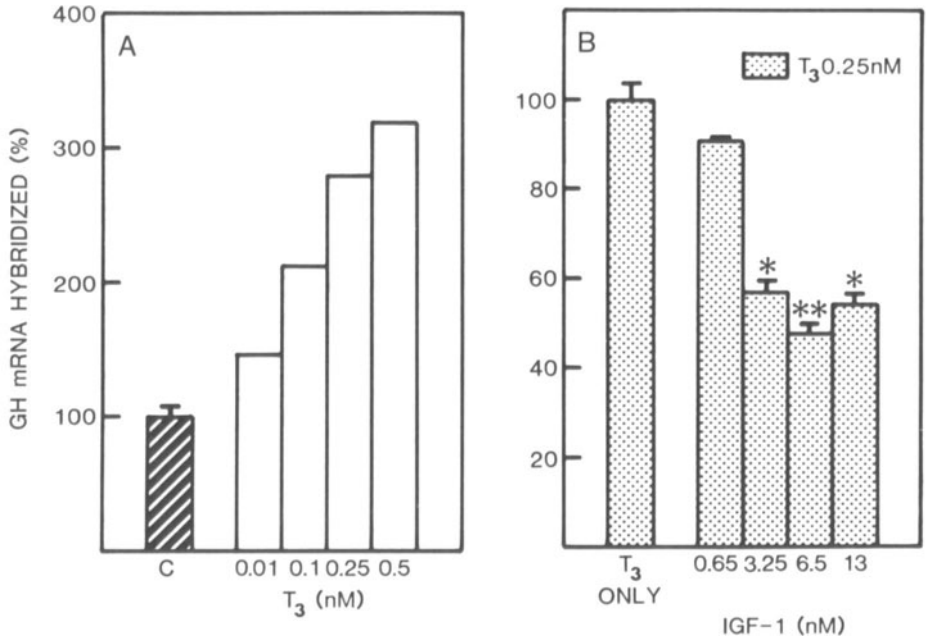

Fig. 2. Cytoplasmic dot blot hybridization of GH mRNA levels after 72 h incubation. A: T_3 dose response. B: IGF-I dose response in the presence of a constant dose of T_3. *$p<0.005$; **$p<0.001$ vs T_3 only.

presence of T_3 is critical for the suppressive action of IGF-I on pituitary GH secretion.

The stimulatory effects of T_3 on GH mRNA sequences have been established (11-15). This mRNA increase has been shown to be due to a direct stimulation of the GH gene transcription by T_3 (12-15). In these studies, IGF-I was shown to inhibit the T_3-induced GH mRNA levels. We have previously shown that in euthyroid rat pituitary cells, IGF-I suppresses both basal and GHRH stimulated GH mRNA levels. GHRH was also shown to stimulate GH gene transcription (16). Therefore, the data suggest that IGF-I may inhibit GH gene transcription. Alternatively, IGF-I may suppress T_3 action GH gene expression at a post-transcriptional site.

REFERENCES

1. Melmed S. J Clin Invest 73: 1425, 1984.
2. Melmed S, Neilson L, and Slanina S. Diabetes 34: 409, 1985.
3. Yamashita S and Melmed S. Clin Res 447A, 1985.
4. Melmed S and Slanina SM. Endocrinology 117: 532, 1985.
5. Peters MA, Lau EP, and Snitman DL. Gene (in press), 1985.
6. Conn PM, Roger DC, and Seag SG. Endocrinology 113: 1592, 1983.
7. Samuels HH, Stanley F, and Casanova J. Endocrinology 105: 80, 1979.
8. Seeburg PH, Shine, and Martial JA. Nature 270: 486, 1977.
9. White BA and Bancroft FC. J Biol Chem 257: 8569, 1982.
10. Yamashita S and Melmed S. Endocrinology (in press), 1986.
11. Seo H, Vassart G, Brocos H, et al. Pro Nat Acad Sci 74: 2054, 1977.
12. Evans RM, Birnberg NC, and Rosenfeld MG. Proc Nat Acad Sci USA 79: 7659, 1982.

13. Spindler SR, Mellon SH, and Baxter JD. J Biol Chem 257: 11627, 1982.
14. Yaffe BM and Samuels HH. J Biol Chem 259: 6284, 1984.
15. Nyborg JK, Nguyen AP, and Spindler SR. Endocrinology 116: 1261, 1985.
16. Barinaga M, Yamamoto G, and River C. Nature 306: 84, 1983.

HYPOTHYROIDISM INHIBITS THE RAPID RESPONSE OF mRNA-S_{14} TO CARBOHYDRATE FEEDING*

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INTRODUCTION

The hormonal and metabolic regulation of hepatic lipogenic enzymes has been under intense investigation for a number of years. These enzymes, and their respective mRNAs, are influenced both by thyroid hormone, as well as those hormones and compounds involved in the metabolism of carbohydrates (1-4). We have recently demonstrated a synergistic relationship between T_3 and carbohydrates in the regulation of several of these enzymes (5), and have used translational assays to demonstrate that this interaction is reflected by the activity of the specific mRNA coding for the enzyme (6-8).

The development of a cDNA to one of the thyroid hormone responsive mRNAs, mRNA-S_{14}, has allowed us to demonstrate that this mRNA responds within 20 minutes to the administration of T_3 in hypothyroid rats (9,10). Furthermore, we have shown that this mRNA is only expressed in those tissues actively engaged in the synthesis, storage, or release of lipids (11), and that it was also responsive to the administration of a high carbohydrate diet (6). Therefore, it became important to determine the kinetics of response of this mRNA to carbohydrate feeding, as well as any possible interaction between thyroid hormone and carbohydrates in the regulation of this mRNA.

MATERIALS AND METHODS

Animal Treatment

Male Sprague-Dawley rats, weighing 150-175 g, were rendered hypothyroid by inclusion of 0.025% methimazole in their drinking water for 3 weeks. All animals had minimal weight gain the week prior to killing. Food was removed from the cages at 1600 hrs the day prior to killing. The morning of the experiment, rats were given either 400 ng T_3 per 100 g BW intravenously, or 1 cc of 60% sucrose by gavage, or both. Control animals received saline. In some animals, 10 units of Regular insulin (U-100, Eli Lilly, Inc.) were administered intraperitoneally.

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Hepatocyte cultures were prepared as described (12), except that cells were maintained on Falcon tissue culture petri plates without collagen coating. The medium was supplemented with sodium selenite (2×10^{-8} M), human transferrin (5 ng/ml), bovine serum albumin (500 mg/L), Aprotinin (2000 Units/L), orotic acid (6.4×10^{-6} M), adenine (8.7×10^{-6} M), and thymidine (6.2×10^{-7} M), in addition to insulin and Dexamethasone as previously noted.

Spot 14 mRNA Quantitation

Total hepatic RNA was isolated from liver in guanidine hydrochloride, or from hepatocyte cultures in guanidine thiocyanate, as described elsewhere (13). Preparation of the [^{32}P]-cDNA probe, and dot-blot hybridization has been reported (10). For these studies, an internal standard containing the same amount of mRNA-S_{14} was applied to all dot-blots to allow comparison between experiments. Dot-blots were quantitated by video densitometry (14).

RESULTS AND DISCUSSION

Since T_3 administration to hypothyroid animals leads to a rapid induction of mRNA-S_{14}, it was necessary to determine the kinetics of response to carbohydrate feeding. To this end, we starved euthyroid rats overnight to minimize the effects of nocturnal eating and to assure a uniform baseline. The following morning, at 0830 hours, 1 cc of 60% sucrose was administered to all rats by gavage. We found a significant increase in the level of mRNA-S_{14} could be measured 30 minutes after the carbohydrate load. Interestingly, the response remained nearly linear for 4 hours, from a baseline of 9.4 OD Units to 66.1 OD Units at 4 hours. This pattern of response is quite similar to that previously noted for mRNA-S_{14} induction by thyroid hormone. The data suggest that some product related to carbohydrate metabolism is also capable of rapidly and directly inducing the formation of mRNA-S_{14}.

The ability of mRNA-S_{14} to respond to both thyroid hormone and carbohydrate feeding led us to querry whether there was any interaction between these two stimuli on the induction of this mRNA. We found that the acute administration of 60% sucrose to overnight-starved hypothyroid rats led to only minimal increases in the level of mRNA-S_{14}. Indeed, the level attained 4 hours after sucrose administration was less than that observed in the starved euthyroid controls (8.42 OD Units vs 9.4 OD Units, respectively). Thus, the hypothyroid-starved rat is markedly resistant to carbohydrate feeding.

In previous studies we have shown that hypothyroidism, starvation, and diabetes mellitus have qualitatively and quantitatively similar patterns of mRNAs, as determined by 2-dimensional gel electrophoresis of *in vitro* translated products (8). In order to test the possibility that the minimal increase in hepatic mRNA-S_{14} elicited by carbohydrate in the hypothyroid animal was due to an inadequate insulin response, we administered 10 Units of Regular Insulin simultaneously with the sucrose. Insulin treatment led to a dramatic lowering of the blood sugar, but there was no significant increase in the level of mRNA-S_{14} above that attained with sucrose alone. Therefore, the diminished response to carbohydrate feeding in the hypothyroid animal cannot be attributable to insufficient insulin secretion.

Because it is known that thyroidal status influences the metabolism of glucose (15), it was possible that the lack of response to carbohydrate in the hypothyroid rat was due to diminished enzymatic machinery responsible for the hepatic metabolism of glucose. This hypothesis is further supported by our earlier findings that a product derived from the mitochondrial

metabolism of pyruvate is responsible for the carbohydrate induction of malic enzyme mRNA, as well as for mRNA-S_{14} (12). If the diminished response of mRNA-S_{14} to carbohydrate in hypothyroidism is due to a reduction of the glucose-metabolizing enzymes, one would anticipate that administration of T_3 will slowly restore the response to carbohydrate feeding. Such a result is based on the assumption that it takes hours to several days to restore all the carbohydrate-metabolizing enzymes to normal. On the other hand, a rapid restoration of the response to carbohydrate following T_3 administration would be most consistent with the hypothesis that preliminary induction of other mRNAs and their proteins are not required for the interaction between T_3 and carbohydrates. A rapid response to carbohydrate immediately after T_3 replacement would suggest that the carbohydrate signal interacts with the T_3 signal in the nucleus of the hepatic cell.

When we administered a replacement dose of T_3 (400 ng per 100 g BW) along with sucrose gavage to hypothyroid rats, we found that the 4 hour response to sucrose was not different from that obtained after feeding sucrose to euthyroid rats. Thus, T_3 immediately restored the ability to respond to carbohydrate feeding. Moreover, the level of mRNA-S_{14} was significantly greater than the sum of the individual responses to T_3 and sucrose. This finding suggests that T_3 multiplies the carbohydrate signal within the nucleus of the cell and that this process does not require a preliminary induction of other proteins.

Lastly, we wished to determine if the interaction between T_3 and carbohydrates required alterations in extrahepatic hormones or metabolites. Therefore, we cultured isolated hepatocytes for 3 days in serum-free medium containing either low glucose (5.5 mM glucose, 0 T_3), maximally effective concentrations of glucose (27.5 mM glucose, 0 T_3), maximally effective concentrations of T_3 (5.5 mM glucose, 5×10^{-7} M T_3), or both (17.5 mM glucose, 5×10^{-7} M T_3). While glucose or T_3 ehnancement alone led to a 2- to 3-fold increase in the level of mRNA-S_{14}, the combination treatment led to a 25-fold increase in the level of mRNA-S_{14}. These data indicate that the synergistic interaction between T_3 and carbohydrates occurs within the hepatic cell without requiring alterations in extrahepatic hormones or metabolites.

SUMMARY

We have shown that spot 14 mRNA responds rapidly to the dietary administration of sucrose in euthyroid rats. On the other hand, when given to hypothyroid rats, sucrose leads to only modest increases in the level of mRNA-S_{14}. The minimal response to sucrose in hypothyroidism cannot be reversed by insulin administration. However, administration of replacement doses of T_3 immediately restores the rapid response to sucrose feeding. The response to both sucrose and T_3 is more than additive. A similar synergistic response to T_3 and glucose was noted in primary hepatocyte cultures.

Our data is most consistent with the hypothesis that T_3-nuclear receptor multiplies a signal generated by carbohydrate metabolism to induce hepatic mRNA-S_{14}. This multiplication occurs directly in the nucleus without the preliminary induction of carbohydrate metabolizing enzymes, or their mRNAs.

ACKNOWLEDGMENTS

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REFERENCES

1. Fitch WM and Chaikoff IL. J Biol Chem 235: 554, 1960.
2. Gibson DM, Lyons RT, Scott DF, et al. Adv Enzyme Regul 10: 187, 1972.
3. Diamant S, Gorin E, and Shafrir E. Eur J Biochem 26: 553, 1972.
4. Roncari DAK and Murthy VK. J Biol Chem 250: 4134, 1975.
5. Mariash CN, Kaiser FE, Schwartz HL, et al. J Clin Invest 65: 1739, 1981.
6. Liaw C, Seelig S, Mariash CN, et al. Biochemistry 22: 213, 1983.
7. Topliss DJ, Mariash CN, Seelig S, et al. Endocrinology 112: 1868, 1983.
8. Carr FE, Bingham C, Oppenheimer JH, et al. Proc Natl Acad Sci USA 81: 974, 1984.
9. Towle HC, Liaw C, and Narayan P. J Cell Biochem Suppl 6: 299, 1982.
10. Jump DB, Narayan P, Towle H, et al. J Biol Chem 259: 2789, 1984.
11. Jump DB and Oppenheimer JH. Endocrinology (in press).
12. Mariash CN and Oppenheimer JH. Metabolism 33: 545, 1984.
13. Mariash CN and Schwartz HL. Metabolism (in press).
14. Mariash CN, Seelig S, and Oppenheimer JH. Anal Biochem 121: 388, 1982.
15. Mariash CN and Oppenheimer JH. Mol Cell Endo (in press).

THYROID HORMONE STIMULATES ALKALINE PHOSPHATASE ACTIVITY IN CULTURED OSTEOBLASTIC CELLS WITH T_3 NUCLEAR RECEPTORS

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INTRODUCTION

It is well known that serum alkaline phosphatase (Al-P) activity is frequently increased in patients with Graves' disease and that the increased Al-P is of bone origin (1). However, the mechanism of hyperphosphatasia had not been elucidated.

We, therefore, investigated the effects of thyroid hormone on Al-P activity in a clonal osteoblastic cell line, ROS 17/2.8 (2). This cell line, originally derived from a transplantable rat osteosarcoma, expresses several osteoblastic traits, such as receptors for 1,25-dihydroxycholecalciferol, PTH, and glucocorticoids (2-4). Furthermore, the cell line contains high Al-P activity which is stimulated by 1,25-dihydroxycholecalciferol and glucocorticoids (2-4). We, therefore, examined thyroid hormone effects on Al-P activity in ROS 17/2.8 cells.

MATERIALS AND METHODS

ROS 17/2.8 cells were kindly provided by Dr. G.R. Mundy, San Antonio, TX. The cells were maintained in F-12 medium supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). When cells became subconfluent, the medium was replaced with F-12 medium supplemented with 10% thyroid hormone-depleted fetal calf serum (5), penicillin, streptomycin, and thyroid hormones at various concentrations. After an additional 3-6 days of culture, cells were rinsed with Hanks' balanced solution, and stored at -20°C in 50 mM Tris-HCl (pH 7.6). Cells were homogenized in a Potter-Elvehjem homonogizer and homogenates were centrifuged at 47,000 g for 20 min. The pellets were resuspended in Tris-HCl solution (50 mM, pH 7.6) and Al-P activity was determined according to a modification of the Lowry method (6). One unit of enzyme activity equals 1 µmol product (p-nitrophenol) formed per minute. Serum-free thyroid hormone concentrations were determined by equilibrium dialysis.

Nuclear receptors for thyroid hormone in ROS 17/2.8 cells were examined according to a modification of the methods of Samuels et al. (7). In brief, confluent cells were cultured in F-12 medium supplemented with 10% thyroid hormone-depleted fetal calf serum. After 24 h, cell monolayers were washed with Hanks' solution twice and incubated with serum-free F-12 medium which

Table 1. Effect of Thyroid Hormones Upon Alkaline Phosphatase Activity in ROS 17/2.8 Cells

	T_4	T_3	rT_3
(-)	0.30 ± 0.010	0.27 ± 0.021	0.22 ± 0.004
10^{-9}	0.32 ± 0.015	0.34 ± 0.032*	-----
10^{-8}	0.38 ± 0.037*	0.59 ± 0.068*	0.23 ± 0.016
10^{-7}	0.46 ± 0.034*	0.63 ± 0.101*	0.23 ± 0.013
10^{-6}	0.54 ± 0.040*	0.57 ± 0.085*	0.25 ± 0.03
10^{-5}	-----	-----	0.33 ± 0.017*

ROS 17/2.8 cells were cultured in F-12 medium supplemented with 10% thyroid hormone-depleted fetal calf serum and thyroid hormone for 5-6 days. Alkaline phosphatase activty was expressed as U/mg of protein. Data are mean ± SD (n=3), *$p<0.05$.

contained [3'-^{125}I] triiodo-L-thyronine. After 2-3 h incubation at 37°C under 5% CO_2 and 95% air, cells were washed with ice-cold Hanks' solution twice and homogenized. The homogenates were centrifuged at 3,000 rpm for 15 min and the pellets were used to prepare nuclei by two successive suspensions and centrifugations in STM-Triton buffer. Hormone specifically bound to the nuclear pellet was counted in a gamma-spectrometer (7).

RESULTS

Al-P activity was increased dose-dependently by T_4, as well as T_3; the minimum T_4 concentration in the medium was 10^{-8} M and free T_4 concentration was about 6 x 10^{-11} M. Maximum stimulation by T_4 was observed at 10^{-7} to 10^{-6} M. The minimum T_3 concentration to elicit a significant increase in Al-P activity was 10^{-10} to 10^{-9} M (free T_3 concentration: 4 x 10^{-12} to 4 x 10^{-11} M). In contrast to T_4 and T_3, rT_3 was ineffective at physiological concentrations, but rT_3 significantly increased the enzyme activity at 10^{-5} M (Table 1). Reverse rT_3 did not inhibit Al-P activity induced by T_3 (Table 2). Enhanced Al-P activity induced by T_4 was inhibited by cycloheximide and actinomycin D (data not shown).

Since ROS cells appear morphologically heterogenous, ROS 17/2.8 cells were subcloned by the limiting dilution method and 7 subclonal cell lines were established. These cells differed in morphology and exhibited variable Al-P activity (Table 3). There was no significant correlation between Al-P activity and cyclic AMP Production induced by PTH in these subclonal cell lines (data not shown). The doubling time of ROS 17/2.8 cells in the logarithmic phase was about 30 h, which was little affected by thyroid hormones.

ROS 17/2.8 cells contained high-affinity, low capacity binding sites for ^{125}I-triiodo-L-thyronine (^{125}I-T_3) in nuclear fraction. The apparent equilibrium dissociation constant, determined by Scatchard analysis, was 70 pM for T_3. ^{125}I-T_3 binding to nuclear fraction was competitively inhibited by T_4 and rT_3. The relative affinities for the nuclear receptor were 10% and <0.3%, respectively, for T_4 and rT_3. Monoiodotyrosine, diiodotyrosine,

Table 2. Effect of rT_3 Upon Alkaline Phosphatase Activity in ROS 17/2.8 Cells

Thyroid hormone concentrations	Al-P activity (U/mg of protein)
(-)	0.132 ± 0.0073
rT_3 (10^{-5} M)	0.193 ± 0.0258*
T_3 (10^{-8} M)	0.283 ± 0.0087**
rT_3 (10^{-5} M) + T_3 (10^{-8} M)	0.313 ± 0.024***

*$p<0.05$, **$p<0.025$, ***$p<0.005$.

and iodide did not displace ^{125}I-T_3 binding to the nuclear fraction at 10^{-7} to 10^{-5} M. The maximum binding capacity was about 2,500 hormone-binding sites per cell nucleus.

DISCUSSION

Al-P activity in osteoblastic cells is regulated by several hormones, such as 1,25-dihydroxycholecalciferol, glucocorticoids, and parathyroid hormone (2-4). We have demonstrated, by using well-differentiated osteosarcoma cells with many osteoblastic features, that thyroid hormone also stimulates Al-P activity. T_4 and T_3 significantly increased Al-P activity at 10^{-8} and 10^{-10} M, respectively. The corresponding free T_4 and T_3 concentrations in the culture medium were on the order of $\sim 10^{-11}$ M and $\sim 10^{-12}$ M, respectively. These free thyroid hormone concentrations are attainable in serum of euthyroid and hyperthyroid patients. In contrast to T_3 and T_4, rT_3 did not elicit a significant increase in Al-P activity at physiological concentrations but could stimulate the enzyme activity at supraphysiological concentrations. Although we have recently found that rT_3 exhibits a strong

Table 3. Induction of Alkaline Phosphatase Activity by T_4 in Subclonal ROS 17/2.8 Cell Lines

T_4 Concentration	Alkaline phosphatase activity (mU/mg of protein) Clone 2	Clone 3	Clone 4	Clone 6
0	330 ± 30	20 ± 2	308 ± 29	58 ± 5
10^{-9} M	344 ± 12	18 ± 1	314 ± 16	71 ± 1*
10^{-8} M	365 ± 19*	19 ± 1	396 ± 42*	75 ± 9*
10^{-7} M	508 ± 34*	22 ± 0.9	433 ± 11*	89 ± 7*
10^{-6} M	545 ± 26*	20 ± 0.8	416 ± 32*	99 ± 5*

ROS subclonal cells were cultured in F-12 medium supplemented with thyroid hormone-depleted fetal calf serum (10%) and T_4 for 6 days. Alkaline phosphatase activity was expressed as milliunit/mg of protein. Data are means ± SD of triplicate; *$p<0.05$.

antagonism (8) against iodothyronine-5'-deiodinase induced by T_3 in cultured fetal mouse liver (9), pharmacological doses of rT_3 exhibit thyromimetic action in ROS 17/2.8 cell activity as it did in GC cells (10). The addition of cycloheximide (1 μg/ml) or actinomycin D inhibited the enhancement of Al-P activity by T_4, suggesting that both RNA and protein synthesis are necessary for this enhancement.

We have obtained 7 subclonal cell lines from ROS 17/2.8 cells. There was no significant correlation between Al-P activity and cyclic AMP production induced by PTH in these subclonal cells. These findings are not surprising since the receptors for thyroid hormone and PTH reside in different cell compartments.

As anticipated from actions of thyroid hormones, ROS 17/2.8 cells contained T_3 nuclear receptors. Affinities of T_3, T_4, and rT_3 for nuclear receptors correspond well to their relative biological activity in inducing Al-P activity in ROS 17/2.8 cells. Therefore, we speculate that thyroid hormones stimulate Al-P activity via the nuclear receptor-mediated mechanisms.

SUMMARY

We have found that ROS 17/2.8 cells (rat osteosarcoma cells with several osteoblastic traits) contain T_3 nuclear receptors and that alkaline phosphatase activity was stimulated by physiological T_3 and T_4 concentrations. Therefore, these in vitro findings may account for an increased serum alkaline phosphatase activity of bone origin in some hyperthyroid patients.

REFERENCES

1. Cooper DS, Kaplan MM, Ridgway EC, et al. Ann Int Med 90: 164, 1979.
2. Majeska RJ, Rodan SB, and Rodan GA. Endocrinology 107: 1494, 1980.
3. Majeska RJ and Rodan GA. J Biol Chem 257: 3362, 1982.
4. Majeska RJ, Nair BC, and Rodan GA. Endocrinology 116: 170, 1985.
5. Samuels HH, Stanley F, and Casanova J. Endocrinology 105: 80, 1979.
6. Majeska RJ and Rodan GA. Calcif Tissue Int 34: 59, 1982.
7. Samuels HH and Tsai JS. Proc Nat Acad Sci USA 70: 3488, 1973.
8. Han DC, Sato K, Fujii Y, et al. Endocrinology (in press).
9. Sato K, Mimura H, Han DC, et al. J Clin Invest 74: 2254, 1984.
10. Papavasilliou SS, Martial JA, Latham KR, et al. J Clin Invest 60: 1230, 1977.

STUDIES ON THE EFFECTS OF THYROID HORMONES AND THEIR METABOLITES ON HUMAN RED CELL ACETYLCHOLINE ESTERASE

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SUMMARY

The activity of acetylcholine esterase of human red cells was studied in the presence or absence of thyroid hormones and their metabolites such as L-T_1, L-T_2, L-T_3, and L-T_4. The activity of the enzyme of intact red cells was not affected by these hormones, though studied under various conditions: the activity of acetylcholine esterase of intact red cells was 19.5 $\pm$ 1.2 units/gHb in the absence of L-T_1, and 18.3 $\pm$ 0.9 units/gHb and 19.8 $\pm$ 1.4 units/gHb in the presence of 50 µg/dl and 500 µg/dl of L-T_1, respectively. Similarly, 3,5-L-T_2, 3,3-L-T_2, rT_3, L-T_3, and L-T_4 had little effect on the enzyme activity of intact red cells.

Furthermore, we studied the acetylcholine esterase of red cell hemolysates in the presence or absence of thyroid hormones and their metabolites and found that the activity of the enzyme was influenced neither by these hormones nor metabolites. The physiological significance of the binding of these hormones to red cell membranes remains to be clarified.

INTRODUCTION

Although acetylcholine esterase (true acetylcholine esterase) is known to exist abundantly in the outer membranes of human red cells, the functions and regulatory mechanism of the enzyme in the membranes are still unclear. For example, the activity of acetylcholine esterase is significantly decreased in the red cells of patients with paroxysmal nocturnal hematuria (1), but the pathophysiological significance of the decreased activity of the enzyme remains unclear.

Thyroid hormones (L-T_3 and L-T_4) and their metabolites (L-T_1 and L-T_2) bind strongly to the plasma membranes of intact red cells (2). However, the physiological significance of this binding is still obscure. It is possible that the activity of acetylcholine esterase in the plasma membranes of the red cells might be modulated by the binding of thyroid hormones to the membranes through some mechanism. On this basis, we investigated the effects of thyroid hormones and their metabolites on human red cell acetylcholine esterase which exists in the plasma membranes of erythrocytes.

EXPERIMENTAL

Heparinized venous blood samples were obtained from normal volunteers by routine venipuncture, after obtaining informed consent. The blood samples were passed through a column of α-cellulose and microcristalline cellulose to isolate human red cells from white cells and platelets (3). The purified red cells were washed with 0.9% NaCl solution twice, and then suspended in a 0.9% NaCl solution (Ht values: 10%).

Thyroid hormones and their metabolites (3-L-T_1, 3,5,-L-T_2, 3,3-L-T_2, rT_3, L-T_3, and L-T_4, which were obtained from Henning, West Germany, and were dissolved in 0.1 N HCl solution and then diluted with 0.9% NaCl solution) were added to an equal volume of red cell suspension. The mixture was incubated at 37°C for 1 hour, and was used for measurement of acetylcholine esterase of red cells. The activity of red cell and hemolysate acetylcholine esterase was measured by the method of Beutler (4). The activity of acetylcholine esterase of red cells or hemolysates was expressed as units/gHb. The Student t test was used to evaluate the statistical significance of the difference.

RESULTS

We investigated the effects of various thyroid hormones and their metabolites (L-T_1, L-T_2, L-T_3, rT_3, and L-T_4) on human red cell acetylcholine esterase at different concentrations. As shown in Fig. 1, these hormones and the metabolites had little effect on the activity of acetylcholine esterase of red cells, though studied at different concentrations. For example, the activity of acetylcholine esterase of intact red cells was 19.5 ± 1.2 units/gHb in the absence of L-T_1, and 18.3 ± 0.9 units/gHb and 19.8 ± 1.4 units/gHb in the presence of 50 μg/dl and 500 μg/dl of L-T_1, respectively. Similarly, 3,5-L-T_2, 3,3-L-T_2, rT_3, L-T_3, and L-T_4 had little effect on the enzyme activity of intact red cells.

Furthermore, we studied the acetylcholine esterase of red cell hemolysates in the presence or absence of thyroid hormones and their metabolites. The activity of the enzyme was influenced neither by these hormones nor metabolites (Table 1).

Fig. 2 shows the effects of incubation time on red cell acetylcholine esterase in the presence or absence of 3'-L-T_1. In the absence of 3'-L-T_1, the enzyme activity was not changed after 1.5 hours. The enzyme activity

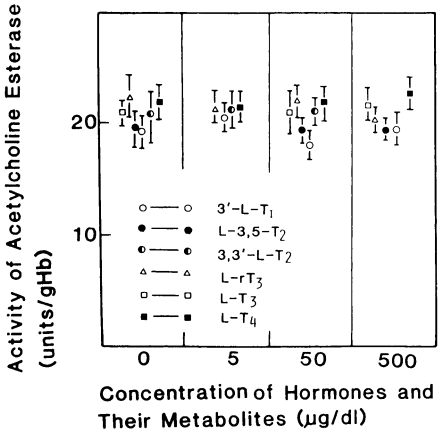

Fig. 1. Effects of thyroid hormones and their metabolites on the acetylcholine esterase activity of intact human red cells (mean ± SD). No statistical differences from 0 concentrations were observed using Student's t test.

Table 1. Effects of Thyroid Hormone Analogues on Activity of Acetylcholine Esterase of Hemolysates

	Concentrations		
	0	50 μg/dl	500 μg/dl
3'-L-T_1	16.1 ± 0.2	16.2 ± 0.2	17.1 ± 0.3
L-3,5 T_2	16.1 ± 0.2	17.1 ± 0.5	17.5 ± 0.4
L-T_3	16.1 ± 0.2	17.2 ± 0.4	16.5 ± 0.3
L-T_4	16.1 ± 0.2	17.1 ± 0.3	16.7 ± 0.2

*Activity of acetylcholine esterase was expressed as units/gHb. Data represents the means (± standard deviation) of triplicate experiments.

was not influenced by the presence of 3'-L-T_1, being consistent with the results in Fig. 1.

DISCUSSION

Thyroid hormones are known to bind to red cell membranes (2). It is, therefore, possible that the modulation of structure proteins and enzymes in the membranes might be caused by their perturbation due to the binding of thyroid hormones to the cell membranes. Davis et al (5) showed that the Ca^{2+}-ATPase activity of the human red cell membrane is stimulated *in vitro* by thyroid hormones, suggesting that the activity of the enzyme was modified by the binding of hormones to the cell membranes. Furthermore, Mendoza and Farias (6) reported that L-thyroxine modulated the Hill coefficient n of

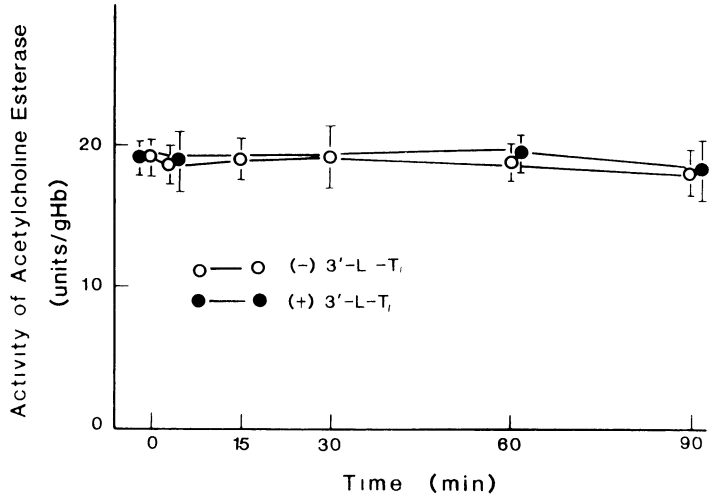

Fig. 2. Changes in the acetylcholine esterase activity of intact human red cells during incubation with or without 3'-L-T_1 (mean ± SD).

acetylcholine esterase in rat red cell membranes, suggesting that cooperativity of the enzyme in rat red cell membranes was modified by the hormone. Though we expected that the activity of acetylcholine esterase of human red cell membranes might be changed by the binding of thyroid hormone to the membranes, the results in Fig. 1, Fig. 2, and Table 1 showed that the enzyme activity of red cell membranes was not influenced by a variety of thyroid hormones and their metabolites under different conditions. We also studied the effects of thyroid hormones on Km values (Michaelis Menton values) of acetylcholine esterase in hemolysates of human red cells, but found little effects (data not shown). These results suggest that the cooperative properties of the enzyme in the human red cell membranes are not changed by the hormone binding. Such differences between our results and the results of Mendoza and Farias (6) might be attributed to interspecies differences.

REFERENCES

1. Tanaka KR. In Erythrocyte Structure and Function, Alan R. Liss, Inc., New York, 1975, p 269.
2. Crispell KR and Coleman J. J Clin Invest 35: 475, 1956.
3. Beutler E, West C, and Blume KG. J Lab Clin Med 88: 328, 1976.
4. Beutler E. In Red Cell Metabolism (2nd Edition), Grune & Stratton, New York, 1976, p 87.
5. Davis FB, Cody V, Davis PJ, et al. J Biol Chem 258: 12373, 1983.
6. Mendoza DD and Farias RN. J Biol Chem 253: 6249, 1978.

EVIDENCE FOR NUCLEAR T_3 RECEPTORS IN NEONATAL CEREBRAL ASTROCYTES

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An important effect of thyroid hormones on brain development has been well documented (1) and T_4 nuclear receptors have been described in whole brain of several animal species (2-5). In developing chick brain, T_3 nuclear receptors are distributed in both neurons and glial cells (6). We have shown that astrocytes from 2 and 12 day old mice in culture produce Nerve Growth Factor (NGF) (7) and that brain NGF concentrations in the developing mouse are thyroid hormone responsive (8). This data suggested that astrocytes may have T_3 receptors. However, recent studies, in abstract form, report the absence of T_3 receptors in 17 day fetal rat brain glial cells (9). No data are currently available regarding T_3 receptors in astrocytes of developing rodent brain. Therefore, the present study was conducted to characterize the T_3 receptor in mouse neonatal astrocytes.

MATERIALS AND METHODS

Astroglial Cell Preparation

Cerebral hemispheres from decapitated Swiss-Webster pups (1 or 12 days old) were removed, minced, washed with cold Honegger's saline D1, pH 7.4, and extracted with 25% Viokase in Ham's saline B, pH 7.4; the suspensions were collected into centrifuge tubes containing resin-treated fetal bovine serum and centrifuged at 800 rpm x 10 minutes. The pellets were resuspended in Fisher's mouse astrocyte medium modified to contain Dulbecco's modified Eagle's medium with 4.5 gm/l glucose, biotin 1 μg/ml, Selenous acid 30 nM, and resin-treated fetal bovine serum (RFBS) 1 mg/ml; final osmolality was 320 mOsm/l. The cell density was 3 x 10^6 per flask incubated at 37°C in a humidified mixture of 10% CO_2 and air. The cultures were refed every third day. The cells were harvested from logarithmic-growing cultures, washed three times with Ham's saline B, and kept on ice until the assay was performed. Astrocytes from 2 day old pups were harvested after 15 days in culture, whereas 12 day old pup cells required 28-30 days in order to obtain a similar yield.

Neuronal cells do not grow in the astrocyte medium. The purity of the astroglial cells in culture was assessed by histochemical and immunofluorescence techniques. Histochemical studies showed similar glutamine

synthetase levels as astrocytes from an adult cell line free of fibroblast contamination. Immunofluorescent studies revealed 80% of cells to contain glial fibrillary protein (GFAP), a glial cell marker. Newly replicating astrocytes may not show a reaction; thus, our cultures were composed predominantly of mouse brain astrocytes.

T_3 Nuclear Binding to Intact Cells

Approximately 5 x 10^6 astrocytes/tube were incubated at 37°C with labeled (^{125}I) T_3 (SA 750-1200 μUci/μg, New England Nuclear) with or without unlabeled T_3 (Sigma Co.) in Eagle's medium, centrifuged, the supernatant radioactivity counted to determine unbound T_3, and the pellets resuspended and washed with the addition of 2 ml of TSM (26 mM Tricine, 0.25 M Sucrose, 1 mM MgCl, 1 mM DTT) with 0.5% Triton X-100 and homongenized (10 strokes) with a rubber pestle. The homogenates were resuspended in TSM and the above wash procedure repeated once. Radioactivity in the pellets was measured to assess bound T_3.

T_3 Nuclear Binding to Isolated Nuclei

All steps were performed at 0-4°C. The cell pellet was homogenized (10 strokes) with a rubber pestle in TSM with 0.5% Triton X-100. The homogenate was centrifuged at 2000 g x 15 minutes. The pellets were washed in TSM buffer without Triton and centrifuged as before. The washing step was repeated once and the final pellet was resuspended in appropriate volume of TSM. Purity of the nuclear suspension was assessed by light microscopy after staining the final suspension with crystal violet. Additionally, the protein/DNA ratio was 2.1. Binding assays were performed by incubating the nuclear suspension with labeled T_3 with and without unlabeled T_3 (10). After incubation, the suspension was centrifuged and pellets were washed twice with Triton-TSM and recentrifuged. First supernatants were saved for free and bound T_3 separation using a resin method, as described elsewhere (10). Radioactivity was determined in the pellets and the supernatants. Maximal binding capacity (MBC) and dissociation constants were calculated using Scatchard analysis (11). For these experiments, tracer amounts of labeled (^{125}I) T_3 were added with different concentrations of unlabeled T_3 (10^{-11} - 10^{-6} M). DNA was determined using the Hill and Whatley method (12). Protein concentrations were measured by the Lowry method using bovine serum albumin as standard (13).

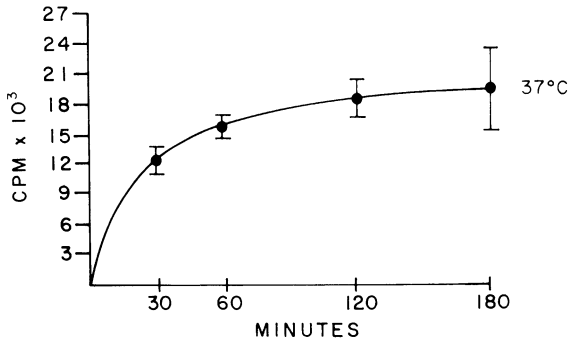

Fig. 1. Specific nuclear T_3 binding in intact astrocytes versus time.

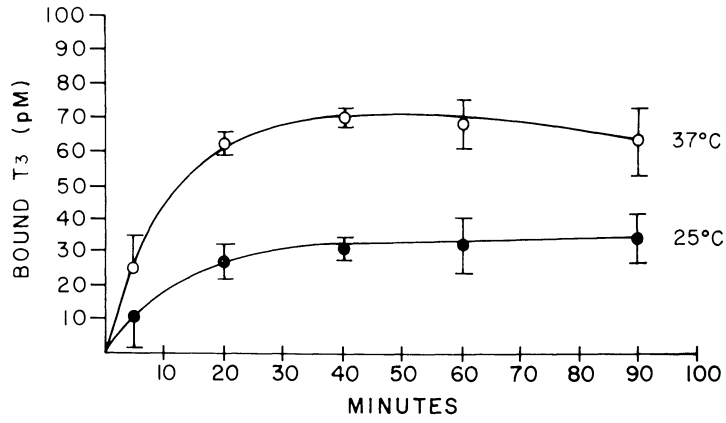

Fig. 2. Specific T_3 binding in isolated nuclei versus time.

RESULTS

Figure 1 shows the kinetics of specific T_3 nuclear binding in the intact cells. The time course shows a specific and saturable uptake of (^{125}I) T_3 to the nuclei at 37°C; equilibrium was obtained at 90 minutes. Therefore, we utilized the 37°C, 90 minute incubation protocol for the steady state studies.

Figure 2 shows the time course of the T_3 specific binding to the isolated nuclei at 25°C and 37°C. Specific binding at 37°C was about twice that at 25°C. Equilibrium occurred more rapidly than in intact cells. Thus, 30 minutes and 37°C were the time and temperature chosen for Scatchard analysis of the isolated nuclei.

A summary of the Scatchard results (Table 1) from the isolated nuclei studies show similar T_3 nuclear receptor binding affinities in the 2 and 12 day mouse astrocyte cultures. This was the case for both intact cells and isolated nuclei. MBC of the isolated nuclei was somewhat greater in astrocytes from the 12 day old than from the 2 day old pups.

DISCUSSION

The present studies indicate high affinity, low capacity, T_3 nuclear receptors in primary cultures of brain astrocytes of the developing mouse. The affinity constants of the receptor in both intact cells and isolated

Table 1. Comparison of T_3 Binding Characteristics in Intact Cells and Isolated Nuclei of 2 and 12 Day Mouse Astrocytes

	Source of cells (days old)	Kd ($\times 10^{-10}$ M)	MBC (M/μg DNA)
Isolated Nuclei	2	6.3	4.6×10^{-16}
	12	9.8	6.0×10^{-16}

nuclei at 2 and 12 days are similar to values previously reported in other cell systems (2-5). MBC in the isolated nuclei was higher than values reported in whole rat brain (14).

The presence of T_3 receptors in astrocytes from 2 and 12 day old mice and the increase in T_3 receptor MBC over this period, if real, are of particular interest since the DNA spurt in rat glial cells has been reported to occur between 4 and 16 days after birth. Kolodny et al (9) could not find T_3 binding in long-term glial cell cultures from 17 day fetal rats. Under the assumption that rat and mouse thyroid hormone physiology are very similar, we speculate that the maturational changes in glial cell T_3 nuclear receptors are important to brain glial cell ontogenesis and may be important in modulating neuronal maturation via glial cell production of growth factors.

REFERENCES

1. Grave GD. Thyroid Hormones and Brain Development, Raven Press, New York, 1977.
2. Oppenheimer HJ, Schwartz HL, and Surks MI. Endocrinology 95: 897, 1974.
3. Bellabarba D, Bedard S, Fortier S, et al. Endocrinology 112: 353, 1983.
4. Schwartz HL and Oppenheimer JH. Endocrinology 103: 943, 1978.
5. Bernal J and Pekonen F. Endocrinology 114: 677, 1984.
6. Haidar MA, Dube S, and Sarkar PK. Biochem Biophys Res Commun 112: 221, 1983.
7. Tarris R, Weichsel Jr ME, et al. Submitted.
8. Walker P, Weil ML. Weichsel Jr ME, et al. Life Sciences 28: 1977, 1981.
9. Kolodny JM, Leonard JL, and Larsen PR. Program of LX Meeting American Thyroid Association, T2, Sept. 1984.
10. Bernal J, Coleoni AH, and DeGroot L. Endocrinology 103: 403, 1978.
11. Scatchard G. Ann NY Acad Sci 51: 660, 1949.
12. Hill BT and Whatley S. FEBS Lett 56: 1975.
13. Lowry OH, Rosebrough NJ, Farr AL, et al. J Biol Chem 252: 6799, 1951.
14. Sokoloff L and Kennedy C. In GE Gaull (ed), Biology of Brain Dysfunction, Vol. 2, Plenum Press, New York, 1973, p 295.

DIRECT THYROID HORMONE STIMULATION OF MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION: THE ROLE OF ADENINE NUCLEOTIDE TRANSLOCASE (AdNT)*

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Since the first reports 30 years ago of direct thyroid hormone action on target cell mitochondria (1-3), Bronk observed that the oxidative capacity of rat liver mitochondria was markedly reduced by thyroidectomy, and substantially restored within three hours after triiodothyronine (T_3) injection in studies on the electron transport system (4,5) with no change whatever in coupling of oxidative phosphorylation; that is, phosphorus/oxygen (P/O) ratios remained constant.

In our laboratory (6), enhanced $AT^{32}P$ formation from inorganic ^{32}P in phosphate was observed, as well as increased oxygen consumption on incubation of isolated liver mitochondria from hypothyroid rats sacrificed 30 minutes after receiving intravenous doses of T_3 (in the nanogram range). The P/O ratios showed no change, and the effects persisted even with blockade of protein synthesis by the inhibitors, cycloheximide and puromycin (6).

A possible basis for direct thyroid hormone action on mitochondria was provided by our earlier finding of a thyroid hormone-binding component in mitochondrial membrane (7), subsequently confirmed by others (8,9). This component, found to arise from the inner mitochondrial membrane, had saturable T_3 binding characteristics with K_a exceeding 10^{11} M^{-1}, the highest of any T_3 binder yet observed (10). A physiological role for this binding component was inferred (10) since it was found in the mitochondria of various rat tissues, including kidney, myocardium, skeletal muscle, lung, intestine, and adipose tissue, but not in the unresponsive tissues (adult brain, spleen, or testes) which exhibit no increased oxygen consumption on administration of thyroid hormone according to the classic work of Barker and Klitgaard (11). Similar binding was observed in human tissues, in liver and kidney mitochondria. Moreover, the binding of hormone analogues was appropriately related to hormonal effect, including tighter binding than T_3 with the more potent synthetic analogue, 3'-isopropyl 3,5-diiodothyronine (10).

Further physico-chemical characterization (12) of this putative mitochondrial T_3 receptor suggested a mass and amino acid composition resembling that of the ADP-ATP translocase of Klingenberg (13).

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The first suggestion of a direct effect of thyroid hormone on the adenine nucleotide translocase system (AdNT) of mitochondrial inner membrane was provided by the observations of Babior and colleagues (14,15) which showed a diminution of carrier-mediated ADP uptake in liver mitochondria from thyroidectomized rats which, however, was restored to normal by T_4 at 2 μg/100 g of body weight and increased above normal by graded increments of hormone. The major reservation against accepting this mechanism as a primary hormone effect was that it seemed to require 48 hours after a large intraperitoneal dose of T_3 to be clearly demonstrable (14). More recent reports, however, suggest a briefer interval depending on the experimental design, thus 14 hours (16), 2 hours (17), and even as brief as 15 minutes (18).

The present study was undertaken to determine whether AdNT might be identical to or at least closely related to the inner membrane mitochondrial receptor. The approach was to isolate AdNT by established methods and then examine its ability to bind the hormone T_3.

The methods of Klingenberg's group (19) were employed using beef heart mitochondria as starting material. Since AdNT is susceptible to proteolysis by mitochondrial proteases unless combined with specific inhibitors of the carrier, such as carboxyatractyloside or palmitoyl CoA (20), we employed the latter with [^{14}C]-labeled palmitoyl CoA as a tracer for AdNT recovery. Hydroxylapatite as a slurry removed most mitochondrial proteins from our mitochondrial protein solution leaving a clear supernatant after centrifugation containing AdNT and the detergent Triton X-100. Triton with no protein whatever present can bind the hormone T_3 (21), and can retain labeled T_3 at a 7,000:1 ratio against dialysate, owing to giant micelles (90,000 daltons).

Removal of Triton was consequently undertaken using [^{3}H]-Triton as a marker with two slurries of Bio-beads Sm-2 followed by passage through a 30 ml column of Extractigel (Pierce Chemical Company, Rockford, IL) which removes Triton X-100. Octyl glucoside solution (shown not to bind T_3) had previously been added to fraction collector tubes to give a final concentration of this detergent of 0.88% (30 mM). Under these circumstances, the AdNT remained in clear solution, whereas clouding soon occurred in the absence of detergent.

Scatchard plots were performed on the purified AdNT as previously described (7,10). Photoaffinity labeling with underivatized [^{125}I]-T_3 was done as described by van der Walt, Nikodem and Cahnmann (22) and the proteins precipitated with cold 80% acetone, then dissolved in the buffer employed by Laemmli (23) for sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis.

The silver-stained proteins of Fig. 1 revealed the band characteristic of AdNT, with mobility the same as that of the 31 kd carbonic anhydrase standard. The bands obtained following photoaffinity labeling were the same as the material prior to this procedure. Covalent binding of the [^{125}I]-T_3 was evident, since radioactivity remained bound after the heat denaturation (22) and was confined exclusively to the stained band except for unbound hormone running ahead with the tracking dye, from counting of 2 mm strips of each lane.

The Scatchard plot shown in Fig. 2 is typical of seven experiments; association constants (K_a) had a mean value of 2.7×10^{11} M^{-1} $\pm$ 1 (S.D.).

The protein band of Fig. 1 with mass approximating 31,000 daltons contains AdNT, the carrier protein transporting ADP into the mitochondrion and

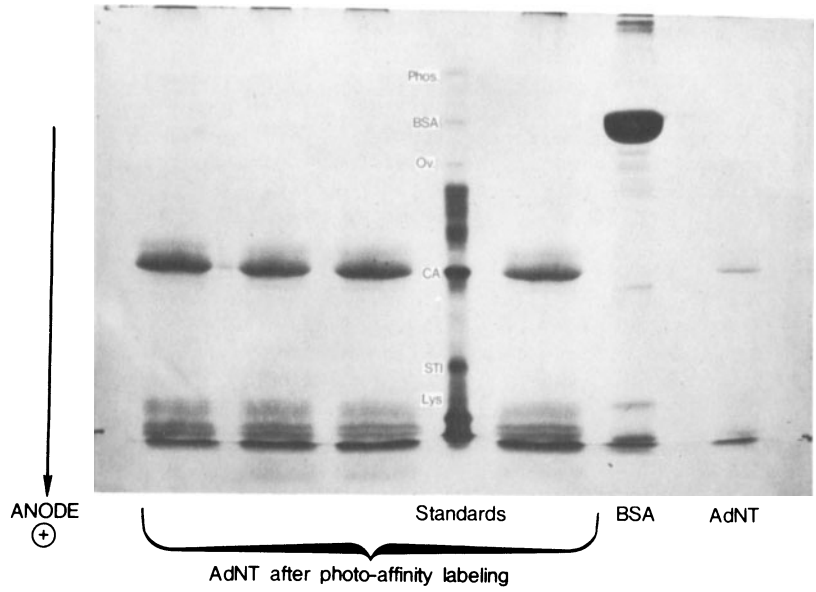

Fig. 1. Mitochondrial adenine nucleotide translocase (AdNT) after sodium dodecyl sulfate (SDS) electrophoresis on polyacrylamide gel, silver stain. The characteristic 31 kd band is evident in the four lanes of AdNT subjected to photoaffinity labeling. Mobility is the same as untreated AdNT and also the 31 kd carbonic anhydrase standard. The standards of known molecular weight run in the middle slab are indicated as follows: Phos = phosphorylase, BSA - bovine serum albumin, Ov = ovalbumin, CA = carbonic anhydrase, STI = soybean trypsin inhibitor, and Lys = lysozyme. A bovine serum albumin (BSA) lane was also run.

ATP out to the cytoplasm where it is available for energy-consuming reactions. The covalently-bound radioactivity of [^{125}I]-T_3 after photoaffinity labeling supports the concept that this may be the receptor site for direct thyroid hormone stimuation of mitochondrial oxidative phosphorylation. This concept is further supported by the "flux control coefficients" (16) signifying that this step of adenine nucleotide transport is a major rate determinant.

It remains, however, to be proven definitively whether the AdNT protein itself, or some closely associated less abundant protein such as the

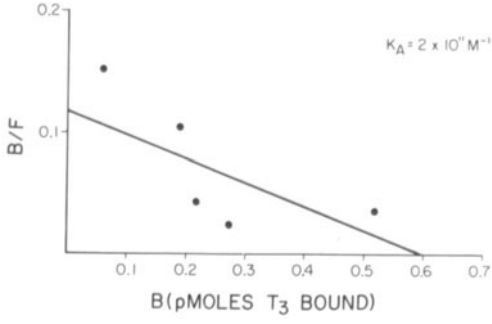

Fig. 2. Binding of triiodothyronine (T_3) by adenine nucleotide translocase (AdNT). Scatchard plot. B/F signifies bound/free. B signifies bound.

phosphate translocase or other inner membrane 31 kd protein, is the actual receptor site. These studies will be crucial, inasmuch as the abscissa intercepts of all Scatchard plots (as in Fig. 2) suggest a number of AdNT molecules far greater than the calculated number of binding sites, even with the minute amount of protein studied. This has also been the case when the receptor has been sought by affinity chromatography (12), hence further examination of this problem is underway. Preliminary work with AdNT inserted in artificial proteoliposomes has, likewise, shown thyroid hormone binding.

Overall, the present findings make the mitochondrial enzyme AdNT, or a closely associated moiety, a strong candidate for the initiating site of thyroid hormone stimulation in mammalian species.

ACKNOWLEDGMENTS

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REFERENCES

1. Martius C and Hess B. Arch Biochem Biophys 33: 486, 1951.
2. Hock FL and Lipmann F. Proc Natl Acad Sci USA 40: 909, 1954.
3. Tapley DF, Cooper C, and Lehninger AL. Biochim Biophys Acta 19: 507, 1955.
4. Bronk JR. Science 153: 638, 1966.
5. Bronk JR and Bronk MS. J Biol Chem 237: 897, 1962.
6. Sterling K, Brenner MA, and Sakurada T. Science 210: 340, 1980.
7. Sterling K and Milch PO. Proc Natl Acad Sci USA 72: 3225, 1975.
8. Goglia F, Torresani J, Bugli P, et al. Pflugers Arch 390: 120, 1981.
9. Hashizume K and Ichikawa K. Biochem Biophys Res Comm 106: 920, 1982.
10. Sterling K, Lazarus JH, Milch PO, et al. Science 201: 1126, 1978.
11. Barker SB and Klitgaard HM. Am J Physiol 170: 81, 1952.
12. Sterling K, Campbell G, and Brenner MA. Acta Endocrinol 105: 391, 1984.
13. Aquila H, Misra D, Eulitz M, et al. Hoppe-Seyler's Z Physiol Chem 363: 345, 1982.
14. Babior BM, Creagan S, Ingbar SB, et al. Proc Natl Acad Sci USA 70: 98, 1973.
15. Portnay GI, McClendon DD, Bush JE, et al. Biochem Biophys Res Comm 55: 17, 1973.
16. Verhoeven AJ, Kamer P, Groen AK, et al. Biochem J 226: 183, 1985.
17. Seitz HJ, Muller MJ, and Sobell S. Biochem J 227: 149, 1985.
18. Mowbray J and Corrigall J. Eur J Biochem 139: 95, 1984.
19. Klingenberg M, Riccio P, and Aquila H. Biochim Biophys Acta 503: 193, 1978.
20. Woldegiorgis G, Yousufzai SYK, and Shrago E. J Biol Chem 257: 14783, 1982.
21. Sterling K, Campbell GA, and Brenner MA. Trans Assoc Amer Phys 96: 324, 1983.
22. van der Walt B, Nikodem VM, and Cahnmann HJ. Proc Natl Acad Sci USA 79: 3508, 1982.
23. Laemmli UK. Nature (London) 227: 680, 1970.

INDUCTION OF LIVER MITOCHONDRIAL α-GLYCEROPHOSPHATE DEHYDROGENASE (α-GPD) BY GLUCOCORTICOID DEFICIT: ROLE OF THE NUCLEAR T_3-RECEPTOR (nT_3-R)*

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It was reported that adrenalectomy in rats induced an increase in mitochondrial α-glycerophosphate dehydrogenase activity (α-GPD) although the mechanism of this activation was not elucidated (1). More recently, we found that the increase in α-GPD activity under a glucocorticoid deficit was accompanied by an increase in the affinity of liver nT_3-R (2). This paper explores further the mechanism involved in the activation of α-GPD under a glucocorticoid deficit. We also attempted to establish a relationship between the changes in the affinity of nT_3-R and the activity of this T_3-dependent enzyme.

MATERIALS AND METHODS

Surgical adrenalectomy in 80 day old and thyroidectomy in 45 day old Wistar rats were performed 10 and 45 days, respectively, before the experiment; drinking water was supplemented with 1% NaCl or 0.9% $CaCl_2$. Nuclei were prepared from 3g-liver by centrifugation through 2.3 M sucrose, 1mM $MgCl_2$, 0.1 mM PMSF, and 2 mM DTT. Mitochondrial fraction was obtained from the initial 700 x g supernatant by centrifugation at 8,500 x g. Affinity constant (Ka) was determined by the Scatchard analysis of the ^{125}I-T_3 binding to the nuclei suspension (3). α-GPD, protein, and DNA were measured by colorimetric techniques (4-6); serum T_3 by RIA (DPC). Statistical evaluation was carried out by analysis of variance (7).

RESULTS AND DISCUSSION

Increasing doses of dexamethasone (D) injected in adrenalectomized rats induced a progressive decrease in α-GPD activity and in the Ka of nT_3-R (Fig. 1). At the highest dose of D (500 µg), a 38% decrease in α-GPD vs 55% in in the Ka value was observed. Although it appears that the Ka was more sensitive than the enzyme activity to the effect of D, this was probably a consequence of the fact that after adrenalectomy, the increase of Ka was proportionally higher than the enzyme activity. Lower doses of D (12 and 25 µg) reduced moderately but significantly the α-GPD activity, while the same

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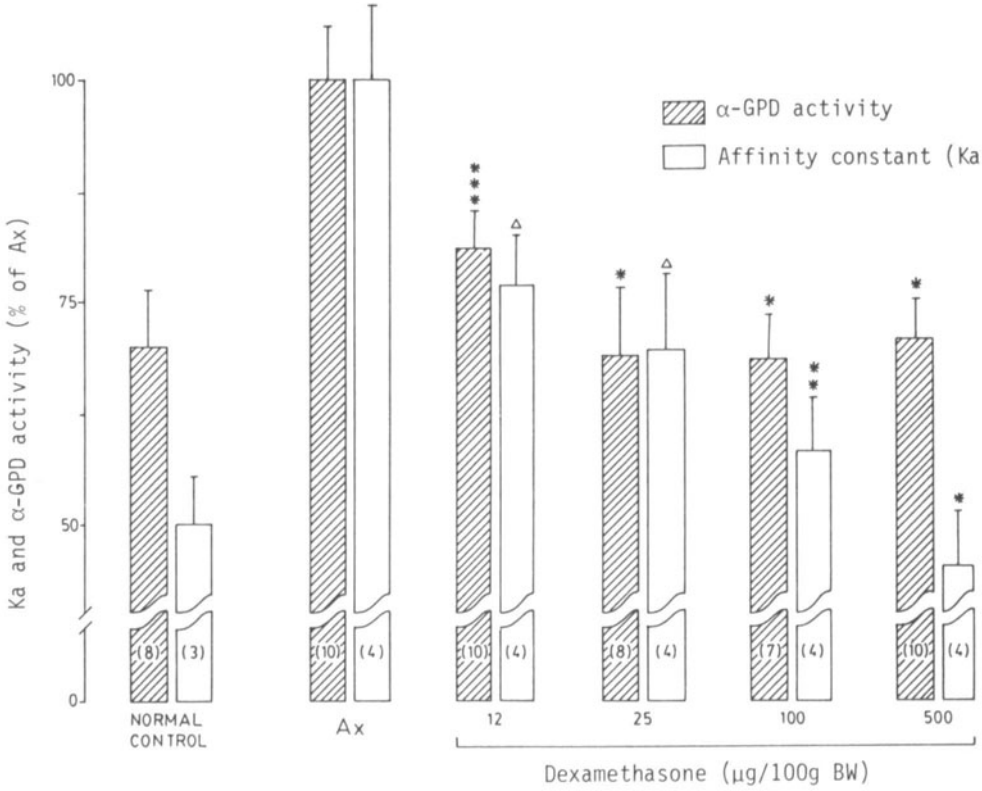

Fig. 1. Effect of progressive doses of dexamethasone (D) in adrenalectomized (Ax) rats, on Ka and α-GPD after the injection of the indicated daily doses of D during 5 days. Values are expressed as a percentage of non-injected Ax animals. Normal controls are included as reference. Bars represent the means ± SE. Number of assays in parentheses. Each D doses were administered with a 12 h interval totalizing the indicated daily doses. P values are compared to the Ax group: *$p<0.01$; **$p<0.02$; ***$p<0.05$; Δ non-significant.

doses had no statistically significant effect on Ka, even when the percentage of reduction was higher than that observed on α-GPD. This may be due to the limited number of observations.

After the injection of D (500 μg/100 g BW) to adrenalectomized rats, a progressive time-related decrease in α-GPD activity and in the Ka value was observed (Fig. 2). Eight hours after the glucocorticoid injection, α-GPD and Ka were decreased by 10 and 22%, respectively, being non-significantly different from the values in the adrenalectomized animals. Twenty-four hours later, both parameters were reduced by 28%, returning thereafter to the values in the normal controls (48-120 h).

Since changes in the affinity of nT_3-R could probably affect the hormone receptor interaction and, consequently, the secondary effect to an earlier intracellular hormonal response, such as enzyme activation, we tried to disclose if the changes in the α-GPD activity under a glucocorticoid deficit were related to similar changes observed at the Ka level. Ka and α-GPD were measured in hypothyroid (Tx) and Tx plus adrenalectomized (Tx + Ax) rats (Table 1). In both cases, serum T_3 was reduced by 70% of the normal

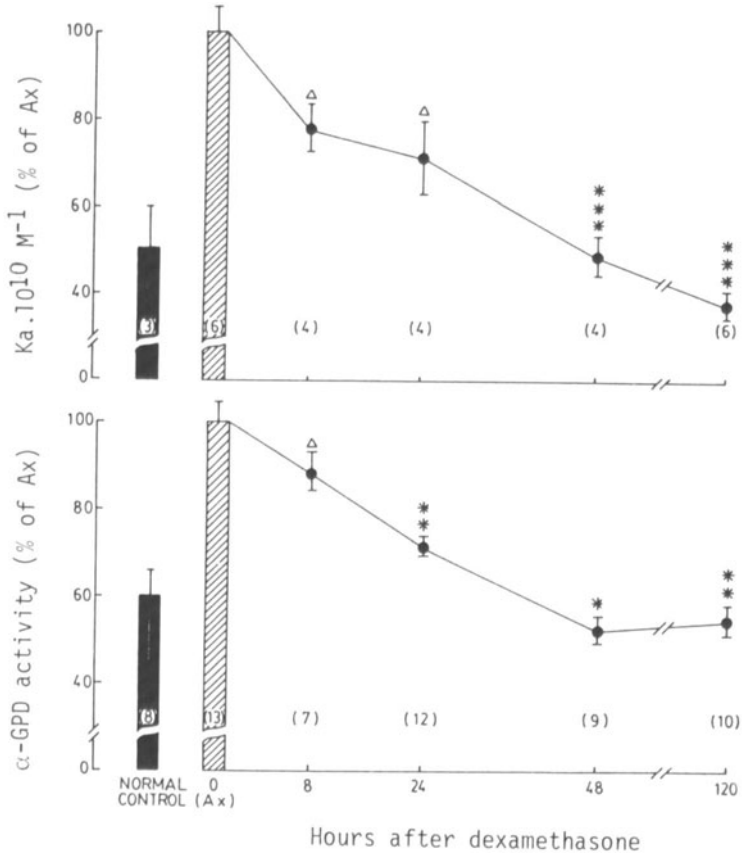

Fig. 2. Temporal effects of dexamethasone (D) (0.5 mg/100 g BW) in adrenalectomized rats (Ax) on Ka of nT_3-R and on α-GPD. Values are expressed as a percentage of the values at time zero. Normal controls are included as reference. Values represent the mean ± SE. Number of assays are in prentheses. The 48 and 120 h values were registered after D injection with a 24 h interval. P values are compared to the Ax group: *$p<0.001$; **$p<0.01$; ***$p<0.02$; Δ non-significant.

controls. As expected, α-GPD in the Tx rats was very low compared to normal controls, while Ka was in the normal range. In the Tx + Ax group, however, α-GPD was higher than in the Tx group, although it did not reach the level in the normal control. In this condition, a simultaneous increase in the Ka value was observed.

The comparison of α-GPD level between Tx and Tx + Ax groups, suggests that the higher enzyme activity under the glucocorticoid and thyroid hormone deficit could be explained considering the increase in the affinity of the nT_3-R observed in this group. On the other hand, in Ax rats where the serum T_3 level was almost in the normal range and Ka was similarly raised as in the Tx + Ax group, a more pronounced increase in α-GPD activity was observed.

Table 1. Effect of the Simultaneous Thyroid and Glucocorticoid Hormone Deficit (Tx and Ax) on α-glycerophosphate Dehydrogenase (α-GPD), Affinity Constant (Ka) of nT_3-R and Serum T_3 Level

	Ka ($x10^{10}$ M^{-1})	α-GPD (ΔA/min mg prot)	T_3 (ng/100 ml)
Normal control	23 ± 5.5 (3)*	0.033 ± 0.003^{ef} (8)	140 ± 10^{g} (3)*
Tx	30 ± 1.4 (3)*	0.009 ± 0.002 (8)	37 ± 5 (3)*
Ax	46 ± 3.3^{ab} (3)*	0.046 ± 0.004^{cd} (8)	102 ± 9.2 (3)*
Ax + Tx	45 ± 3.3^{ab} (3)*	0.022 ± 0.002 (8)	42 ± 5.8 (3)*

Values are $\bar{x}$ ± SE. Number of assays in parentheses. *Pool of 3 animals each. a: p value vs Tx < 0.01; b: p < 0.02 vs normal control; c: p < 0.001 vs Ax + Tx and vs Tx; d: p < 0.05 vs normal control; e: p < 0.01 vs Tx; f: non-significant vs Ax + Tx; g: p < 0.001 vs Ax + Tx and vs Tx.

This finding suggests that α-GPD is primarily under the T_3 control and that the affinity of the nuclear receptors could potentially modulate the enzyme response.

Fig. 3 compares the levels of α-GPD before and 24 h after the injection of 200 μg T_3/100 g BW, a dose designed to fully saturate the nT_3-R for 24 h, in Ax and normal rats. As might be expected, basal level of α-GPD in Ax animals was higher than in the normal controls, a finding consistent with

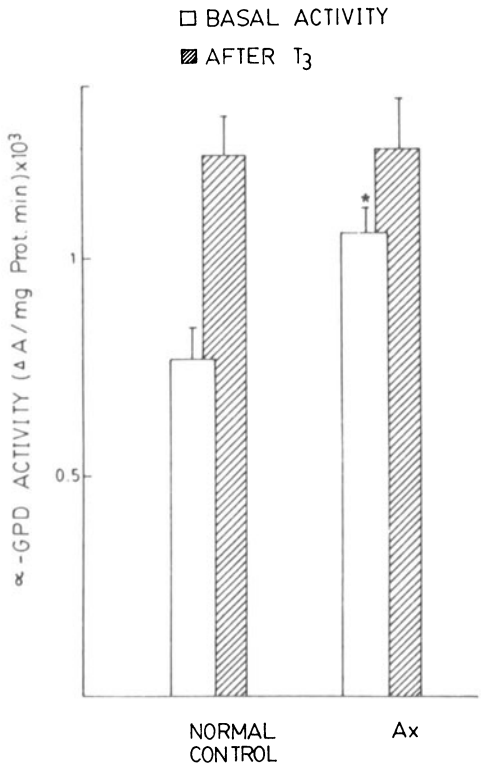

Fig. 3. Maximal T_3-induced -GPD activity in Ax and normal controls. The enzyme activity was measured 24 h after the ip injection of 200 μg T_3/100 g BW. Bars are the mean ± SE from at least 10 animals. *p<0.01 vs the basal normal control.

an increased affinity of nT_3-R found in these animals. However, when the receptors were totally occupied after a large dose of T_3, both groups attained the same maximal response. This indicates that the effect of the higher affinity on the tissue metabolic response was only expressed when the nT_3-R were not completely saturated.

In conclusion, these results seem to indicate that under a glucocorticoid deficit, the affinity of the nT_3-R could be involved in the mechanisms of α-GPD activation. This dependence becomes evident when the receptors are not fully saturated.

REFERENCES

1. Sellinger OZ, Lee K, and Fesler KW. Biochem Biophys Acta 124: 289, 1966.
2. Recupero AR, Coleoni AH, Cherubini O, et al. Acta Endocrinol 104: 485, 1983.
3. Bernal J, Coleoni AH, and DeGroot LJ. Endocrinology 103: 403, 1978.
4. Lee YP and Lardy HA. J Biol Chem 240: 1427, 1965.
5. Lowry OH, Rosebrough NJ, Farr AL, et al. J Biol Chem 193: 265, 1951.
6. Burton K. Biochem J 62: 315, 1956.
7. Sokal RR and Rohlf FJ. In Biometria: principios y metodos estadisticos en la investigacion biologica. Blume H. Ediciones, Madrid, Espana, 1969.

ONTOGENY OF NUCLEAR T_3 RECEPTOR IN PRIMARY CULTURED ASTROCYTES*

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Thyroid hormones play an essential role in assuring normal development and optimal function of the mammalian brain (1-3). It is generally admitted that most of the physiological action of T_3 is mediated by its interaction with a specific nuclear receptor (NTR) (4). Ontogenesis of T_3 receptor in the developing rat brain is well documented (5,6). However, few data are available about its distribution in specific cell types. Previous work in our laboratory has shown that in the central nervous system of adult rats, the NTR is located in neuronal cells and absent in glial cells (7). Since no astrocytes were present in our preparations, we further investigate in vitro the localization of the receptor in this cell line.

MATERIALS AND METHODS

Cell Culture

Cerebral hemispheres from 2 day old Sprague Dawley rats were dissociated in Puck's solution by passing through a nylon mesh (48 µm pore size). Cells were seeded at a density of 4×10^5 cells/cm^2 in 100 mm diameter plastic petri dishes previously coated with L-polylysine. The cultures were incubated in DME medium containing 10% fetal calf serum free of thyroid hormone at 37°C in a humidified 10% CO_2, 90% air atmosphere (8).

Binding Assay

Cells were suspended in HPC 100 buffer (0.5 M hexylene glycol, 0.5 M PIPES, 100 µM $CaCl_2$, pH 7) and homogenized with a 5 ml syringe (needle 27) for 5 strokes. After centrifugation at 1000 g for 10 min at 4°C, the pellet was washed twice with SM buffer (0.32 M sucrose, 3 mM $MgCl_2$) containing 0.5% Triton x 100 and finally washed once with 0.14 M NaCl, 3 mM $MgCl_2$. The rereceptor was salt-extracted and incubated with various concentrations of ^{125}I-T_3 (0.25, 0.5, 1, 2, 5, 10, 20 x 10^{-10} M) at 30°C for 45 min followed by 18 h at 4°C as described by Bernal et al. (9). The relative binding affinities of thyroid hormone analogs were determined by incubating aliquots of solubilized receptors (0.3 ml) with ^{125}I-T_3 (0.25 x 10^{-10} M) concomitantly with increasing doses of L-T_3 (1 - 200 x 10^{-10} M), Triac (0.5 - 200

*Supported by MRC grant MT-5730.

Table 1. Ka and MBC of the Nuclear T_3 Receptors in Cultured Astrocytes (M ± SEM)

Days of culture (N)	7 (3)	14 (4)	21 (3)	28 (3)	33 (3)
Ka ($\times 10^{10}$ M^{-1})	1.80 ±0.73	1.01 ±0.09	1.24 ±0.06	1.28 ±0.52	1.58 ±0.17
MBC (ng T_3/mg DNA)	0.095 ±0.024	0.145 ±0.020	0.198 ±0.048	0.119 ±0.024	0.122 ±0.012

x 10^{-10} M), DT_3 (2 - 200 x 10^{-10} M), and L-T_4 (10 - 2000 x 10^{-10} M). The relative binding affinities of the analogs were calculated as the molar ratio of the concentration of T_3 and the analog that caused 50% displacement of ^{125}I-T_3.

RESULTS

Table 1 shows the equilibrium association constant (Ka) and maximum binding capacity (MBC) at various days of culture. The equilibrium association constant ranged between 1.01 - 1.80 x 10^{10} M^{-1}. At the seventh day of culture, the concentration of receptors was low (0.095 ± 0.024 ng T_3/mg DNA), it increased progressively until the 14th day (0.145 ± 0.02 ng T_3/mg DNA), and reached a peak at the 21st day (0.198 ± 0.048 ng T_3/mg DNA). Thereafter, the receptor concentration was less at day 28 and 33 (0.119 ± 0.024 and 0.122 ± 0.012 ng T_3/mg DNA, respectively) (Fig. 1).

The results indicated also that the relative affinities of the analogs are in the following order: TRIAC ≥ L-T_3 > DT_3 > LT_4.

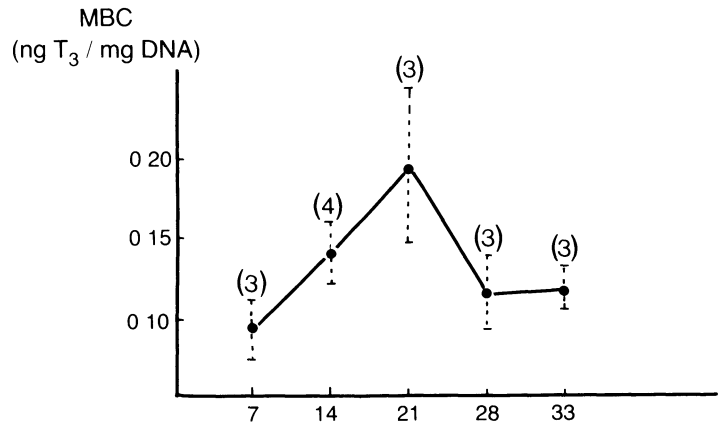

Fig. 1. Binding capacity of nuclear T_3 receptor per mg DNA as a function of days of culture. Values are derived from the data in Table 1.

DISCUSSION

Primary cultures of astrocytes have been achieved according to Schousboe et al. (8). It is well documented that no neurons were detected in these cultures (10). Our results demonstrated that NTR is present in cultured astrocytes with a level evidently elevated in the late neonatal period (after two weeks of culture). Considering the rapid proliferaration of astrocytes in this period, the total binding capacity of NTR *in vivo* manifested by a progressive decline (5,6) may be due to the relative elevated population of astrocytes while the content of NTR in the astrocytes was low. Although the total content of NTR is localized predominantly in the neurons, the role of the astrocytes cannot be neglected and the question which arises is whether the effects seen in hypo- and hyperthyroidism result from a direct action of thyroid hormone on neurons and/or is secondary to an action on other types of cells.

REFERENCES

1. Grave GD. Thyroid Hormones and Brain Development, Raven Press, New York, 1977.
2. Legrand J. J Physiol 78: 603, 1982-83.
3. Sokoloff L and Kennedy L. In GE Gaul (ed), Biology of Brain Dysfunction, Plenum Press, New York, 1973, p 295.
4. Oppenheimer JH. Ann Intern Med 102: 374, 1985.
5. Schwartz HL and Oppenheimer JH. Endocrinology 103: 943, 1978.
6. Valcana T and Timiras PS. Mol Cell Endocrinol 11: 31, 1978.
7. Ruel J, Faure R, and Dussault JH. American Thyroid Association (1982) and J Endocrinol Invest (in press).
8. Schousboe A, Fosmark H, and Formby B. J Neurochem 26: 1053, 1976.
9. Bernal J, Coleoni AM, and DeGroot LJ. Endocrinology 103: 403, 1978.
10. Hansson E. Neurochem Res 9: 153, 1984.

PURIFICATION AND CHARACTERIZATION OF T_3-RECEPTOR FROM BEEF LIVER PLASMA MEMBRANE

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Over the past two decades, it has been believed that the transport of thyroid hormones into cells was by simple diffusion. However, recent evidence indicates that the uptake of T_3 into the cells occurs by receptor-mediated endocytosis (1), and that this mode of entry is physiologically significant (2). It is also known that the plasma membrane receptor is highly stereospecific for L-T_3 (3). Two classes of T_3-receptor (high affinity - low capacity and low affinity - high capacity sites) were determined in GH_3 cells (3), swiss 3T3 cells (4) and human erythrocytes (5). Using affinity labeling with Bromoacetyl [^{125}I]T_3 (BrAc[^{125}I]T_3, the binding site of the T_3-receptor was identified by SDS-PAGE to be a 55K dalton band (3). However, its structural details and the role of the plasma membrane receptor on the mechanism of T_3 action have not been clearly defined.

The aim of this paper was to purify the receptor from beef liver plasma membrane in order to elucidate the basic structure of the receptor.

MATERIALS AND METHODS

Beef liver was obtained from the slaughter house and the plasma membrane was isolated from minced liver by a slightly modified method described previously (3). The plasma membrane fraction that sedimented to the 37/43% sucrose interface of step-gradient centrifugation (100,000 x g, 90 min) was used for the following purification of the receptor. In each purification step, a [^{125}I]T_3 binding assay was performed using a plasma membrane, as previously described (3). In order to follow the T_3-receptor, affinity labeling was done in some experiments on the purified plasma membrane with 0.1 nM Bromoacetyl [^{125}I]T_3 (BrAc[^{125}I]T_3) in Dulbecco's PBS at 15°C before solubilization. BrAc[^{125}I]T_3 was synthesized by a modified method of Nikodem et al. (6).

Beef liver plasma membranes (0.4-1 g of protein) were solubilized with 0.5% CHAPS in 20 mM Tris-HCl buffer, pH 7.4, with 1 mM $CaCl_2$ at 4°C for 15 min with stirring. After centrifugation at 100,000 x g for 30 min, the supernatant was directly applied on the gel filtration column (Cellulofine GCL-2000-sf, 2.5 x 85 cm, Seikagaku Kogyo, Japan). The column was eluted with 20 mM Tris-HCl buffer, pH 7.4, containing 0.1% CHAPS and 1 mM $CaCl_2$. The fractions containing [^{125}I]T_3 binding activity and radioactivity of

BrAc[^{125}I]T_3 were pooled and directly applied to a column (6 x 330 mm) of hydroxyapatite (Kawasaki-Bernardi Column, Koken, Japan). The column was thoroughly washed with 0.001 M phosphate buffer, pH 6.8, and then eluted with the gradient of 0.001 to 0.2 M phosphate buffer, pH 6.8. The active fractions were pooled and applied to Mono Q ion exchange column (6 x 100 mm, Pharmacia, Sweden). After washing the column with 20 mM Imidazole buffer, pH 6.5, the column was eluted with the gradient of 0 to 1 M NaCl in 20 mM Imidazole buffer, pH 6.5.

The active fractions were applied to a reverse-phase HPLC column (Widopore 7c18, 4 x 150 mm, Nagel) equilibrated with 0.1% trifluoroacetic acid, and eluted with the gradient of 36 to 65% 2-Propanol:Acetonitrile (1:1) containing 0.1% trifluoroacetic acid for 60 min at 40°C.

The peak fractions of the purification process were analyzed by SDS-polyacrylamide slab gel electrophoresis according to Laemmli (7). The proteins were visualized by Coomassie blue staining and the labeled proteins were identified by autoradiography.

RESULTS AND DISCUSSION

Since the T_3 receptor may occur in association with the nucleus (8), as well as mitochondria (9) and cytosol (10), particular care was taken to

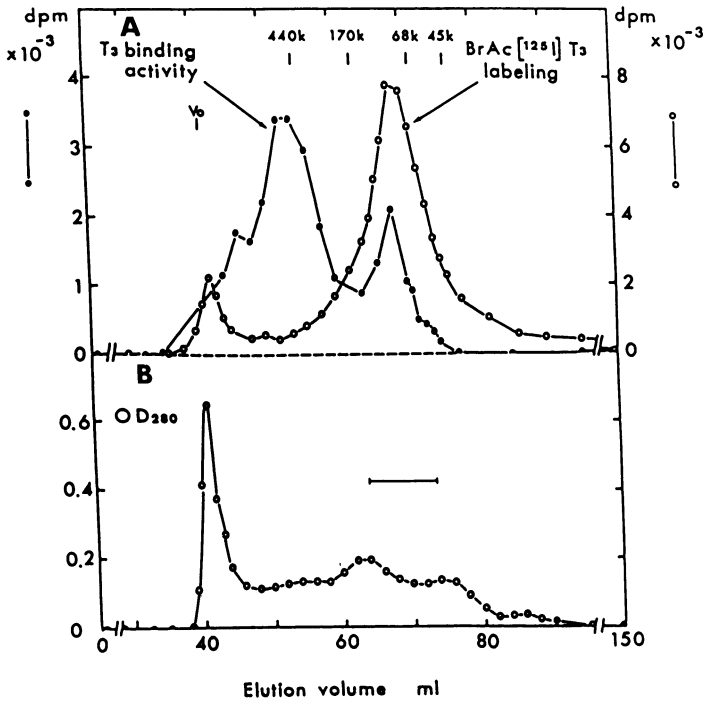


Fig. 1. Gel filtration chromatography of solubilized beef liver plasma membrane. Column: Cellulofine GCL-2000-sf, Eluate: 20 mM Tris-HCl containing 0.1% CHAPS and 1 mM CaC_2, pH 7.4.
A) T_3 binding activity (●) and the profile of affinity labeling with BrAc[^{125}I]T_3 (○).
B) Profile of absorption at 280 mμ.

avoid such subcellular contaminants during the purification of the plasma membrane.

In some experiments, in order to follow the T_3-receptor protein during purification, affinity labeling was done on the plasma membrane with 0.1 nM BrAc[^{125}I]T_3 at 15°C for one hour. About 90% of the affinity-labeled proteins gave a 55K dalton band on SDS-PAGE. The same sample prepared for SDS-PAGE (treated with 3% SDS and 5% mercaptoethanol and denatured at 100°C for five minutes) was applied to a Cellulofine GCL-2000-sf gel filtration column. The molecular weight of the major labeled protein was calculated to be 80K dalton under these gel filtration conditions. The elution profile was identical with the sample solubilized by CHAPS. These results confirm that the 80K dalton protein by gel filtration and the 55K dalton protein by SDS-PAGE is the same one.

The plasma membrane was solubilized by 0.5% CHAPS in 20 mM Tris-HCl buffer, pH 7.4, at 4°C for 15 minutes with stirring. About 70% of affinity-labeled protein was solubilized and recovered in 100,000 x g supernatant.

Fig. 1 shows the elution profile of the solubilized plasma membrane by Cellulofine GCL-2000-sf column. The affinity-labeled protein showed only one major peak at 80K dalton. However, two major peaks appeared with [^{125}I]T_3 binding activity at 80K and approximately 500K dalton which had different protein band profiles by SDS-PAGE. The 55K dalton protein, which is the major protein labeled with BrAc[^{125}I]T_3, was shown mostly in 80K fraction.

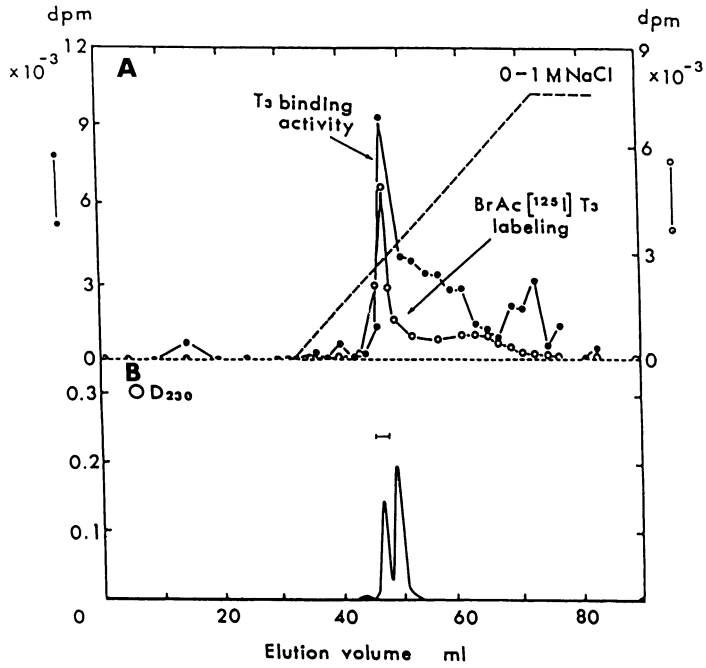
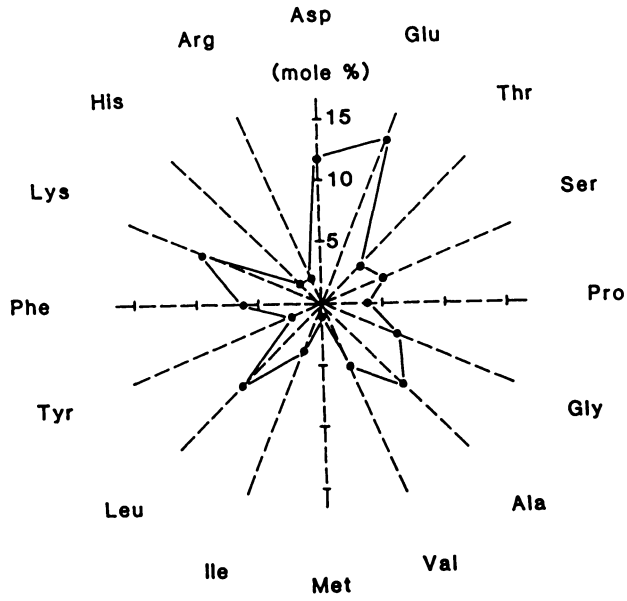


Fig. 2. Mono Q ion exchange chromatography of T_3 receptor on beef liver plasma membrane. Eluate of Hydroxyapatite at 0.12 M phosphate was applied to Mono Q column. A) Profiles of T_3 binding activity (●) and affinity labeled protein (○). B) Absorption at 230 mμ.

Accordingly, we selected the 80K fraction on gel chromatography for the further purification.

Included within the 80K fraction were many contaminating proteins. However, these contaminating proteins were effectively removed by KB column (hydroxyapatite column) and the affinity-labeled protein was eluted with 0.12 M phosphate. This fraction gave two adjacent bands on SDS-PAGE of approximately 55K dalton by Coomassie blue staining. The fractions eluted with 0.12 M phosphate were pooled and directly applied to Mono Q ion exchange chromatography. Fig. 2 shows the elution profile and [^{125}I]T_3 binding activity. The two protein peaks were separated clearly and T_3 binding activity coincided to the first peak eluted with 0.35 M NaCl. The affinity-labeled protein with BrAc[^{125}I]T_3 was eluted to the completely same position. This protein was identified to be a single band by SDS-PAGE, and its purity was further confirmed by eliciting a single peak on reverse-phase HPLC.

Under reducing conditions, the 80K protein had a single 55K band on SDS-PAGE. The sedimentation constant was 5.4S, and the pI was determined to be 4.5 by solid phase isoelectric focusing. Amino acid composition of the purified receptor eluted from reverse-phase HPLC was determined (Fig. 3). Note that approximately 25% of the amino acid residues were acidic (14.4% Glu and 11.8% Asp).



Try,Cys:not determined.

Fig. 3. Amino acid components of purified T_3 receptor. Amino acid components of eluate from reverse-phase HPLC were analyzed. One fourth of amino acid residues were acidic (14.4% Glu and 11.8% Asp).

In conclusion, we have isolated from beef liver plasma membrane a protein with T_3-receptor binding activity. The activity of the T_3 receptor on the plasma membrane was localized to an 80K dalton protein (55K dalton by SDS-PAGE) as the basic structure containing T_3 binding activity.

REFERENCES

1. Cheng, S-y, Maxfield FR, Robbins J, et al. Proc Natl Acad Sci USA 77: 3425, 1980.
2. Horiuchi R, Cheng S-y, Willingham M, et al. J Biol Chem 257: 3139, 1982.
3. Horiuchi R, Johnson ML, Willingham M, et al. Proc Natl Acad Sci USA 79: 5527, 1982.
4. Cheng S-y. Endocrinology 112: 1754, 1983.
5. Yamauchi K and Horiuchi R. In preparation.
6. Nikodem VM, Cheng S-y, and Rall JE. Proc Natl Acad Sci USA 77: 7064, 1980.
7. Laemmli UK. Nature (London) 227: 680, 1970.
8. Oppenheimer JH. Ann Intern Med 102: 374, 1985.
9. Sterling K, Brenner MA, and Sakurada T. Science 210: 340, 1980.
10. Rao ML and Rao GS. Biochem J 206: 19, 1982.

EFFECT OF THE THYROID STATUS ON THE PROPERTIES OF THE β-RECEPTORS OF ADIPOCYTES AS RELATED TO LIPOLYSIS*

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INTRODUCTION

Catecholamine-induced lipolysis in adipocytes decreases with age and appears, at least in young animals, to be influenced by thyroid hormones. In the myocardium, the effets of catecholamines are modulated by thyroid hormones through alterations of the β-adrenergic receptor-adenylate cyclase complex (1,2). The aim of this work was to document the influence of thyroid hormones on the properties of the β-receptor-adenylate cyclase complex and to examine whether the sensitivity to thyroid hormones is altered during aging.

METHODS

Three month and 12 month old male Wistar rats were used. Hypothyroid animals were produced by administration of propylthiouracil (1 mgr/100 gr of B.W. for 14 days) and hyperthyroid rats were induced by injection of T_3 (2.5 - 5 µg/100 gr B.W. for 4 days). After sacrifice, epididymal fat pads from 3 to 6 animals in each age group were removed. Isolated fat cells were prepared according to the method of Rodbell (3).

Packed fat cells were diluted in Krebs Ringer bicarbonate buffer (pH 7) containing 3% defatted bovine albumin (fraction V) and 5 µM glucose, and incubated for 90 min at 37°C with various concentrations of epinephrine (5.10^{-8} M to 2.10^{-4} M). The rate of lipolysis was measured as glycerol release by a commercially available enzymatic method (Boehringer Mannheim, GmbH, FRG).

Crude membranes were prepared according to the method of McKeel and Jarett (4) with only minor modifications for homogenization (5).

Membranes were used immediately. Binding experiments with [^{3}H]-dihydroalprenolol (DHA), a β-receptor antagonist, were performed according to the method of Williams et al. (6) with only minor modifications; non-specific binding was estimated with isoproterenol (10^{-4} M) and incubations were conducted for 30 min at 37°C. The data were analyzed according to the method of Scatchard. Competition curves of isoproterenol for DHA sites were

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Table 1. Rat Body Weights and Adipose Tissue Weight and Diameter Data On Pooled Samples (Mean ± SD)

	Body weight (gr)	Fat pad weight (gr)	Adipocyte diameter (μm)
3 months			
Control	294 (28)	3.48 (0.95)	80.9 (6.0)
Hypothyroid	287 (18)	3.53 (0.52)	82.1 (4.9)
Hyperthyroid	287 (26)	3.14 (0.82)	78.2 (6.3)
12 months			
Control	462 (40)	8.31 (2.15)	104.0 (5.0)
Hypothyroid	454 (35)	8.91 (1.12)	109.1 (8.1)
Hyperthyroid	407 (31)	7.98 (1.41)	102.2 (8.0)

generated by incubating the samples for 20' at 22°C with and without Gpp(NH)p 100 μM. These results were analyzed by the method of Hill.

RESULTS

No effect of thyroid status was observed on total body weights, epididymal fat pad weights, and mean adipocyte diameters at 3 months or at 12 months (Table 1). Only slight decreases in B.W. were noted in 12 month old hyperthyroid rats.

Maturation between 3 and 12 months decreased the lipolytic response of fat cells. In 3 month old hypothyroid rats, the stimulation of lipolysis is by epinephrine was significantly decreased. At 12 months, this effect was still observed but slightly reduced. In 3 month old hypoerthyroid rats, basal lipolysis was slightly increased. A higher sensitivity to lower doses of epinephrine was noted. In 12 month old hyperthyroid rats, the stimulation of the glycerol release was of the same magnitude as that in 3 month old rats.

Scatchard analysis of the binding of [^{3}H]-DNA by adipocyte membranes demonstrated a single class of binding sites in each age group. Aging had no effect on β-receptor number or on Kd. A decrease ($p<0.05$) in receptor number in 3 month old hypothyroid rats was observed, with an increase in 3 month old hyperthyroid rats ($p<0.05$). No effect of thyroid status was observed on these parameters in older rats (Table 2).

Competition for [^{3}H]-DNA binding to β-receptors by isoproterenol was studied in the presence and absence of Gpp(NH)p. Hill plot analysis of dose-response curves revealed the presence of more than one class of binding sites for the agonist in the absence of Gpp(NH)p. In 3 month old euthyroid rats, the EC50 for isoproterenol was 72 nM (Table 2). Gpp(NH)p decreased in affinity of the receptor for the agonist, wherein the EC50 increased by about one log unit. According to Hill plots, only one class of low affinity binding sites for the agonist was present when the guanine nucleotide was added.

Thyroid status modified the isoproterenol competition curves. In hypothyroid rats, the EC50 was increased and the Gpp(NH)p effect was altered.

Table 2. Age-related Effects of Thyroid Status on β-adrenergic Receptors On Pooled Samples (Mean ± SD)

	Kd (DHA) (nM)	Bmax (fmol/mg prot)	EC50 (nM) -Gpp(NH)p	EC50 (nM) +Gpp(NH)p
3 months				
Control	1.87 (0.72)	178 (19)	71.6 (25)	701 (223)
Hypothyroid	1.47 (0.36)	142 (11)	159.2 (87)	1122 (167)
Hyperthyroid	2.18 (0.18)	254 (54)	150.2 (57)	701 (352)
12 months				
Control	2.44 (0.68)	154 (3)	323 (184)	239 (94)
Hypothyroid	2.54 (0.29)	160 (30)	169.5 (57)	312.9 (169)
Hyperthyroid	2.96 (1.21)	196 (48)	236.5 (188)	875 (765)

EC50: Isoproterenol concentration displacing 50% of bound [^{3}H]-DHA (nM).

Hill analysis of the curves in the presence of Gpp(NH)p revealed more than one single class of binding sites, in contrast to euthyroid animals. The affinity of the β-receptor for isoproterenol was slightly decreased in each group of older animals. Interestingly, the sensitivity to Gpp(NH)p was completely lost and no significant effect of the guanine nucleotide was demonstrated on the EC50 of isoproterenol in rats of any thyroid status.

DISCUSSION

As reported earlier, hypothyroidism reduced the lipolytic response of fat cells in young animals (7,8). This effect was also observed, but to a lesser extent, in old animals. In agreement with Malbon et al. (7), we observed an increase of glycerol release in hyperthyroid animals with lower doses of epinephrine and no change at higher doses in either the young or old animals.

Scatchard analysis of DHA binding demonstrated no change in Kd for DHA, but revealed a slight decrease of β-receptor number in hypothyroidism and an increase in hyperthyroidism. These data contrast with those reported by others (7,8), but are in agreement with Giudicelli (9). The existence of two classes of binding sites for isoproterenol, one of high affinity and one of low affinity, has been demonstrated (10-12). High affinity binding sites can be converted to low affinity binding sites in the presence of Gpp(NH)p, which shifts competition curves to the right. This GTP non-hydrolyzable analog binds to the N unit of the β-receptor adenylate cyclase complex. As reported by Malbon (13) in young rats, thyroid hormones modified the affinity of the receptor for the agonist and reduced the sensitivity to Gpp(NH)p. Our data show, in addition, that in aging rats, independent of thyroid status, the affinity of the β-receptor for the agonist was reduced and the sensitivity to Gpp(NH)p was lost. As suggested for the effect of thyroid hormones, one may speculate that a coupling defect between the β-receptor and the regulatory protein Ns could be the underlying mechanism to explain the loss of sensitivity to Gpp(NH)p.

In conclusion, these data indicate that thyroid status may indeed influence the catecholamine-induced lipolysis through changes occurring at

the β-receptor level. This study also suggests that the effects of thyroid hormones are related to the association of the regulatory subunit with the β-receptor. Alterations of this association could explain the loss of thyroid hormone effects in aging animals.

REFERENCES

1. Bilezikian JP and Loeb JN. Endocrine Rev 4: 378, 1983.
2. Stiles GL, Caron MG, and Lefkowitz RJ. Physiol Rev 64: 661, 1984.
3. Rodbell M. J Biol Chem 239: 375, 1964.
4. McKeel DW and Jarett L. J Cell Biol 44: 417, 1970.
5. Belsham GJ, Denton RM, and Tanner MJA. Biochem J 192: 457, 1980.
6. Williams LT, Jarett L, and Lefkowitz RJ. J Biol Chem 251: 3096, 1976.
7. Malbon CC, Moreno FJ, Cabelli RJ, et al. J Biol Chem 253: 671, 1978.
8. Goswami A and Rosenberg IN. Endocrinology 103: 2223, 1978.
9. Giudicelli Y. Biochem J 176: 1007, 1978.
10. Kent RS, De Lean A, and Lefkowitz RJ. Mol Pharmacol 17: 14, 1980.
11. Stiles GL and Lefkowitz RJ. Life Sci 28: 2529, 1981.
12. Williams RS and Bishop T. Am J Physiol 243: E345, 1982.
13. Malbon CC. Mol Pharmacol 18: 193, 1980.

RECEPTOR INDUCTION BY THYROID HORMONES IN TADPOLE ERYTHROCYTES IN VIVO AND IN VITRO*

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This paper describes the continuation of studies of the binding of the thyroid hormone (TH) or triiodothyronine (T_3) to nuclei of red blood cells (RBCs) of Rana catesbeiana tadpoles (1-4). Nuclei of tadpole liver and tail contain specific binding sites for TH which may affect the dramatic changes in these tissues during metamorphic climax. Moriya et al. (4) have detected saturable, high affinity binding of T_3 in tadpole RBC nuclei. They also found that the number of binding sites per nucleus increased four to five times during the approach to metamorphic climax or in response to an injection of T_3. Similar results were reported independently by Galton et al. (5,6). Suggestions that the nuclear binding sites in tadpole RBCs are TH receptors have been based primarily on their binding characteristics and the presumption that the receptor increase is of physiological significance. Therefore, we have investigated receptor induction, the proposal that the increase in T_3 binding sites is a direct response of the RBCs to T_3, and that the binding sites are T_3 receptors (1-6).

In this work, we have addressed two basic questions on the induced increase of T_3 receptors: 1) Are RNA and protein synthesis required for the increase in T_3 receptor number? and 2) Is the observed increase in T_3 receptors a direct response to T_3 binding?

In Vivo Experiments with Actinomycin-D and Cycloheximide

In order to assess the role of RNA and protein biosynthesis, we used the classical biosynthetic inhibitors, actinomycin-D and cycloheximide. We have found that injection of actinomycin-D (0.3 μg/g) or immersion in cycloheximide solutions (2 mg/L) inhibited the increase in T_3 receptors observed in control tadpoles after T_3 treatment. When the dose of either inhibitor was decreased tenfold, the inhibition was eliminated. If either inhibitor was administered 48 hours or more after T_3 injection, the number of receptors increased to about the same level as with T_3 alone. Only if the inhibitor was administered before T_3 or within 24 hours after T_3 was the increase in T_3 receptors inhibited. These data demonstrate that the inhibitors do not interfere with the ability to detect an increase in T_3 binding sites, but rather prevent the response to T_3 which occurs after a lag time of about 24 hours. Apparently, both RNA synthesis and protein synthesis are required

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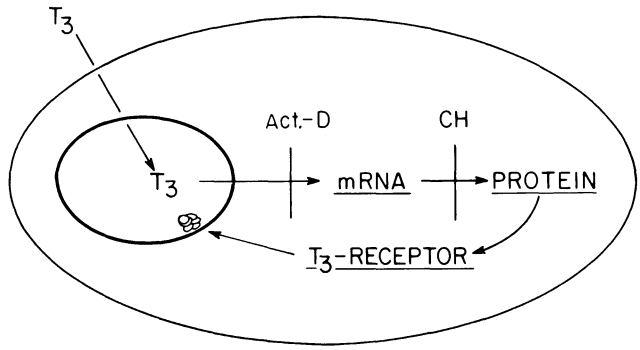

Fig. 1. Diagram showing receptor induction in the nucleated bullfrog tadpole red cell and how it might be inhibited by actinomycin-D and cycloheximide.

for the increase in T_3 receptors which occurs on the second day after T_3 injection at 20C (Fig. 1).

In Vitro Receptor Induction

A possible limitation in interpreting the above results is that the experiments were performed *in vivo*. While the lag time of 24 hours for the RBC response is relatively short, it does not exclude the possibility that the RBC responds not to T_3, but to other humoral factors induced by T_3. To test whether RBCs respond directly to T_3, we attempted to elicit an *in vitro* increase in T_3 binding sites in RBCs incubated with T_3 in a variety of media. When the tadpole RBCs were incubated with T_3 in a phosphate-saline buffer, no increase in T_3 receptors was observed in 48 hours. However, when tadpole RBCs were incubated with T_3 in M199, a nutrient-rich medium, the number of nuclear T_3 binding sites began to increase after 24 hours and continued to increase for the next 24 hours. This increase in T_3 binding was inhibited by cycloheximide at 1.0 μM. The sensitivity to cycloheximide was about the same as Schaefer and Theil (7) observed for the induction of ferritin synthesis by excess iron injection in tadpole RBCs and about tenfold more sensitive than they observed for total protein synthesis. Actinomycin-D inhibited the receptor increase at .04 micrograms/ml; a concentration which preferentially inhibits ribosomal RNA synthesis. Whether this reflects a requirement for ribosomal RNA synthesis or perhaps the involvement of an actinomycin-D-sensitive messenger RNA synthesis in this response has not been determined. These results demonstrate that tadpole RBCs respond directly to T_3 and that the increased T_3 binding requires synthesis of RNA and protein in the RBCs (Fig. 1).

Properties of the In Vitro System

The *in vitro* T_3-RBC system has provided a useful model for the further study of receptor induction. We observed a T_3 dose-dependent *in vitro* increase in T_3 binding sites in RBC nuclei. A half maximal increase in the number of sites occurred at a T_3 concentration of 2.8 pM. This value is

comparable to a K_d of 3.5 pM, the concentration at which one-half of the T_3 binding sites are occupied. These data suggest that occupation of the T_3 binding sites is the initiating event in the increase in the number of sites, an event characteristic to T_3 receptors.

We also tested the specificity of the nuclear T_3 binding sites for binding and the ability of T_3 analogs to inhibit ^{125}I-T_3 binding. An exploratory study was performed to determine T_3 analog concentrations which partially inhibited binding of 10 pM ^{125}I-T_3. The ^{125}I-T_3 binding was assayed in the presence of a constant dose of analog and the observed K_d obtained from Scatchard analysis was used to estimate the inhibitor constant, K_I, for the analog. The results were in good agreement with the relative potencies of the analogs for various metamorphic responses compiled from the recent literature (8). The most potent compounds, T_3 and its acetic acid chain analog, "Triac", showed the highest binding affinity. T_2 and rT_3, which differ from T_3 only by the absence and different position of a single iodine, respectively, have very low potencies and also had very low binding affinities for the RBC nuclei. T_4 and D-isomer of T_3 have intermediate potencies and displayed intermediate binding affinities. The T_3 binding sites of RBC nuclei show the specificity for binding active TH analogs expected to TH responses (8,9).

SUMMARY

Using the biosynthetic inhibitors, actinomycin-D and cycloheximide, we have shown that RNA and protein biosynthesis are necessary for receptor induction in bullfrog tadpole RBC both *in vivo* and *in vitro*. An increase in T_3 receptors can be induced in RBC suspensions if a nutrient-rich medium is used. The model system described here appears to have the characteristics of an increase in T_3 receptor synthesis in direct response to T_3. We also confirmed the specificity of the nuclear T_3 binding sites for binding various TH analogs.

REFERENCES

1. Yoshizato K and Frieden E. Nature 254: 705, 1975.
2. Kistler A, Yoshizato K, and Frieden E. J Biol Chem 250: 8337, 1975.
3. Moriya T, Thomas CR, and Frieden E. Endocrinology (Baltimore) 114: 170, 1984.
4. Moriya T, Thomas CR, and Frieden E. Aool Sci 1: 477, 1984.
5. Galton VA. Endocrinology (Baltimore) 114: 735, 1984.
6. Galton VA and St Germain DL. Endocrinology (Baltimore) 114: 99, 1985.
7. Schaefer FV and Theil EC. J Biol Chem 256: 1711, 1981.
8. Jorgensen EC. In CH Li (ed), Hormonal Proteins and Peptides, Academic Press, New York, 1978, p 107.
9. Frieden E. In LI Gilbert and E Frieden (eds), Metamorphosis. A Problem in Developmental Biology, Plenum Press, New York, 1981, p 545.

PUTATIVE NUCLEAR TRIIODOTHYRONINE RECEPTORS IN TADPOLE LIVER DURING METAMORPHIC CLIMAX

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It has been shown previously that the maximum binding capacity (MBC) of the putative 3,5,3'-triiodothyronine (T_3) receptor in red blood cells (RBCs) and tail tissue of Rana catesbeiana tadpoles is increased during development (1,2), and can be stimulated in RBCs by treatment with thyroid hormone (TH) (3). The present study was performed to determine if the MBC of tadpole liver nuclei is also increased during development or following treatment with TH. Because of the relatively high levels of endogenous TH in tadpoles during climax and the slow rate of dissociation of the hormone-receptor complex (4), the use of an in vivo saturation assay employing [^{125}I]T_3 was not feasible. Thus, a method was developed for determining MBC by injecting sufficient stable T_3 to just saturate the receptors, and then quantifying total nucleus-bound T_3 and plasma T_3 concentrations by RIA. Non-saturable (NS) binding was determined by injecting tadpoles with 10 nmol T_3. Under these conditions, the contribution of the saturable binding sites to total binding was expected to be insignificant. Thus, when total T_3 bound to nuclei was plotted against plasma T_3 concentration, the NS binding could be represented as a line drawn between the origin and the point designating the nucleus-bound T_3 after injection of 10 nmol T_3. The quantity of T_3 bound to the saturable sites after injection of a lower dose of T_3 was then obtained by subtracting from the value for total nucleus-bound T_3, the amount of T_3 bound to NS sites at the same plasma T_3 concentration. Preliminary studies using isotopic techniques in premetamorphic tadpoles indicated: 1) that comparable values for MBC were obtained when data were analyzed by this method and by Scatchard analysis; 2) that saturation of the nuclear receptors was almost complete following injection of 200 pmol T_3/tadpole.

METHODS

Groups of tadpoles (13 and 16 at Taylor and Kollros (5) stages XII-XIV and XIX-XXIII, respectively) were injected with 200-300 pmol stable T_3; corresponding groups received 10 nmol T_3 or vehicle. After 16-20 h, plasma, liver, and RBCs were obtained. Liver and RBC nuclei were obtained as previously described (3,6). Nuclei were extracted for 2 x 1 hour at 45°C with 2 x 1 ml of absolute ethanol. The mean extraction efficiency was 70% and 51% in nuclei of tadpoles given 200-300 pmol and 10 nmol T_3, respectively. The DNA content of the residue was measured; it was not influenced by the extraction procedure. The T_3 contents of the nuclear

Table 1. Maximum Binding Capacity (MBC) of Hepatic T_3 Nuclear Receptors in Rana Catesbeiana Tadpoles During Early Prometamorphosis and Metamorphic Climax

Stage	MBC[a] (plasma) (pmol T_3/mg DNA)	MBC[a] (plasma) (sites/nucleus[b])	MBC[a] (cytosol) (pmol T_3/mg DNA)	MBC[a] (cytosol) (sites/nucleus)
XII-XIV	0.308[c] ± 0.024	2411 ± 187	0.310 ± 0.041	2427 ± 328
XIX-XXIII	0.260 ± 0.035	2035 ± 275	0.275 ± 0.032	2153 ± 251
p value	NS	NS	NS	NS

[a]MBC was determined using plasma or liver cytosol total T_3 concentrations.
[b]Based on 13 pg DNA/nucleus.
[c]Mean ± SE.

Data used to assess NS fraction: (obtained in tadpoles given 10 nmol T_3)	Nuclear T_3 (pmol/mg DNA)	Plasma T_3 (pmol/ml)
XI-XIV	8.92 ± 1.17	247 ± 27.4
XIX-XXIII	13.7 ± 1.51	367 ± 24.2

extracts, liver cytosol (the 800 x g supernatant of the liver homogenate), and plasma were determined by RIA using the T_3 RIA established in this laboratory (7). The data were corrected for extraction efficiency and analyzed as described above.

RESULTS

Maximum Binding Capacity of Hepatic T_3 Receptors During Metamorphosis

As shown in Table 1, the MBC of the hepatic nuclei was not increased between early prometamorphosis and metamorphic climax. Moreover, values for MBC determined using plasma T_3 levels were comparable to those obtained using cytosol T_3 concentrations. Data used to determine the NS fraction are also given in Table 1. It can be calculated from these data that the basic assumption used in this study, namely that following 10 nmol T_3 most of the T_3 was bound to non-saturable sites, is justified.

Receptor Occupancy During Metamorphic Climax

Endogenous nucleus-bound and plasma T_3 levels were also determined by RIA in tadpoles undergoing metamorphic climax (prior to stage XIX, nucleus-bound T_3 was undetectable.) These data, together with those obtained following saturation of the receptors with T_3, permit an estimation of the percentage of the sites that are occupied during climax. As shown in Table 2, 80% of the hepatic receptors (57% as estimated using liver cytosol T_3 levels) and almost all the RBC receptors are occupied with T_3 during this phase.

Effect of T_4 on the MBC of Hepatic Nuclear Receptor

This study was carried out in tadpoles in early prometamorphosis and, thus, MBC could be determined using the [^{125}I]T_3 technique and Scatchard analysis. However, the RIA method was used in one study. As shown in Table 3, pretreatment with T_4 increased the MBC in RBCs an average of 2.4-fold in 4 experiments but had no effect on MBC in hepatic nuclei.

Table 2. Occupancy of Nuclear Receptors by T_3 During Metamorphic Climax

Nuclei	Endogenous T_3 (pmol/mg DNA)	MBC (pmol/mg DNA)	% Occupancy
Liver[a]	0.208 ± 0.029	0.260 ± 0.035[c]	80
Liver[b]	0.158 ± 0.025	0.275 ± 0.032[c]	57
RBC	0.233 ± 0.060	0.238 ± 0.046	94

[a]Values calculated using plasma T_3 concentrations. [b]Values calculated using liver cytosol T_3 concentrations.

Table 3. Effect of Pretreatment with T_4 on MBC of Tadpole Liver and RBC Nuclei

Exp.	T_4-treatment[a]	MBC (pmol/mg DNA) RBC Scat. Anal.	RBC RIA	Liver Scat. Anal.	Liver RIA
1	-	0.042		0.194	
	+	0.125		0.203	
2	-	0.046		0.225	
	+	0.146		0.242	
3	-	0.110		0.255	
	+	0.186		0.248	
4	-		0.143		0.212
	+		0.263		0.202

[a]1 nmol T_4 injected 14 days prior to study.

DISCUSSION

These studies indicate that MBC did not increase in tadpole liver between early prometamorphosis and metamorphic climax. This is very different to the situation in RBC and tail; in both these tissues, MBC increases substantially during prometamorphosis (1,2). However, the finding does substantiate the previous report that MBC was comparable in hepatic nuclei from tadpoles at stages X and XIX (3). MBC was also found to be unaffected by development when determined in isolated liver nuclei (Galton, unpublished observations).

Another difference between the liver and RBC putative T_3 receptor was observed. MBC can be increased in tadpole RBC nuclei by pretreatment of the tadpoles with TH (3). In the present study, it was found that treatment of tadpoles with T_4, although it resulted in the expected increase in MBC of RBCs, had no effect on the MBC of tadpole liver nuclei.

The putative T_3 receptors in liver and RBCs of tadpoles undergoing metamorphic climax were found to be 57-80% and more than 90% occupied with T_3, respectively. This substantiates a previous suggestion that occupancy is substantial during this phase (1,3), and also indicated that, as in mammals, the majority of TH on the receptor is T_3.

In seeking an explanation for why receptor number increases during development in RBCs and tail tissue but not in liver, it is important to note that there are marked differences in the ultimate expression of TH action in RBCs and tail on the one hand, and liver on the other. In the former tissues, TH-dependent metamorphosis results in cell death, a dramatic effect which usually occurs in a very short space of time. A very different situation occurs in the liver where metamorphosis is accompanied by the differentiation of cells to adult function. Here, in contrast to tail and RBCs, biochemical differentiation occurs in a proliferating population of cells. If, thus, appears that although hepatic differentiation can be accomplished solely through an increase in plasma TH levels, the events which mediate

cell destruction in some tissues require a concomitant increase in receptor number.

REFERENCES

1. Galton VA and St Germain DL. Endocrinology 116: 99, 1985.
2. Yoshizato K and Frieden E. Nature 254: 705, 1975.
3. Galton VA. Endocrinology 114: 735, 1984.
4. Galton VA. Endocrinology 107: 1910, 1980.
5. Taylor AC and Kollros JJ. Anat Rec 94: 7, 1946.
6. Galton VA and Schaafsma J. Endocrinology 112: 1999, 1983.
7. St Germain DL and Galton VA. J Clin Invest 75: 679, 1985.

ACTIVITY OF T_3-RESPONSIVE α-GLYCEROPHOSPHATE DEHYDROGENASE (α-GPD) AND THE CHARACTERISTICS OF NUCLEAR T_3-BINDING IN THE MALE AND FEMALE RAT*

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The present study explores the possible sex-related difference in the metabolic effects of thyroid hormones by studying the activity of the liver mitochondrial T_3-responsive α-glycerophosphate dehydrogenase (α-GPD). In parallel experiments we characterized the liver nuclear T_3-receptors in male and female rats. We also attempted to determine whether such differences are related to the different sex-hormone content.

MATERIALS AND METHODS

Male and female Wistar rats, 75 days old unless otherwise indicated, were used.

Nuclei and mitochondrial fractions were prepared from 3 g of liver. Nuclei were isolated by centrifugation through 2.3 M sucrose, -1 mM $MgCl_2$, -2 mM DTT, -0.1 mM PMSF, as previously described (1). Maximal binding capacity (MBC) and affinity constant (Ka) were obtained by incubating the nuclear suspension with ^{125}I-T_3. Parallel tubes contained the same amount of ^{125}I-T_3 and 3 x 10^{-7} M T_3 to assess non-specific binding (2). Mitochondrial fraction was obtained from the 700 xg supernatant of the total liver homogenate by centrifugation at 8,500 xg for 10 min (3). α-GPD was measured by the method of Lee and Lardy (4), DNA by the method of Burton (5), and protein by the method of Lowry et al. (6). Statistical comparisons were made with the analysis of variance (7).

RESULTS AND DISCUSSION

As depicted in Table 1, adult female rats exhibited a higher basal α-GPD activity compared to age-matched males. When the enzyme activity was related to DNA concentration, the same difference was observed, indicating a net increase per cell.

The difference in the enzyme activity was also evident in immature rats (20 days) (ΔA/min mg prot; mean ± SE; male 0.0737 ± 0.005; female 0.1038 ± 0.008; $p<0.01$; n:13).

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Table 1. Basal Activity of Liver Mitochondrial α-glycerophosphate Dehydrogenase (α-GPD) in Adult Male and Female Rats

	n	Male	Female
α-GPD			
ΔA/min/mg protein	10	0.0525 ± 0.0022	0.0744 ± 0.0013*
ΔA/min/mg DNA	10	0.484 ± 0.045	0.723 ± 0.058*

Values given are the mean ± SEM; n, number of animals.
*Significantly different from male group, $p<0.01$.

The basal α-GPD activity and the characteristics of the nuclear T_3-receptors studied during the different stages of the ovarian cycle (data not shown) revealed no significant differences in MBC, Ka and α-GPD during the four days of the ovarian cycle, indicating that the physiological hormonal changes in the female did not induce modifications neither at the receptor level nor in the metabolic response of T_3 measured by α-GPD activity.

The effect of apparent physiological replacement doses of testosterone (T) administered to gonadectomized male and female rats on α-GPD activity is shown in Table 2. A T deficit or a replacement dose did not modify the

Table 2. The Effect of Testosterone Administration in Castrated Male and Female Rats, on Liver Mitochondrial α-glycerophosphate Dehydrogenase (α-GPD) Activity

Treatment	α-GPD (ΔA/min/mg protein)
Male	
sham surgery	0.0430 ± 0.0010
castrated	0.0390 ± 0.0033
castrated + Testosterone	0.0388 ± 0.0033
Female	
sham surgery	0.0732 ± 0.0035
castrated	0.0688 ± 0.0088
castrated + Testosterone	0.0681 ± 0.0057

Castrated male and female rats (75 days old) were injected sc with 0.33 mg Testosterone in 0.1 ml corn oil daily for 13 days, or vehicle. Values are the mean ± SEM of a group of 4 animals. The significance of the difference between sham vs castrated, sham vs castrated + Testosterone, or castrated vs castrated + Testosterone, was in all cases non-significant.

Table 3. Characteristics of the Liver Nuclear T_3-receptor in Adult Male and Female Rats

	n	MBC* (fmoles T_3/100 µg DNA)	KA** (x 10^9 M^{-1})
Male	6	76.5 ± 8.1	5.30 ± 0.94
Female	5	63.0 ± 18.4	5.78 ± 0.71
		NS	NS

Values given are the mean ± SEM; n, number of samples assayed (pool of 3 animals each); *MBC, maximal binding capacity; **Ka, affinity constant; NS, not significant.

enzyme activity compared with normal control males. In the females, ovariectomy or T injection to ovariectomized rats did not induce modifications in α-GPD activity.

To clarify the mechanism of the different levels of α-GPD in males and females, we studied the characteristics of T_3 receptors in purified liver

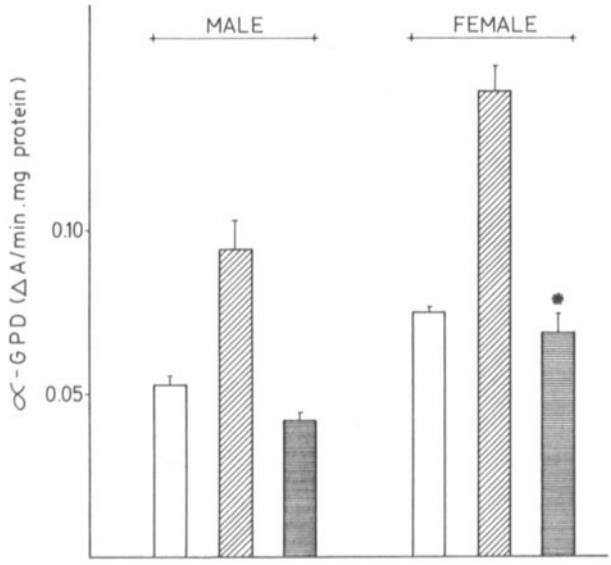

Fig. 1. Response of α-GPD to T_3 in adult male and female rats. Open bars: basal level of α-GPD; diagonal lined bars: activity of α-GPD 24 h after the iv administration of 500 µg T_3/100 g BW; horizontal lined bars: T_3-induced α-GPD activity. *Statistically significant differenct T_3-induced enzyme activity female vs male rats, $p<0.01$. Bars ± SEM. Each group consisted of seven animals.

Table 4. Kinetics of α-glycerophosphate Dehydrogenase (α-GPD) in Adult Male and Female Rats

	n	Km ($\times 10^{-3}$ M)	Vmax (ΔA/min/mg protein)
Male	4	48.9 ± 5.62*	0.0534 ± 0.0062**
Female	4	44.6 ± 7.68	0.1015 ± 0.0170

Values are given as mean ± SEM; n, number of samples assayed (pool of 3 animals each); *p value male vs female, not significant; **p value male vs female <0.05.

nuclei. No significant difference in MBC or in the Ka value was observed between the groups (Table 3).

Since the extent of the tissue metabolic response to T_3 also depends on the tissue hormone concentration, and more specifically on the saturation of the hormone receptor, we measured basal α-GPD and its response to a dose of T_3 designed to fully saturate the nuclear receptors. Twenty-four hours after the ip injection of 500 μg T_3/100 g BW, the enzyme activity showed a marked increase in both groups (Fig. 1). However, the T_3-induced α-GPD activity was significantly higher in the females. This indicates that the different basal enzyme activity was not related to differences in the endogenous saturation of nuclear T_3-receptors.

Table 4 shows the kinetics of α-GPD in adult male and female rats. Km was identical in both groups, while Vmax was significantly higher in the females, suggesting an increase in the enzyme mass.

In conclusion, since we did not observe any modification at the receptor level, the different α-GPD activity in male and female rats probably results as a consequence of a post-receptor modification of the initial signal, generated by the T_3-nuclear receptor interaction. The factors leading to the different activity of α-GPD in both sexes, although unknown, do not seem to be related to the sex-hormone content.

REFERENCES

1. Bernal J, Coleoni AH, and DeGroot LJ. Endocrinology 102: 452, 1978.
2. Bernal J, Coleoni AH, and DeGroot LJ. Endocrinology 103: 403, 1978.
3. Recupero AR, Coleoni AH, Cherubini O, et al. Acta Endocrinol 104: 485, 1983.
4. Lee YP and Lardy HA. J Biol Chem 240: 1427, 1965.
5. Burton K. Biochem J 62: 315, 1956.
6. Lowry OH, Rosebrough NJ, Farr AL, et al. J Biol Chem 193: 265, 1951.
7. Sokal RR and Rohlf FJ. Biometria: principios y metodos estadisticos en la investigacion biologica. Blume H Ediciones, Madrid, Espana, 1969.

ROLE OF T_4 IN THE MODULATION OF PITUITARY T_3 NUCLEAR RECEPTORS (T_3nR) IN HYPOTHYROID RATS*

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It is now well documented that cellular responses to thyroid hormones are mediated by a nuclear receptor, the concentration of which is known to vary with tissue, as well as with physiopathological conditions (see 1 for review). Regarding possible changes in T_3nR with variations of thyroid function, there is still controversy which may be due to the fact that such variations would be tissue dependent. Indeed, no changes in the concentration of T_3nR have been reported in the liver, kidney, and heart of hypothyroid animals (2-4), whereas more recently an increase in the liver and the brain (5-7), and a reduction in the lung, liver, and the pituitary gland (8-11) have been shown in hypothyroid rats. In pituitary tumor cells and in the rat pituitary gland, T_3 has been found to induce a decrease in the concentration of nuclear receptors (12,13).

The factors involved in the regulation of T_3 receptors are still unknown, but in the pituitary gland we have obtained some evidence that T_3 may be one of these factors. Indeed, we have previously shown that the concentration of T_3nR in the anterior pituitary gland of rats thyroidectomized for two weeks is half that found in intact adult male rats (14). We have also shown that a single injection of T_3 (0.5 µg/100 g b.w.) to thyroidectomized rats could restore a normal concentration of T_3nR within one hour and at least for three hours. Fifteen hours later, the receptor density was again at its initial low concentration (14).

These results are supportive of the hypothesis that T_3 modulates the concentration of its own nuclear receptors in the pituitary gland, and they are suggestive of a direct effect of T_3 on the synthesis of the receptors. We have, therefore, tested whether the induction of the nuclear receptors by T_3 can be inhibited by cycloheximide (Cy) (15). When Cy (1.0 mg) is given to hypothyroid rats before T_3, the T_3 induced synthesis of nuclear receptors is completely suppressed. Administration of Cy alone to thyroidectomized rats has no effect on the concentration of T_3nR for the three hour period studied.

These data are, therefore, supportive of the view that T_3 induces a rapid synthesis of its nuclear receptors in the pituitary gland which may

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consequently influence the secretion of TSH. Indeed, an inverse relationship is found between the density of T3nR and plasma TSH levels measured in various conditions ($r = -8128$, $n = 36$, $p<0.001$).

All these data pertain to the effects of T_3 on T3nR. The possible effects of T_4 on these receptors needed to be investigated. Indeed, the respective roles of T_4 and T_3 on TSH secretion are still matters of controversy, although the experiments of Obregon et al. (16) and those of Larsen et al. (4) have clearly demonstrated that in the pituitary gland T_4 acts on TSH secretion only after its conversion to T_3. As regards the nuclear receptors, the question arises whether T_4 can by itself modulate pituitary T3nR or whether T_4 acts only after deiodination into T_3.

The aim of the present study is to answer this question using the following experimental protocol. Adult male rats, thyroidectomized for two weeks, were divided into two groups. One group of animals received T_4, one injection (2 or 5 µg/100 g b.w.), and the other group received iopanoic acid (IOP, 5 mg/100 g b.w.) four injections at 12 hour intervals. This drug is known to inhibit the conversion of T_4 to T_3 in the pituitary gland (4,16, 17). The IOP treated rats received the same dose of T_4, nine hours before the last injection of IOP. All animals were killed by rapid decapitation 12 hours after T_4. A group of thyroidectomized rats injected with saline served as controls. Hormones were measured in plasma by RIA, and T3nR were determined in nuclear extracts from pools of six anterior pituitary glands as previously reported (14).

Values for plasma T_4, T_3 and TSH, and for pituitary T3nR are shown in Table 1. The very low concentrations of T_4 observed in thyroidectomized rats are increased 12 hours after T_4 administration in a dose-dependent manner, the T_4 concentration being markedly greater than that found in normal rats. A similar dose-dependent increase in plasma T_3 levels is observed. With the 5.0 µg dose, the concentration of plasma T_3 is similar to that found in normal animals. The increased concentrations of T_4 and T_3 are associated with a significant decrease in the concentrations of plasma TSH which remain, however, still higher than those of the normal rats.

As regards the density of pituitary T3nR after T_4, a dose-dependent increase is found restoring a normal concentration of T3nR. However, since the injection of T_4 resulted in a normal plasma concentration of T_3, the induction of T3nR by T_4 may be secondary to the conversion of T_4 into T_3 in the pituitary gland. To eliminate this possibility, T_4 deiodination into T_3 was blocked by a pretreatment with IOP. The effects of IOP on plasma T_4, T_3 and TSH, and pituitary T3nR are shown in Table 1. Pretreatment with IOP does not alter plasma T_4 levels measured after T_4 injection. In contrast, plasma T_3 and TSH levels are affected by pretreatment with IOP, T_3 levels being significantly less and TSH greater in the IOP + T_4 groups than in the group treated with T_4 only (Table 1). Therefore, these data attest to the blocking effect of IOP on T_4 deiodination at the pituitary and peripheral levels. As regards the pituitary T3nR, pretreatment with IOP reduces the receptor concentration when compared to that observed in rats treated with T_4 only. The concentration of T3nR remained similar to that of hypothyroid animals (Table 1). The induced synthesis of pituitary T3nR observed after T_4 administration can, therefore, be prevented by blockade of T_4 deiodination into T_3, thus supporting the view that T_4 effects observed on the density of T3nR are not due to T_4 itself, but to T_3 which has been formed locally.

Finally, the pituitary GH content, which is known to be T_3-dependent, was found to be very low in thyroidectomized rats (60 $\pm$ 15 µg/AP). It increased 12 hours after T_4 (165 $\pm$ 56 µg/AP) and was not changed when the rats were pretreated with IOP before T_4 (72 $\pm$ 10 µg/AP).

Table 1. Effects of T_4 and Iopanoic Acid (IOP) on the Concentrations of Plasma T_4, T_3, TSH, and Pituitary T_3nR in Hypothyroid Rats

Animals	Treatment T_4 µg / 100 g b.w.	Treatment IOP 4x5 mg / 100 g b.w.	Plasma T_4 ng/ml	Plasma T_3 ng/ml	Plasma TSH ng/ml	Pituitary T_3nR fmol/AP
Normal	0	-	**40.0 ± 0.9 (114)	**0.74 ± 0.01 (155)	**1.89 ± 0.12 (60)	*24.0 ± 2.3 (23)
Hypothyroid	0	-	6.9 ± 0.3 (59)	0.27 ± 0.01 (59)	10.19 ± 0.36 (39)	11.6 ± 2.3 (8)
"	2	-	**100.4 ± 4.9 (48)	**0.50 ± 0.02 (51)	**5.76 ± 0.23 (46)	*17.7 ± 1.5 (5)
"	2	+	107.0 ± 6.3 (35)	++0.39 ± 0.01 (35)	++8.03 ± 0.36 (25)	+10.9 ± 0.6 (5)
"	5	-	**232.1 ± 17.1 (29)	**0.78 ± 0.04 (29)	**3.55 ± 0.32 (25)	*17.4 ± 3.8 (4)
"	5	+	209.2 ± 18.2 (17)	++0.43 ± 0.02 (17)	+5.08 ± 0.54 (17)	11.1 ± 3.2 (3)

Mean ± SEM; ( ) number of rats or experiments; *$p<0.01$; **$p<0.001$ vs hypothyroid rats; +$p<0.01$; ++$p<0.001$ T_4 + IOP vs T_4.

In conclusion, T_4 deiodination into T_3 in the thyrotrope cells is required not only for the control of TSH secretion, but also for the modulation of the T_3 nuclear receptor. Therefore, at the pituitary level, T_4 definitely acts as a prohormone.

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REFERENCES

1. In JH Oppenheimer and HH Samuels (eds), Molecular Basis of Thyroid Hormone Action, Academic Press, New York and London, 1983.
2. Oppenheimer JH, Schwartz HL, Surks MI, et al. Recent Prog Horm Res 32: 529, 1976.
3. Bernal J, Coleoni AH, and DeGroot LJ. Endocrinology 103: 403, 1978.
4. Larsen PR, Silva JE, and Kaplan MM. Endocr Rev 2: 87, 1981.
5. Thrall CL. Brain Res 279: 177, 1983.
6. Dozin B and De Nayer P. Neuroendocrinology 39: 261, 1984.
7. Hamada S and Yoshimasa Y. Endocrinology 112: 207, 1983.
8. Das DK and Ganguly M. Endocrinology 109: 296, 1981.
9. Coulombe P, Ruel J, Faure R, et al. Am J Physiol 245: E81, 1983.
10. Gordon A and Spira O. Endocrinology 96: 1357, 1975.
11. Lim VS, Passo C, Murata Y, et al. Endocrinology 114: 280, 1984.
12. Samuels HH, Perlman AJ, Raaka BM, et al. Recent Prog Horm Res 38: 557, 1982.
13. Franklyn JA, Ramsden DB, and Sheppard MC. Mol Cell Endocrinol 40: 145, 1985.
14. Von Overbeck L and Lemarchand-Beraud T. Mol Cell Endocrinol 33: 281, 1983.
15. Von Overbeck K and Lemarchand-Beraud T. Ann Endocrinol 45: 35, 1984.
16. Obregon MJ, Pascual A, Mallol J, et al. Endocrinology 106: 1827, 1980.
17. Kaplan MM. Neuroendocrinology 38: 254, 1984.

HUMAN NUCLEAR THYROID HORMONE RECEPTORS

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Nuclear thyroid hormone receptors are acidic, non-histone, chromatin associated proteins (1) with molecular weights of 47,000 - 57,000 (2,3) and bind T_3 with high affinity in the presence of sulfhydryl groups (4). It is generally believed that nuclear receptors mediate biological responses after binding thyroid hormone and induce activation of specific gene expression. Although nuclear thyroid hormone receptors exist in human tissues (5,6), most studies have been performed on rat tissues, including liver, kidney, and cultured pituitary cells. To further study nuclear thyroid hormone receptors in human tissues, we developed an improved isolation method for nuclei of cultured human hepatoma cells (Hep G2) and cultured human fibroblasts. The characteristics of nuclear receptors were compared in these cell lines.

MATERIALS AND METHODS

The established human liver cell line Hep G2 was derived from a caucasian child with primary hepatoblastoma (7). All experiments were performed after 110 transfer in cell culture. Fibroblast cultures were established from skin biopsy samples from 5 normal subjects and used after 4-12 transfers. Cells were cultured in 100 mm diameter plastic petri dishes in 10 ml of modified Eagle's medium containing 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). Forty-eight hours before the experiment, the growth media was changed to serumless Ham's F-10 media and this was also repeated 24 hours before the experiment. For nuclear isolation, cells harvested by trypsinization were washed with PBS and homogenized in 0.25 M sucrose, 1 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.85, 1 mM DTT, 0.1 mM PMSF (SMTD-PMSF). They were re-homogenized in 1% Triton X-100 and incubated for 15 minutes at 0°C. After centrifugation at 1,000 x g for 10 minutes, the pellet was suspended in SMTD-PMSF with 0.5% Triton X-100 and was gently homogenized. The pellet was then suspended in 2 M sucrose, 1 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, and centrifuged at 220,000 x g for 30 minutes. The resultant pellet was washed with SMTD-PMSF containing 1 mM $CaCl_2$ and used as purified nuclei. This preparation of nuclei gave 40-60% of recovery of DNA, a protein/DNA ratio of 2.5 - 3.0, and intact nuclei with no cytoplasmic contaimination by phase contrast microscopy. For preparation of nuclear extract (NE), nuclei were incubated with 0.3 M KCl, 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.0, 1 mM DTT (KMTD), for 60 minutes at 0°C, followed by centrifugation at 135,000 x g for 60 minutes to remove residual chromatin.

T_3 binding assays were performed on nuclei by incubating them with [^{125}I]-T_3 in SMTD, at room temperature for 2 hours, which was optimum for T_3 binding. Parallel duplicate tubes containing 3 x 10^{-7} M unlabeled T_3 were used for determination of nonspecific binding. The conditions for gel filtration were Sephadex G-150, column of inner diameter 1.5 cm, total volume 180 ml, elution buffer 0.3 M KCl + 2 mM EDTA + 10 mM Tris-HCl pH 8.0 + 10 mM 2-mercaptoethanol, and elution rate 6.6 ml/cm^2/h. For density gradient sedimentation, a 5 ml 8 - 35% wt/vol linear glycerol gradient in KMTD was centrifuged at 0 C at 60,000 rpm for 18 hours in a SW-65 rotor. For isoelectric focusing (IEF), 5% polyacrylamide gels containing 5% glycerol + 2% Pharmalyte + 1 mM DTT were utilized. [^{125}I]-T_3 labeled NE was deprived of free [^{125}I]-T_3 by Dowex resin. In IEF, the gel was prefocussed 500 Vh, and samples were focused at 3 C to 3,500 Vh with a separation distance of 9 cm. The pH gradient of the gel was determined by surface electrode and the gel was dried without fixation and radioautographed.

RESULTS

Table 1 summarizes studies performed on Hep G2 and cultured human fibroblast nuclear thyroid hormone receptors. T_3 binding studies show that Hep G2 cells have less receptors (21 fmol/100 µg DNA) than fibroblasts (55 fmol/100 µg DNA), but the affinity (Ka) is the same (2.1 x 10^{10} M^{-1}). 6 - 20% of the receptors were released during nuclear incubation without changing its affinity to T_3. T_4 showed 10 - 20 times less affinity but the same capacity in comparison to T_3 in both nuclei and released receptors. Triiodothyroacetic acid is the most potent competitor for [^{125}I]-T_3 binding to receptors followed by L-T_3, D-T_3, and L-T_4. Molecular size of receptor was indistinguishable between these cell lines when studied by gel filtration (Stokes radius of 34 Å and molecular weight of 59,000) and density gradient sedimentation analysis (sedimentation coefficient of 3.4 S). Heat inactivation studies revealed almost identical half-life (t-1/2) of T_3 binding activity at 45 C for both receptors. In this study, the velocity of the inactivation process at any moment of time depends upon the remaining binding activity, suggesting that it is a chemical reaction of the first order. Isoelectric focusing (IEF) studies showed pI of receptors at 5.3 - 5.5, 5.7, and 5.9 in Hep G2, and 5.3 - 5.4, 5.7, and 5.9 in human fibroblasts.

DISCUSSION

Aside from lower binding capacity in Hep G2 and minor difference in the IEF pattern, the characteristics of nuclear thyroid hormone receptors in Hep G2 cells and human fibroblasts are near identical, as assessed by Ka for T_3, relative affinities to iodothyronine analogues, molecular size, and heat lability, suggesting a similar structure of receptor protein in these cells. Hep G2 nuclear T_3 binding capacity per cell is half of that of human fibroblasts and 1/4 of rat liver nuclei, which capacity is almost identical to human liver nuclei (6). Lower T_3 binding capacity in Hep G2 nuclei may be related to malignant transformation of liver cells or difference in conditions under which studies were performed (cell culture vs surgically removed liver tissues). Multiple receptor forms shown by IEF may represent structurally different receptor molecule, post translational modification of receptor protein, or association of receptor with other molecules such as DNA or histones. Although we have no clear explanation for slight differences in the acidic part of the receptor, the overall IEF pattern is quite similar between fibroblast and Hep G2 nuclear receptors.

Table 1. Characteristics of Nuclear Thyroid Hormone Receptors in Cultured Human Hepatoma Cells (Hep G2) and Cultured Human Fibroblasts

Cells	Binding Characteristics for T_3	Relative Affinity for Iodothyronines	Sephadex G-150 Gel filtration		Glycerol Gradient Sedimentation	Heat Inactivation Study
	Isolated Nuclei		Stokes Radius	Molecular Weight	Sedimentation Coefficient	t-1/2 at 45°C
Hep G2	Ka = 2.1 x 10^{10} M^{-1} MBC = 21 fmol/100 µgDNA (16%)	Triac ∨ L-T3 ∨ L-T4	34 Å	59,000	3.4 S	7.9 minutes
Fibroblast	Ka = 2.1 x 10^{10} M^{-1} MBC = 55 fmol/100 µgDNA (12%)	Triac L-T3 ∨ D-T3 ∨ L-T4	34 Å	59,000	3.4 S	7.2 minutes

Numbers in parentheses indicate amount of receptors released into media during nuclear incubation expressed as % of total nuclear receptors.

SUMMARY

An improved isolation method was developed for nuclei of cultured human fibroblast and Hep G2 cells. Using this nuclear preparation, Ka for T_3, relative affinity of iodothyronine analogues, molecular size, and heat inactivation pattern of the nuclear receptors were indistinguishable between these cell lines. However, Hep G2 cells have a binding capacity significantly below fibroblasts and normal liver.

REFERENCES

1. Surks MI, Koerner D, Dillman W, et al. J Biol Chem 248: 7066, 1973.
2. Latham KR, Ring JC, and Baxter JD. J Biol Chem 251: 7388, 1976.
3. Pascual A, Casanova J, and Samuels HH. J Biol Chem 257: 9640, 1982.
4. DeGroot LJ, Refetoff S, Strausser J, et al. Proc Natl Acad Sci USA 71: 4042, 1974.
5. Bernal J, Refetoff S, and DeGroot LJ. J Clin Endocrinol Metab 47: 1266, 1978.
6. Schuster LD, Schartz HL, and Oppenheimer JH. J Clin Endocrinol Metab 48: 627, 1979.
7. Knowles BB, Howe CC, and Aden DP. Science 209: 497, 1980.

ONTOGENY OF THYROID DURING EMBRYOGENESIS: EVIDENCE OF TWO THYROXINE BINDING SITES*

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INTRODUCTION

Evidences gathered over the past ten years indicate that triiodothyronine (T_3) is the thyroid hormone responsible for the metabolic effect (1). However, there are particular situations in which little or no T_3 is produced by the organism and, therefore, it is not clear how the thyroid action will be expressed. One of these situations is the fetal or neonatal period, in humans and in various experimental animals. Very low amounts of T_3 are produced during this time, since the 5'-monodeiodinase is not yet fully developed (2-4). Therefore, thyroxine (T_4) is the only thyroid hormone present in large quantities during a period of great hormonal need because of the rapid maturation of several target tissues. Such a situation is particularly evident in chick embryo where most of the target organs, particularly the nervous system, develop between 7-12 days (5), when the concentration of T_3 in the serum is very low (6).

In an attempt to better our understanding of the action of T_4 during chick embryogenesis, we performed more detailed studies on the ontogeny of T_4 association constants and maximal binding capacities by solubilized nuclear receptors from various target tissue.

MATERIALS AND METHODS

Golden Comet chick embryo were obtained from a local hatchery. ^{125}I-T_4 (S.A.: 1.2 - 1.8 Ci/gm) was purchased from Amersham Corp. (Oakville, Ont., Canada). Radioinert T_4 and T_3 were from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade. The experiments were performed on liver, brain, and lungs from embryos of 9, 12, 17, and 19 days of age. The solubilized nuclear receptor was obtained according to the technique of Silva et al. (7). Purified nuclei were prepared as previously described (8). The binding assays were carried out on aliquots of 150 µl of nuclear extracts, which were incubated with ^{125}I-T_4 (1×10^{-10} M, final concentration) and increasing amounts of unlabeled hormone (10^{-10} - 10^{-5} M) as described in the respective techniques (7,8). ^{125}I radioactivity was

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determined in a Beckman L-9000 (Beckman Instr., Palo Alto, CA) gamma spectrometer. The binding data were assessed by plotting the dose-displacement data, expressed as the bound to free ratio (B/F) vs the bound (B) hormone. The nonlinear Scatchard plots were analyzed according to a two-site model by an iterative process to nonlinear regression on an IBM 155 computer (9). The analysis yielded the two association constants (K_1, K_2) and the maximal binding capacities (N_1, N_2). In a series of experiments we determined the binding parameters on the same nuclear preparation, before and after the 0.4 M NaCl extraction, in order to determine the extraction efficiency for the two sites. This efficiency varied between 52-100% with hepatic extracts, 36-75% with brain, and 47-56% with lung extracts. The values of N_1 and N_2 were corrected for DNA content (10).

RESULTS

Nuclear Binding of T_4

Fig. 1 shows representative Scatchard plots obtained with the binding data of brain nuclear extracts from embryo of 9 to 19 days. T_4 was bound

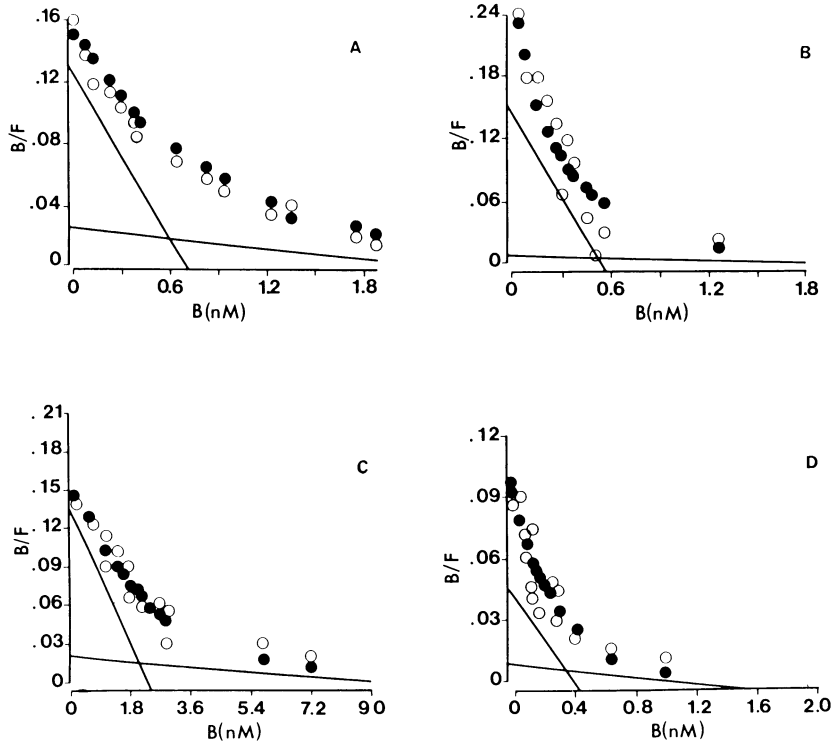

Fig. 1. T_4 binding to nuclear extracts from brain of chick embryo. Scatchard plots obtained with a two-site model on a computer system (9) showed two distinct binding sites. K_1: x 10^8 M^{-1}; K_2: x 10^7 M^{-1}; N_1, N_2: fmol/µg/DNA. Day 9 (A): K_1 = 1.7, N_1 = 1.1, K_2 = 3.2, N_2 = 3.2; Day 12 (B): K_1 = 2.7, N_1 = 2.0, K_2 = 3.3, N_2 = 6.6; Day 17 (C): K_1 = 5.8, N_1 = 0.74, K_2 = 1.8, N_2 = 8.6; Day 19 (D): K_1 = 1, N_1 = 0.9, K_2 = 1.4, N_2 = 4.4.

Table 1. Ontogeny of T_4 Binding Sites During Chick Embryogenesis

	9 days				12 days				17 days				19 days			
Organ	K_1	K_2	N_1	N_2	K_1	K_2	N_1	N_2	K_1	K_2	N_1	N_2	K_1	K_2	N_1	N_2
Liver	3±2	2.3±2	2.4±2	14±6	4±1	1±0.5	1±0.7	5±1	4±2	3±1	0.9±0.6	7±3	5±3	1.5±0.4	0.7±0.5	5±3
Brain	4±2	2±1	0.9±0.6	5±2	3±1	3±0.7	2±0.8	7±2	8±3	2±1	0.7±0.2	8±3	1.3±0.4	1.2±0.5	1±0.2	5±2
Lungs					4±1	0.4±0.1	2±0.2	4±1	5±3	3±2	0.3±0.2	4±1	2±1	2±0.2	0.5±0.2	16±5

K_1 = x 10^8 M^{-1}
K_2 = x 10^7 M^{-1}
N_1, N_2 = Fmol/µg DNA

to two different sites. K_1 varied between 1.1 - 6.6 x 10^8 M^{-1} and K_2 between 1.4 - 3.2 x 10^7 M^{-1}. N_1 increased from 1.1 fmol/µg DNA at day 9 to 22 at day 12 and they fell to 0.7 at day 19. N_2 went from 3.2 fmol/µg at day 9 to 6.6 at day 12, 8.6 at day 17, and 4.4 at day 19.

Ontogeny of T_4 Receptors

Table 1 shows the variations of T_4 K_1, K_2, N_1, and N_2 during chick embryogenesis. K_1 was of the order of 10^8 M^{-1} in all organs, whereas K_2 was 10^7. Both association constants showed little variations during embryogenesis, except for a slight increase of K_1 in brain at day 17. In liver the concentrations of both sites were high during the early phase of embryogenesis and then they decreased until the hatching period. In brain they increased from day 9 to day 12 and 17 and then they also fell, whereas in lungs, N_1 decreased and N_2 increased towards the end of embryogenesis.

DISCUSSION

Our data clearly indicate that T_4 binds to two distinct binding sites in target tissue during chick embryogenesis. Although previous reports (1) have proposed the existence of only a single binding site for both thyroid hormones, recent reports suggest that there is more than one site (11,12). Of interest is that such observations have all been made in another developmental model, the tadpole. Furthermore, chromatographic studies have demonstrated the existence of two T_4 binding proteins in the brain of the neonatal rat (13). Therefore, our data confirm such previous findings and reaffirm the demonstration that studies conducted in developmental models may give further insight into the mechanism of action of thyroid hormone. The presence of a T_4 first site with high affinity and very low capacity during early embryogenesis, when T_3 concentration is low, T_4 is high, and tissue development very rapid, suggests that the latter is most likely the active hormone. As for the second site which has a lower affinity and a very high capacity, it could represent a mean for concentrating the maximum amount of hormone at the nuclear level during the period of rapid maturation of target tissues. The ontogenic pattern showing a decrease of both sites toward the end of the embryogenesis when T_3 starts to increase lend support to this hypothesis. In conclusion, our data, as well as those observed in other experimental models, suggest that during early fetal life T_4 may be the hormone mainly responsible for promoting growth of target tissues.

REFERENCES

1. Oppenheimer JH. Science 203: 971, 1979.
2. Fisher DA, Dussault JH, Sack J, et al. Rec Prog Horm 33: 59, 1972.
3. Dubois JD and Dussault JH. Endocrinology 101: 435, 1975.
4. Borges M, Labourene J, and Ingbar SH. Endocrinology 107: 1751, 1980.
5. Hanaway J. J Comp Neurol 131: 1, 1967.
6. Millette F, Lehoux JG, and Bellabarba D. Endocrinology 108: 254, 1981.
7. Silva ES, Astier H, Thakare U, et al. J Biol Chem 248: 7066, 1977.
8. Bellabarba D and Lehoux JG. Endocrinology 109: 1017, 1981.
9. Rodbard D. Adv Exp Med Biol 36: 289, 1973.
10. Giles KW and Myers A. Nature 206: 93, 1965.
11. Galton VA and Schaafsma J. Endocrinology 112: 1999, 1983.
12. Moriya T, Thomas CR, and Frieden E. Endocrinology 114: 170, 1984.
13. Margarity M, Matsokis N, and Valcana T. Mol Cel Endocrinol 31: 333, 1983.

INSULIN, GLUCOSE, AND GLUCAGON ARE POTENTIAL MODULATORS OF THYROID HORMONE ACTION THROUGH BROWN ADIPOSE TISSUE 5'-DEIODINASE IN RATS

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Brown adipose tissue (BAT) contains Type II iodothyronine 5'deiodinase (5'D) (1). At variance with other tissues containing this enzyme, BAT 5'D is markedly stimulated by the sympathetic nervous system (2). This stimulation is physiologically relevant since it results in several-fold increments in locally-produced BAT T_3 and generation of circulating T_3 (3). These results, previously reported by us, suggest that BAT may become an important source of circulating T_3 and, further, that T_3 may have an important physiological role in the function of this tissue. Probably the main function of BAT is to generate heat through the oxidation of fatty acids uncoupled from ATP generation, which in turn is important for body temperature regulation and for dissipation of excess calories in the overfed state. Conversely, fasting and carbohydrate deprivation depress thermogenesis and the metabolic activity of BAT. We observed earlier that an adequate glucose supply was necessary for the elevation of BAT 5'D to occur following hypophysectomy. Altogether, these observations prompted us to examine the influence of insulin, glucagon, carbohydrate feeding, and fasting on BAT 5'D and its responses to various stimuli.

5'D was measured in homogenates of BAT after floating the fat by low speed centrifugation. The assay conditions were the same as those reported repeatedly by our group, namely 2 nM rT_3 in the presence of 20 mM dithiothreitol (DTT) and 1 mM propylthiouracil (PTU) (4,5). Although the absolute values of enzyme activity may vary depending on the relative amounts of PTU and DTT, the relative physiological responses are independent of these particular assay conditions. Other methodological aspects will be addressed with the description of the various experiments.

Although the first observation that led us to perform these studies was the failure of BAT 5'D to increase after hypophysectomy when the supplement of glucose in the drinking water was withheld (6), we will examine first the effects of single injections of insulin or glucagon on BAT 5'D. Doses of insulin and glucagon were chosen that would elicit maximal or near maximal responses. One U insulin/100 g BW or 50 μg glucagon/100 g BW were injected intravenously and the responses of BAT 5'D were measured at various intervals thereafter. Glucagon exhibited a more prompt response with a significant increase in BAT 5'D 1 hour and a maximal response 2 hours after the injection. No detectable response to insulin was observed during the first hour and the maximal response occurred at 4 hours. Although the dose of insulin was subsequently found to be maximal, that of glucagon was

Table 1. Effect of Cycloheximide on Insulin- or Glucagon-induced BAT 5'D Stimulation

Hormone	Control	Stimulated	Stim + Cyclh
Insulin (Expt. 211)	100 ± 7	850 ± 88	112 ± 25
Glucagon (Expt. 288)	100 ± 25	451 ± 79	25 ± 5

All values are the mean ± SEM from 4-5 rats where the enzyme activity has been expressed as percent of the values in untreated rats (15-50 fmol I/h/mg prot).

submaximal but greater than the ED_{50}. Nonetheless, even using maximal doses of both hormones, the responses to glucagon were always smaller than to insulin. The differences in time of onset and maximal responses suggest that these hormones operate via different mechanisms.

Based on these results, the dose-response relationships were examined two hours after the intravenous injections of glucagon or four hours after intravenous injections of insulin. Maximal responses were observed with 1 U insulin or 100 µg glucagon per 100 g BW. The response to various doses of glucagon appeared biphasic, with lower responses following doses greater than 100 µg/100 g BW.

To explore further the mechanism of insulin- and glucagon-induced elevation of BAT 5'D, we examined the effect of pretreating the rats with cycloheximide. The drug was given at 10 mg/100 g BW intraperitoneally one half hour prior to the insulin or glucagon. The results are shown in Table 1. In this, as in subsequent tables, the results (most of the time obtained on separate days for each of the stimuli) are presented as percent of the corresponding controls, which almost invariably fell within the 15-50 fmoles of I^-/h/mg protein. In the experiment in Table 1, the rats were injected either with 1 U insulin or 100 µg glucagon/100 g BW intraperitoneally. We observed the same 8-9 fold response to insulin and 4-5 fold response to glucagon. Most importantly, BAT 5'D responses to both hormones were abolished by the preadministration of cycloheximide.

Table 2. Effects of Carbohydrate Overfeeding or Catecholamine Depletion on BAT 5'D Response to Insulin

Control	Sucrose	Insulin	INS + SUC	INS + MPT
100 ± 7	94 ± 19	850 ± 88	1381 ± 206	1043 ± 219

All the values are the mean ± SEM from 5-6 rats where the enzyme activity has been expressed as percent of the control (15-50 fmol I/g/mg prot). Sucrose: 10% in drinking water. Insulin: 1.00 U/100 g BW subcutaneously 4 hours before. MPT: alpha methyl-p-tyrosine, 20 mg/100 g BW intraperitoneally 24, 16, and 4 hours before.

Table 3. Effects of Fasting or Feeding Carbohydrates on the Response of BAT 5'D to Various Stimuli

Expt	Stimulus	Fed	Fasted	Carbohydrates
290	Norepinephrine	2959 ± 506	299 ± 28*	2763 ± 678
292	Isoproterenol	370 ± 28	246 ± 14*	482 ± 71
288	Glucagon	279 ± 79	63 ± 6*	451 ± 80
211	Insulin	380 ± 38	---	613 ± 93†

All values are the mean ± SEM from 3-5 rats where the enzyme activity has been expressed as percent of the enzyme activity in normal untreated rats (15 fmol I/h/mg prot). *$p<0.05$ vs Fed group; †$p<0.001$ vs Fed group. Doses (per 100 g BW) and timing before killing (hours): NE: 40 µg sbc, 3 h; ISO: 20 µg sbc, 3 h; GLUC: 100 µg ip, 3 h; INS: 1 U sbc, 4 h; CHO: 10% sucrose (insulin) or 10% glucose (rest) in drinking water for 24-36 h.

Since glucose has been reported to increase the norepinephrine turnover in BAT through hypothalamic stimulation and insulin-induced hypoglycemia elicits a rather marked and generalized adrenergic response, we explored the possibility that insulin could exert its effect via such mechanisms. The results of such an experiment are shown in Table 2. The administration of carbohydrate as 10% sucrose in the drinking water for 48 hours did not affect BAT 5'D. Insulin, 1 U/100 g BW subcutaneously, induced ∿8-fold increase in enzyme activity, but a ∿14-fold increase in animals given sucrose ($p<0.05$). On the other hand, catecholamine depletion induced by the administration of alpha-methyl-p-tyrosine did not affect the response to insulin. The regimen of MPT used has proven to be sufficient to abolish the BAT response to cold stress (2) and depletes the BAT of norepinephrine (kindly measured by Dr. J. Young at Beth Israel Hospital, Boston). These results indicate that the effect of insulin is not mediated through hypoglycemia or the sympathetic nervous system. Furthermore, the concomitant administration of carbohydrates enhances the response to insulin.

We would like to examine now the effect of fasting or feeding carbohydrates on the responses of BAT 5'D to various stimuli. The results are presented in Table 3. In the fed state, we observed a 30-fold increase in BAT 5'D activity following the injection of norepinephrine (NE), a modest but significant response to isoproterenol, and responses to insulin and glucagon somewhat lower than those shown previously, probably due to slightly different experimental conditions (Table 3). Fasting resulted in a marked reduction in the responses to all of these stimuli ($p<0.001$) (insulin was not tested in the fasted state). The administration of carbohydrates, 10% glucose in the drinking water for 36 hours, did not augment the response to the various stimuli except for insulin as shown previously (Table 2). These findings suggest that carbohydrates and/or fed state are a permissive condition for the stimulation of BAT 5'D. However, under these circumstances at least, carbohydrate overfeeding cannot amplify the responses of the enzyme. We have observed that carbohydrates do increase enzyme activity, albeit modestly, in the absence of exogenous stimulation, provided they are given for 48 hours or longer, an effect which is probably mediated through augmented insulin secretion.

Table 4. Effect of Streptozotocin-induced Diabetes on BAT 5'D Responses to Various Stimuli

Stimulus	Normal	Diabetic	P
Untreated	100 ± 10	38 ± 3	<0.001
Norepinephrine	1638 ± 296	706 ± 47	<0.001
Isoproterenol	967 ± 93	300 ± 70	<0.001
Glucagon	467 ± 83	63 ± 10	<0.001
Insulin	---	3090 ± 486	---

All values are the mean ± SEM from 5 rats where the enzyme activity has been expressed as percent of the control values (30 ± 3 fmol I/h/prot). Plasma glucose was 365 ± 39 and 94 ± 5 in diabetic and normal rats, respectively. Insulin was given 24 h (2 U) and 5 h (1 U) before. Plasma glucose in these animals was 235 ± 49 mg/dl.

It also seemed of interest to explore the responses of BAT 5'D in diabetes mellitus, since this condition combines reduced levels of insulin with high blood sugar and elevated plasma glucagon. Diabetes was induced by the injection of 5 mg of streptozotocin/100 g BW. Only the rats with documented glucosuria were used between 7-10 days after the injections. The results are summarized in Table 4. The basal BAT 5'D in the diabetic rats was reduced. Whereas in the normal animals we observed the usual responses to NE, isoproterenol, and glucagon, in the diabetic animals the activity levels of BAT 5'D after these manipulations were significantly lower. Interestingly, the response to insulin, if anything, was exaggerated in the diabetic animals (compared with 5-10 fold increases observed in normal animals as shown above). These results suggest that the intracellular availability of glucose is essential for the BAT 5'D responses. Note that the insulin regimen given to these animals did not normalize the plasma glucose, although it improved it significantly. This decreases the chances that the lack of response to other stimuli was a nonspecific consequence of the decompensated diabetes, although this possibility cannot be entirely excluded.

In summary, we can conclude that: 1) glucose and insulin are essential for maximal BAT 5'D responses to various stimuli. 2) The effect of insulin is potentiated by the availability of glucose and is not mediated through the sympathetic nervous system. 3) Glucagon is also a stimulus for BAT 5'D by mechanisms seemingly different from insulin, although both hormones require protein synthesis for their effects. 4) Given the thermogenic response to overfeeding and glucagon, and the increments in BAT T_3 concentration that follow these degrees of stimulation of BAT 5'D, these studies suggest that T_3 and BAT 5'D play a key role in the thermogenic response of BAT.

REFERENCES

1. Leonard JL, Mellen SA, and Larsen PR. Endocrinology 112: 1153, 1982.
2. Silva JE and Larsen PR. Nature 305: 712, 1983.
3. Silva JE and Larsen PR. J Clin Invest, 1985 (in press).

4. Visser TJ, Leonard JL, Kaplan MM, et al. Proc Nat Acad Sci 79: 5080, 1982.
5. Silva JE, Leonard JL, Crantz FR, et al. J Clin Invest 69: 1176, 1982.
6. Silva JE and Larsen PR. 1985-86 (submitted for publication).

EFFECT OF THYROID HORMONES, FOOD DEPRIVATION, AND COLD EXPOSURE ON THYROID T_4 OUTER RING MONODEIODINATING ACTIVITY (5'MDI) IN MICE

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Active iodothyronine inner-ring and outer-ring monodeiodinases (5 and 5'MDI) are present in thyroid (1-3). The evidence suggests they are maintained and can be stimulated by TSH (3,4). More recently, we have shown that they are extremely sensitive to TSH stimulation and the increase is dose-dependent (5). Furthermore, we have demonstrated that LATS-rich patient serum stimulates T_4 5'MDI in mouse thyroid (6), and appears to correlate well with the kinetic data (7,8) which suggest a preferential secretion of T_3 from thyroid in hyperthyroid patients. The present studies examine the effect of thyroid hormones, starvation, and cold on thyroid 5'MDI in comparison with those in liver, kidney, or brown adipose tissue (BAT).

MATERIALS AND METHODS

Female Swiss Webster albino mice (20-25 g) were maintained on regular animal chow except in the study of effect of starvation in which chow was removed 72 h prior to sacrifice. Water was available to all animals *ad libitum*. TSH (0.02 - 0.2 IU/d), T_4 (2 µg/g BW/d), or T_3 (1 µg/g BW, tid) was given intraperitoneally for 1-7 days as specified in each experiment. Puromycin (0.4 mg/g BW, IP) was given one hour prior to cold exposure. Controls received equivalent volume of solvent (saline). T_4 5'MDI in thyroid, liver, and renal cortex was assayed according to the methods reported previously (5). BAT T_4 5'MDI activity was examined *in vitro* by modification of a method reported earlier (9). The assay mixture (final volume, 250 µl) consisted of 10 mg wet tissue weight equivalent of tissue homogenate (BAT infranatant) suspended in 256 mM sucrose - 8 mM HEPES buffer (pH 7.0) containing 200 mM dithiothreitol (DTT) and 1.25 µM T_4. Incubations were carried out at 37°C for 60 min, after which 2 volumes of 95% ethanol were added. T_3 produced in the incubation reactions was measured by radioimmunoassay as previously described (5). Results are expressed as mean $\pm$ SEM picomoles T_3 produced per mg protein (p) per h. Statistical analyses were performed with Student's two-tailed t test for unpaired data modified with Bonferroni's inequality for multigroup comparison (10).

RESULTS

Effect of T_3 and T_4 on T_4 5'MDI in Thyroid, Liver, and Kidney

In mice treated with pharmacological doses of T_4 or T_3 for 1 week, thyroid 5'MDI decreased significantly (60 ± 3.4 and 65 ± 3.1 pmol T_3/mg p/h in T_4- and T_3-treated mice, respectively, vs 95 ± 9.4 pmol T_3/mg p/h in saline control, $p<0.02$). Hepatic 5'MDI, on the other hand, increased markedly (696 ± 10 and 384 ± 18 pmol T_3/mg p/h in T_4- and T_3-treated mice, respectively, vs 130 ± 8.8 pmol T_3/mg p/h in saline, $p<0.0001$). Similarly, renal cortical 5'MDI activity was stimulated by T_4 treatment (128 ± 7.2) as compared to saline control 13 ± 1.2 pmol T_3/mg p/h, $p<0.001$).

Effect of Starvation on T_4 5'MDI in Thyroid, Liver, and Kidney

As shown in Fig. 1, a 3-d fast reduced the thyroid 5'MDI to 80% of fed control (97 ± 7.1 vs 121 ± 6.5 pmol T_3/mg p/h, $p<0.05$). The decrease induced by fast was not reversed by the supplementation of SH-reagent (DTT, 0.2 - 4 mM) (unpublished observation). The thyroidal activities, however, were normalized by TSH administration (0.2 IU/d, Fig. 1). Similar changes were observed in liver and renal cortex.

Effect of Cold Exposure on Thyroid and BAT T_4 5'MDI Activity

A 3- or 72-h cold (6-8°C) exposure did not alter thyroid 5'MDI (123 ± 5.6 and 128 ± 5.4 pmol T_3/mg p/h, respectively, vs control, 115 ± 5.4 pmol

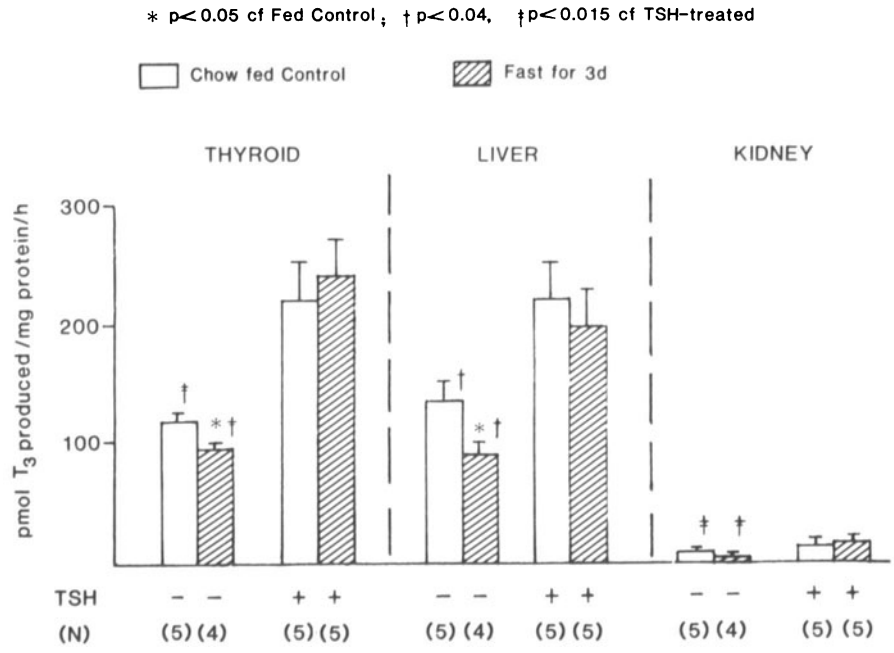

Fig. 1. Effect of 3 d fast, with or without TSH treatment, on thyroid, liver, and kidney T_4 5'MDI. Mice were given saline or TSH (0.02 IU/d ip) during the 3 d fast. The tissue T_4 5'MDI were studied *in vitro* in a reaction mixture containing 0.5 mg equivalent tissue homogenate, 4 mM DTT, 2.5 μM T_4, and 120 mM phosphate buffer (pH 7.0). (N), the number of groups of mice (usually three mice per group). Values given are the mean ± SEM.

T_3/mg p/h, $p>0.05$). While BAT 5'MDI increased markedly (1.91 ± 0.15 and 1.14 ± 0.13 pmol T_3/mg p/h on 3 and 72 h cold exposure, respectively, vs control 0.39 ± 0.05 pmol T_3/mg p/h, $p<0.0001$).

The Effect of T_4 Treatment (2 μg/g BW/d for 7 d) on BAT and Thyroid 5'MDI Response to Cold or TSH

As shown in Fig. 2, BAT 5'MDI was significantly higher following cold exposure for 3 h (2.05 ± 0.45 vs 0.59 ± 0.25 pmol T_3/mg p/h of room temperature control, $p<0.0001$). Puromycin (0.4 mg/g BW) prevented the cold-induced increase in BAT 5'MDI when given to mice one hour prior to cold stress (0.66 ± 0.08 and 0.41 ± 0.07 pmol T_3/mg p/h in cold exposure plus puromycin and room temperature control, respectively, vs 1.87 ± 0.20 pmol T_3/mg p/h in cold exposure without puromycin). Pretreatment of mice with pharmacological dose of T_4 for 1 wk also completely suppressed the BAT 5'MDI in response to cold (Fig. 2, lower panel). By contrast, thyroid 5'MDI preserved its 5'MDI preserved its capacity in response to a single dose of TSH stimulation (0.2 IU) following T_4 treatment for 1 wk (72 ± 3.2 and 45 ± 3.4 pmol T_3/mg p/h in with and without TSH-treated mice on T_4, respectively, $p< 0.001$) (Fig. 2, upper panel).

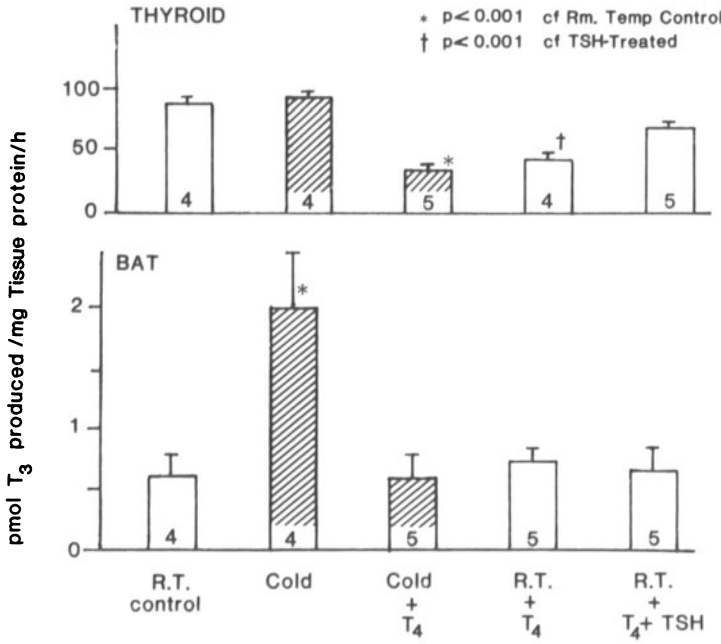

Fig. 2. Effect of 3 h cold exposure on BAT and thyroid T_4 5'MDI in mice with or without T_4 treatment (2 μg/g BW/d for 7 d). In one study, mice also received a single dose of TSH (0.2 IU) 24 h prior to sacrifice in addition to T_4. The shaded bar represents groups of mice being exposed to cold (6-8°C) for 3 h. The number in each bar represents groups of mice (usually three mice per group) in each study. Values given are mean ± SEM.

DISCUSSION

The TSH-induced increase of thyroid iodothyronine monodeiodinase is associated with increased serum T_3 and T_4, as well as hepatic and renal deiodinase activities (5). Both T_3 and T_4 are potent inducers for hepatic 5'MDI and 5MDI (data not presented) as shown in the present study and in a previous report (11). The pharmacological doses of T_3 or T_4, however, significantly reduce thyroid 5'MDI. The present study demonstrates conclusively that the TSH-mediated induction of thyroid 5'MDI is not a result of TSH-induced increase of circulating thyroid hormones, but a direct effect of TSH. Excess circulating T_3 or T_4 stimulates, while the hypothyroid state reduces the hepatic and renal 5'MDI (12). The enzyme activity in other tissues, i.e., thyroid (4), BAT (13), brain (14), and pituitary gland (15), in contrast, increases significantly in the hypothyroid state. Considering that the body would need less of the more potent thyroid hormone, T_3, in the hyperthyroid state, teleological arguments would suggest that liver deiodinates all the way to inactive compounds, thus behaving as an organ of detoxication, presumably protecting the organism from being exposed to potentially toxic levels of T_4 and T_3 in hyperthyroidism.

Kinetic studies of thyroid hormones in total caloric deprivation have revealed that the T_4 production rate is unimpaired and T_3 production is markedly decreased (16). Hepatic and renal 5'MDI are decreased as shown in the present study, as well as in others, and are thought to be responsible for the resulting "low T_3 syndrome" in starvation. The present study, however, provides a possible additional mechanism, i.e., decreased thyroidal 5'MDI and, consequently, a possible decreased secretion of T_3 from the thyroid. Furthermore, the conversion defect can be corrected by exogenous TSH, suggesting that it may be mediated through TSH which is decreased in starvation (17). Even though TSH appears to reverse 5'MDI deficiency in liver and kidney as shown in the present study (Fig. 1), the reversals in liver and kidney are likely due to a secondary rise of circulating thyroid hormones following TSH administration, and not a direct effect of TSH.

Acute cold exposure is associated with increased TSH secretion as a result of augmented production of TRH (18). The present study, however, showed that 3 and 72 h cold exposure in mice resulted in increased 5'MDI in BAT but not in thyroid. This result suggests that thyroid 5'MDI may not be involved in acute cold adaptation.

SUMMARY

Thyroid T_4 5'MDI is stimulated by TSH and suppressed by pharmacological doses of T_4 or T_3, while the thyroid hormone treatment markedly stimulates T_4 5'MDI in liver and kidney. In mice treated with pharmacological doses of T_4 for 7 days, the thyroid 5'MDI remains responsive to a single dose of TSH. Starvation significantly decreased thyroid 5'MDI and the reduction is not related to the status of sulfhydryl group and is reversed by TSH. Cold exposure for 3-72 h does not alter thyroid 5'MDI but markedly stimulates BAT 5'MDI.

CONCLUSIONS

1) Increased 5'MDI in thyroid after TSH is a direct effect of TSH rather than a secondary effect of increased circulating thyroid hormones as in liver and in kidney. Therefore, thyroid 5'MDI, not liver or kidney, may be important to an animal's adaptation to primary hypothyroidism.

2) The decrease of thyroid 5'MDI in starvation, which is mediated through TSH, may be involved in the pathogenesis of "low T_3 syndrome" in total caloric deprivation.

3) BAT, rather than thyroid, 5'MDI may be involved in the acute cold adaptation in adult mice.

REFERENCES

1. Erickson VJ, Cavalieri RR, and Rosenberg LL. Endocrinology 108: 1257, 1981.
2. Ishii H, Inada, M, Tanaka K, et al. J Clin Endocrinol Metab 57: 500, 1983.
3. Wu, SY. Endocrinology 112: 417, 1983.
4. Erickson VJ, Cavalieri RR, and Rosenberg LL. Endocrinology 111: 434, 1982.
5. Wu SY, Reggio R, and Florsheim WH. Endocrinology 116: 901, 1985.
6. Wu SY, Reggio R, Florsheim W, et al. Prog Endocrine Soc, 64th Annual Meeting (abstract), 1982, p 396.
7. Inada M, Kasagi K, Kurata S, et al. J Clin Invest 55: 1337, 1975.
8. Faber J, Lumkoltz IB, Kirkegaard C, et al. Endocrinology 16: 199, 1982.
9. Glick Z, Wu SY, Lupien J, et al. Am J Physiol 249: E000, 1985.
10. Glantz SA. Primer of Biostatistics, McGraw-Hill, New York, 1981, pp 30-93.
11. Grussendorf M and Hefner M. Clin Chimica Acta 80: 61, 1977.
12. Kaplan MM and Utiger RD. Endocrinology 103: 156, 1978.
13. Leonard JL, Mellen SA, and Larsen PR. Endocrinology 112: 1153, 1983.
14. Kaplan MM and Yaskowski KA. Endocrinology 110: 761, 1982.
15. Visser TJ, Kaplan MM, and Leonard JL. J Clin Invest 71: 992, 1983.
16. Eisenstein A, Hagg S, Vagenakis A, et al. J Clin Endocrinol Metab 47: 889, 1978.
17. Croxson MS, Hall TD, Kletsky OA, et al. J Clin Endocrinol Metab 45: 560, 1977.
18. Hefco E, Krulich L, Illner P, et al. Endocrinology 97: 1185, 1975.

3,3',5'-TRIIODOTHYRONINE (rT_3) INHIBITS IODOTHYRONINE-5'-DEIODINATING ACTIVITY INDUCED BY EQUIMOLAR CONCENTRATION OF 3,5,3'-TRIIODOTHYRONINE (T_3) IN CULTURED FETAL LIVER OF MOUSE

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INTRODUCTION

rT_3 is generally considered to be without thyroid hormone activity at physiological concentrations and to play no significant physiological role. However, several agonistic and antagonistic effects of rT_3 were reported at supraphysiological doses (1,2). As is well known, there exists little or no iodothyronine-5'-deiodinating activity (I-5'-DA) in the fetal liver of rodents (3). The enzyme activity rapidly increases after birth and peaks at 3-4 weeks in the case of mouse (4). We recently demonstrated that this ontogenetic development of iodothyronine-5'-deiodinase in the liver can also be induced *in vitro* by physiological concentrations of glucocorticoid and thyroid hormones (5). To investigate the rT_3 effect on I-5'-DA, fetal liver of mouse on the 19th day of gestation, in which little or no I-5'-DA was detected and, therefore, rT_3 was very stable, was cultured for 3-15 days, and I-5'-DA was determined in liver homogenate.

MATERIALS AND METHODS

Fetal liver on the 19th day of gestation was cultured as described previously (5). Livers of 20-24 fetuses from two pregnant mice were cut into explants of less than 1 mm^3 and were cultured at 37°C in 12 ml of Dulbecco-Vogt medium supplemented with 6% thyroid hormone-depleted fetal calf serum (6), penicillin, streptomycin sulfate (100 µg/ml), hydrocortisone hemisuccinate (0.2 µg/ml), and various concentrations of thyroid hormones. At the end of incubation, hepatocytes were homogenized in ice-cold 0.25 M sucrose solution containing 10 mM Tris-HCl buffer and I-5'-DA was assessed as described elsewhere (7). In brief, hepatocyte homogenates (10-100 µg) were incubated in 200 µl of sodium phosphate buffer (100 mM, pH 7.0) containing 10 mM DTT, 1.3 mM EDTA, and 10^{-7} M or 10^{-6} M [3',5'-^{125}I] rT_3 (∿30,000 cpm). After 15 min (for 10^{-7} M ^{125}I-rT_3) or 120 min (for 10^{-6} M ^{125}I-rT_3) incubation at 37°C, the reaction was stopped by adding 10% TCA. The enzyme activity was expressed at picomoles of ^{127}I-/mg of protein/min.

RESULTS

As reported previously (5), little or no I-5'-DA was detected in the fetal liver cultured in medium supplemented with insulin and hydrocortisone

but without thyroid hormone (Table 1). Addition of T_3 distinctly induced the enzyme activity in the fetal liver. The activity was detectable after 2-4 days culture and peaked at 12-15 days culture. rT_3 per se could not induce the enzyme activity in the fetal liver at 10^{-7} - 10^{-6} M. However, when rT_3 was added to the medium supplemented with insulin, hydrocortisone, and T_3 (10^{-8} M) throughout the culture period (for 10-12 days), rT_3 distinctly inhibited I-5'-DA induced by 10^{-8} M T_3, and this inhibitory effect was statistically significant by paired t analysis ($p<0.025$). Antagonistic effect of rT_3 on the enzyme activity was dose-dependent: 10^{-6} M rT_3 decreased I-5'-DA by 68% and 10^{-5} M rT_3 by 80%. When fetal liver was cultured with 10^{-8} M T_3 for 9 days and the rT_3 (10^{-6} M) was added to the culture medium, I-5'-DA induced by T_3 decreased rapidly for the first 22 h, with a half life of about 16 h.

The inhibitory effect of rT_3 on T_3 stimulation seems competitive; I-5'-DA induced by 1×10^{-9}, 2×10^{-9}, and 1×10^{-7} M T_3 was significantly inhibited by 5×10^{-9}, 1×10^{-8}, and 10^{-7} M rT_3. rT_3 also inhibited the enzyme activity induced by T_4 (10^{-7} M) in a dose-dependent manner (data not shown).

Lineweaver-Burk analysis of I-5'-DA in the homogenates of fetal liver cultured with T_3 (10^{-8} M) and rT_3 (0-10^{-5} M) for 12 days revealed that a dose-dependent decrease in I-5'-DA (Table 1) was due to a decrease in Vmax, whereas no alteration in Km (7.1×10^{-8} M) was detected, suggesting that rT_3 inhibits the increase in the amount of the enzyme induced by T_3.

Free T_3 concentration in the medium supplemented with 10^{-8} M T_3, when determined by equilibrium dialysis, was about 7×10^{-10} M. When 10^{-8} M rT_3 was added to this medium, free T_3 concentration was 6×10^{-10} M. Therefore, rT_3 could elicit an anti-T_3 effect in inducing I-5'-DA at almost equimolar concentration on the basis of free thyroid hormone concentrations. Free

Table 1. Inhibitory Effect of rT_3 on I-5'-DA Induced by T_3 in Cultured Fetal Liver

Indothyronines	I-5'-DA (picomoles of ^{127}I/mg protein/min)
T_3 (-), rT_3 (-)	N.D.
T_3 (-), rT_3 (10^{-6} M)	N.D.
T_3 (10^{-8} M), rT_3 (-)	1.77 ± 0.37
T_3 (10^{-8} M), rT_3 (10^{-8} M)	1.45 ± 0.38*
T_3 (10^{-8} M), rT_3 (10^{-7} M)	1.08 ± 0.49**
T_3 (10^{-8} M), rT_3 (10^{-6} M)	0.56 ± 0.18**
T_3 (10^{-8} M), rT_3 (10^{-5} M)	0.37 ± 0.17**

Fetal liver of mouse on the 19th day of gestation was cultured in Dulbecco-Vogt medium supplemented with thyroid hormone-depleted fetal calf serum (6%), insulin (1 μg/ml), hydrocortisone hemisuccinate (0.2 μg/ml), T_3 (10^{-8} M), and various concentrations of rT_3 (0-10^{-5} M). After 10-12 days of culture, hepatocytes were homogenized and I-5'-DA was determined. Data are mean ± SD of 8 different experiments. *$p<0.025$, **$p<0.0025$. Statistical analysis was performed by paired Student's t test.

rT_3 concentrations in the medium containing 1 x 10^{-9} and 4 x 10^{-9} M rT_3, a minimal rT_3 concentration which slightly or significantly decreased I-5'-DA induced by 10^{-9} M T_3, were 6 x 10^{-11} and 2.4 x 10^{-10} M, respectively.

DISCUSSION

In a number of studies, rT_3 has elicited agonistic or antagonistic effects in vitro or in vivo at supraphysiological doses, but could exert no biological effect at physiological doses. However, these studies were performed in adults or mature animals in which iodothyronine-5'-deiodinase was well developed. In mature animals, rT_3, the best substrate for iodothyronine-5'-deiodinase in the liver and kidney, is so rapidly degraded in the peripheral tissues that biological effects of rT_3, if any, will be substantially obscured. Using fetal liver of mouse, in which little or no I-5'-DA was detected and, therefore, rT_3 is very stable, we have demonstrated that rT_3 could exert a very strong antagonistic effect against T_3 induction of I-5'-DA.

The mechanism by which rT_3 inhibits I-5'-DA induced by T_3 is unknown. However, a contamination of liver homogenate with rT_3 brought from the culture medium into the assay tube is most unlikely; the amount of rT_3 in 20 µl of homogenate in the liver cultured with 10^{-6} or 10^{-5} M rT_3 was 0.12 and 0.60 picomoles, respectively. This is too small to affect the substrate concentration in the test tube, in which 20 or 200 picomoles of [3',5'-^{125}I] rT_3 were present. In addition, I-5'-DA assessed at low (10^{-7} M) and high (10^{-6} M) substrate concentrations was almost identical. Furthermore, a gradual decrease in I-5'-DA with a half life of 16 h also suggests that the decrease in I-5'-DA induced by rT_3 is due to a real decrease in the enzyme activity. Lineweaver-Burk analysis revealed that a decrease in I-5'-DA was due to a decrease in Vmax, but not due to alteration of Km. These data suggest that rT_3 inhibits I-5'-DA by decreasing the amount of iodothyronine-5'-deiodinase induced by T_3. Previously we reported that iodothyronine-5'- as well as -5-deiodinase is synthesized and degraded continuously in monkey hepatocarcinoma cells (7). Therefore, we speculate that the inhibitory effect of rT_3 on I-5'-DA induction by T_3 is due to inhibition of enzyme synthesis, stimulation of enzyme degradation, or both.

The inhibitory action of rT_3 on the effect of 10^{-8} M T_3 was observed at 10^{-8} M rT_3, and the corresponding free T_3 and rT_3 were 7 x 10^{-10} M and 6 x 10^{-10} M, respectively. Therefore, rT_3 antagonized T_3 at almost equimolar concentrations on the basis of free thyroid hormone concentration. To our knowledge, this is the most potent anti-T_3 effect of rT_3 ever reported. It should be pointed out that rat hepatocytes contain nuclear receptors which bind not only for T_3, but also for rT_3 (8,9). Since the Ka values of the binding of rT_3 to each receptor are similar (0.65 x 10^9 M^{-1} for T_3, 0.68 x 10^9 M^{-1} for rT_3), we speculate that the rT_3 effect on I-5'-DA is probably mediated through a rT_3-nuclear receptor interaction.

When fetal liver was cultured at 10^{-9} M T_3, rT_3 could elicit an anti-T_3 effect at 1-4 x 10^{-9} M (free T_3: 7 x 10^{-11} M, free rT_3: 6-24 x 10^{-11} M). These free rT_3 concentrations may be attainable in vivo, for example in amniotic fluid in which high rT_3 concentrations are reported (132-605 ng/dl) (10).

SUMMARY

Using fetal mouse liver explants, in which little or no iodothyronine-5'-deiodinase is present and, therefore, rT_3 is very stable, we have demonstrated that rT_3 exhibits a very strong antagonistic effect against induction by T_3 of iodothyronine-5'-deiodinase.

REFERENCES

1. Papavasilliou SS, Martial JA, Latham KR, et al. J Clin Invest 60: 1230, 1977.
2. Coiro V, Harris A, Goodman HM, et al. Endocrinology 106: 68, 1980.
3. Harris ARC, Fang SL, Prosky J, et al. Endocrinology 103: 2216, 1978.
4. Sato K and Robbins J. Horm Metab Res Suppl 14: 30, 1984.
5. Sato K, Mimura H, Han DC, et al. J Clin Invest 74: 2254, 1984.
6. Samuels HH, Stanley F, and Casanova J. Endocrinology 105: 80, 1979.
7. Sato K and Robbins J. J Biol Chem 255: 7347, 1980.
8. Smith HC, Robinson SE, and Eastman CJ. In JR Stockigt and S Nagataki (eds), Thyroid Research VIII, Pergamon Press, 1980, p 283.
9. Wiersinga WM, Chopra IJ, and Solomon DH. Endocrinology 110: 1052, 1982.
10. Chopra IJ and Crandall BF. New Engl J Med 293: 740, 1975.

DESIGN OF ACTIVE SITE-DIRECTED IODOTHYRONINE DEIODINASE LIGANDS USING TBPA AS A BINDING SITE MODEL

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INTRODUCTION

Rat liver microsomal type I iodothyronine deiodinase (ITH-D) accounts for the major part of the production of thyromimetically active L-T_3 from L-T_4 in euthyroid conditions. Therefore, structure-activity-relationships (SAR) were evaluated for various classes of enzyme ligands (ITH-analogues, dyes, flavonoids) which serve either as ITH-D substrates or inhibitors in vitro (1). We have previously reported that flavonoids, and other phenolic secondary metabolites of plants used in folk medicine for treatment of thyroidal diseases, are potent ITH-D inhibitors (2). Analysis of SAR for ITH-metabolites and ITH-analogues revealed that the ligand-binding site of the ITH-D exhibits high analogy to the serum ITH-transport protein, thyroxine-binding prealbumin (TBPA), with respect to essential structural requirements for ligands, in contrast to TBG or the nuclear T_3-receptor, both of which show only minor similarity (3). The x-ray structures of ITHs, TBPA, and the T_4-TBPA-complex were solved in high resolution (4-6). Therefore, these data for the T_4-binding site of TBPA could be used for computer-graphic molecular modeling of the putative enzyme ligand binding site of the ITH-D. In order to test our previously developed hypothesis concerning the high similarity of both ligand binding sites (3,7-9), we tried to displace ^{125}I-labeled L-T_4 from binding to hTBPA by competition with representative effectors of the ITH-D-reaction.

METHODS

ITH-D assays were performed for different pathways of the ITH-monodeiodination cascade using various ITH-substrates or analogues in the presence or absence of test substances with rat liver microsomal or detergent-solubilized membrane fractions, rat cerebellum homogenates, or intact, freshly isolated rat hepatocytes in suspension serving as ITH-D enzyme sources (7-9). Dose-response curves or Lineweaver-Burk kinetic analyses were established for inhibitors and/or substrates. Competition of binding of ^{125}I-L-T_4 to TBPA was analyzed with modifications (9) according to Sommack et al. (10). Computer-graphic analysis on a MMS-X-system was performed as described (9). ITH-D ligands used are either commercially available or obtained from various non-commercial sources (7-9).

RESULTS

Compounds consisting of only a single aromatic ring are potent ITH-D inhibitors if they are able to mimic the characteristics of either the phenolic or the tyrosyl ring of iodothyronines. However, the monomeric parts of the ITH-diphenylether structure are not active as inhibitors. The most potent derivatives with ID-50% values in the range of the apparent Michaelis-Menten constant of L-T_4 (2 µM) are 3,5-diiodo-4-hydroxy-benzoic acid-butyl-ester (1 µM), 3,5-diiodo-salicylate (2 µM), and iopanoic acid (4 µM) (7). These compounds contain at least two iodine atoms in meta-position and bulky and/or hydrogen-bonding residues in the 1- (and 2-) positions. Iodine-free cinnamic and benzoic-acid derivatives consisting of only a single aromatic ring are almost inactive as ITH-D inhibitors (7,8). In contrast, dimers and polymers derived from cinnamic acid, the precursor in the biosynthesis pathway of phenolic secondary metabolites of plants, are potent iodine-free inhibitors of the ITH-D (1,2,7-9). The minimal essential inhibitor structures derived from systematic SAR-analysis of more than 70 analogues of the flavonoid classes [(dihydro-)chalcones, aurones, and other flavonoids] are summarized in Fig. 1a-c (8,9). Potent two-ring phenolic compounds are able to partially or completely adopt an ITH-analogue conformation (Fig. 1d), and to expose residues essential for inhibitory activity at molecular positions which correspond to essential parts of the natural ITH-D substrate ligands, the iodothyronines and their metabolites. These positions are complementary to the putative essential pockets of the ITH-D, where enzyme-ligand interactions may occur (schematized in Fig. 1). Favorable interactions result

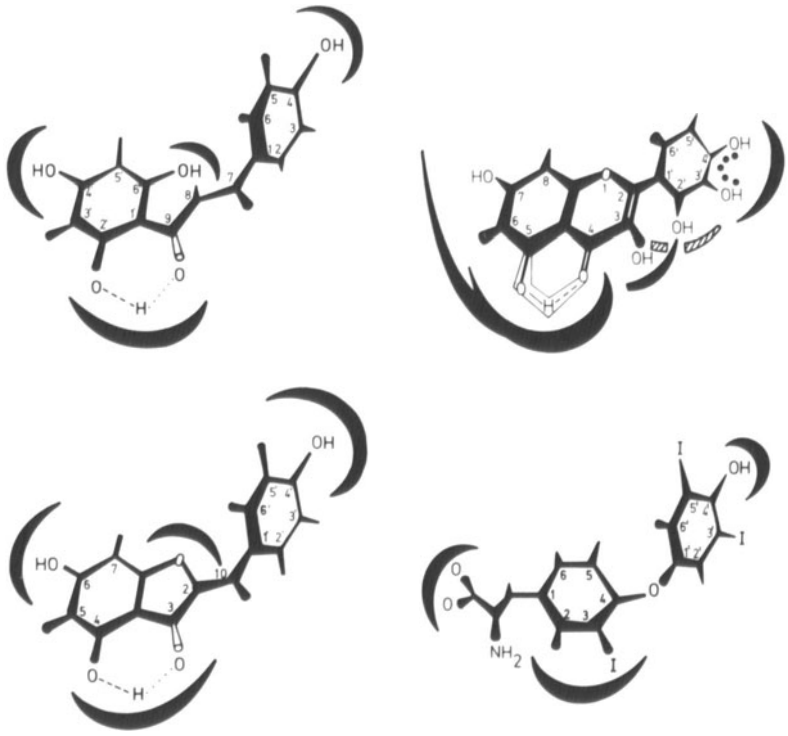


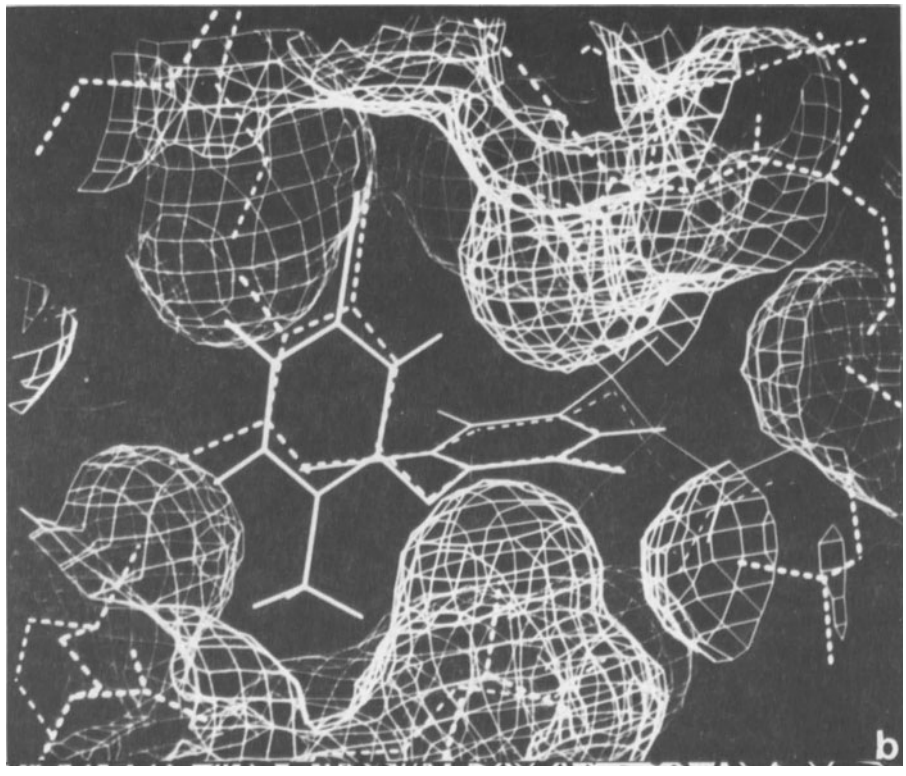
Fig. 1. Minimal essential inhibitor structures for flavonoids in comparison to the antiskewed structure of rT3. The essential ligand-enzyme interactions are schematized for chalcones (upper left), flavon(ol)s (upper right), aurones (lower left), and the iodothyronine substrate rT3 (lower right).

from ionic contacts at both distal phenolic groups of flavonoids, the side chain of ITH-analogues, as well as from a putative intramolecular hydrogen-bonding, keto-hydroxy-group arrangement in the flavonoids which might substitute for an iodine atom of the ITH-analogues in the corresponding binding pocket (Fig. 1). Furthermore, high inhibitory potency is achieved for compounds which have non-planar, i.e., skewed (T_4-analgue) or antiskewed (rT_3-analogue) (Fig. 1d), conformations (4,5,8,9). Bulky sugar residues, methylation, or the introduction of neural apolar substituents at appropriate essential molecule positions abolish inhibitory potency. For compounds consisting of two aromatic ring systems (flavonoids, iodothyronine analogues), partial or complete fulfillment of the following requirements is essential to reach Ki-values equal to or lower than the Km-values of the iodothyronine substrates: 1. Negative charges at both distal ends of the molecule. 2. An iodothyronine analogue conformation fixed by bulky (halogen-) substituents in ortho-position to the bridge structure connecting the two aromatic rings (corresponding to the 3,5-position of iodothyronines). 3. A substituent at this bridge structure which is isosteric to the oxygen atom and carries lone electron pair(s). 4. Substituents replacing the phenolic region of the iodothyronines exhibiting polar or hydrogen-bonding properties. 5. Halogen atoms at the phenolic ring (not essential but increase ligand affinity).

Also, tricyclic non-planar halogenated phenolic indicator- or food dyes are potent ITH-D inhibitors with ID-50% values equal to or lower than the Km-value of rT_3 in the 5'-D-reaction. Highest activity is reached by iodinated xanthenes (erythrosin, rose bengal; ID-50% = 0.2 μM). This indicates again that halogenation, especially iodination, increases the ligand affinity for the ITH-D as already found for the flavonoids and monomeric phenols (1,7-9). Indicator and food dyes with a coplanar orientation of the three aromatic rings are inactive as ITH-D inhibitors.

The most potent ITH-D inhibitor among the flavonoid-analogues, 3'-iodo-4,4',6-trihydroxy-aurone (Ki=0.05 μM), has an almost optimal conformation similarity to the antiskewed conformation of rT_3 (8), that naturally occurring ITH-D ligand with the highest enzyme affinity. Computer-graphic studies illustrate the T_4-binding pockets of the model protein TBPA with the electron densities of the protein backbone (Fig. 2a). The phenolic ring of the skewed T_4-structure (Fig. 2a,b) (dotted line, upper molecule) is overlapped by the phenolic ring of the antiskewed structure of rT_3 (dotted line, lower molecule). Therefore, the tyrosyl-ring of rT_3 points into a free pocket of the TBPA binding site which is not occupied by the tyrosyl-ring of T_4. 3'-iodo-4,4',6-trihydroxy-aurone (the solid line molecule) is also overlapped with its phenolic ring similar to those of the iodothyronines. The benzofuran-ring of the aurone occupies a space just between the two tyrosyl rings of the ITHs. This orientation produces no conflicting interactions with the TBPA-backbone and enables favorable contacts between essential functional groups of the aurone and the TBPA backbone amino acid residues.

An example of a potent ITH-D inhibitor, 2'-CH_3-3,3',5'-triiodo-L-thyronine (ID-50% = 7 μM) is given in Fig. 2b. The introduction of a 2'-substituent fixes this compound in the skewed T_4-analogue conformation by completely blocking the rotation of the phenolic ring around the diphenyl-ether bond, which is already highly restricted by the two 3,5-diiodo-substituents. Fib. 2b illustrates that the 2'-substituent is able to occupy a pocket in this region of TBPA which is not filled by naturally-occurring iodothyronines. This pocket can also be filled by substituents with more polar (-OH) or more hydrophobic (-benzyl) or also more voluminous character (-halogenated phenol), indicating that there exists more space in this region of the ligand-binding site pocket of the model protein TBPA and of the ITH-D available for substituents than normally occupied by the 3'-iodine atom of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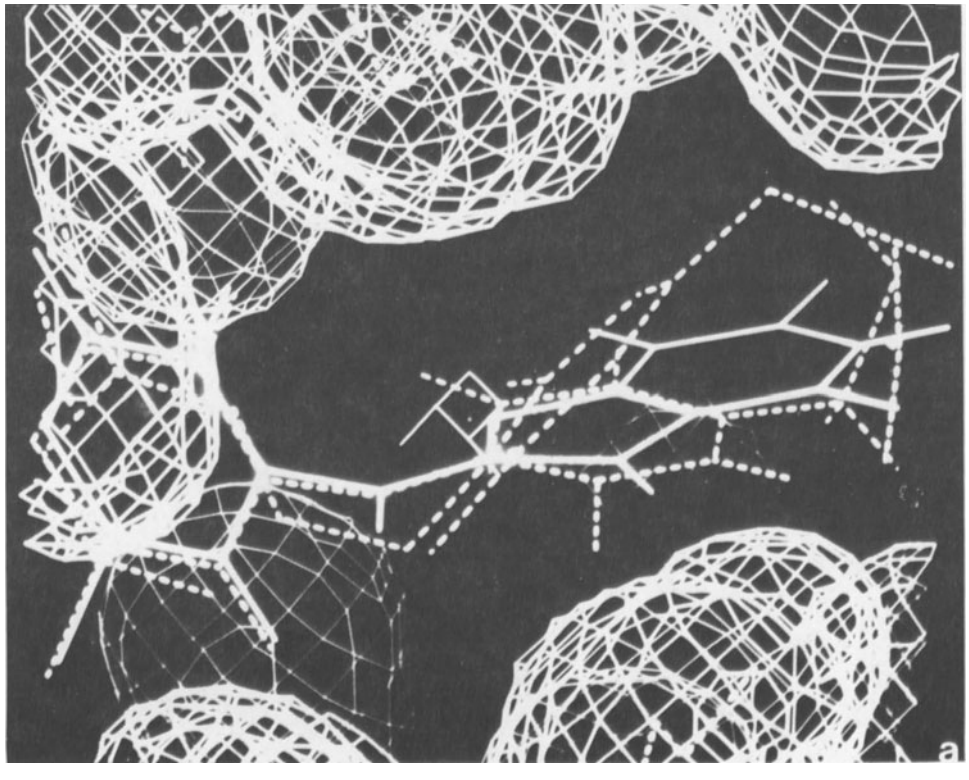
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Fig. 2a. Computer graphic presentation of the crystallographic data of the TBPA-T_4-complex showing the protein van der Waals surface focusing on the tyrosyl-ring region of the T_4-binding site of TBPA. The phenolic ring of the skewed T_4 (dotted, upper molecule) is overlapped by the phenolic ring of the antiskewed rT_3 (dotted, lower molecule) and the phenolic ring of 3'-iodo-4,4',6-trihydroxy-aurone (solid molecule), illustrating the close conformational similarity of ITH-D substrates and inhibitors.

2b. TBPA protein surface as in 2a. The ITH-D inhibitor 2'-CH_3-3,3,5'-triiodothyronine (solid molecule) is superimposed on T_4 (dotted). The free 2'-region pocket of TBPA is not occupied by the natural ligand T_4 in contrast to potent ITH-D inhibitors with a skewed conformation fixed by 2'-substituents.

naturally-occurring ITHs; e.g., two of the aromatic rings of the dye tetrabromophenolblue (ID-50% = 10 µM) can occupy the phenolic and the tyrosyl-ring pockets of the T_4-binding site of TBPA and the third aromatic ring of the non-planar dye can be accommodated in the 2'-pocket without producing conflicting interctions with the protein backbone as found for structurally related inactive compounds (flavonoid-glycosides).

A comparison of the ID-50% values for inhibition of various routes of the ITH-monodeiodination cascade, as well as for the inhibition of T_4-binding to hTBPA, is given in Fig. 3. Representative inhibitors of the ITH-D-reactions tested with either rat liver microsomal fractions, rat cerebellum homogenates, or freshly ioslated intact rat hepatocytes in suspension, are able to inhibit these reactions to a similar extent and with similar SAR. Furthermore, the last column of Fig. 3 shows that inhibitor concentrations necessary for half-maximal displacement of T_4 from binding to hTBPA are very similar to those values found for ITH-D inhibition, and similar SAR are found. This constitutes strong evidence in favor of our model underscoring the high similarity of the ligand binding sites of type I ITH-D and of TBPA. This similarity is not restricted to ITH-metabolites and analogues but is also found for phenolic secondary metabolites of plants, especially

	rat liver microsomes				hepato-cytes	cerebellum homogenate	TBPA-binding
ITH-D-reaction	T4 → T3 / rT3	T4 → T3 / rT3	T4 → T3 / rT3	T4A → T3A / rT3A	T4 → T3 / rT3	T4 → T3 / rT3	
compound	3,3'-T2	3,3'-T2	3,3'-T2	3,3'-T2A	3,3'-T2	3,3'-T2	
phloretin	2.5		2	2.5	2.5	3	2
4,4',6-trihydroxy-aurone	1.5	2		5	2	1.5	0.7
3'iodo-4,4',6-trihydroxy-aurone	0.5				0.5		
EMD 46806	1.5	2		2	5	3.5	
Luteolin	5						0.7
Luteolin-7-β-glucoside	150						~15

Values indicate halfmaximal inhibitory doses (ID 50, µM) in the different reactions.
Solid arrows indicate the deiodinase reaction under study.

Fig. 3. Flavonoid inhibition of various ITH-deiodinase pathways and of binding of ^{125}I-labeled L-T_4 to human TBPA.

flavonoids, as well as for food and indicator dyes. Furthermore, these data show that computer-graphic molecular modeling of ligand-protein interaction is a useful tool to analyze and explain SAR data and to develop optimized ligands for the affinity-labeling of the ITH-D and for the design of new types of "antithyroidal" iodothyronine deiodinase-directed drugs.

REFERENCES

1. Hesch RD and Koehrle J. In SH Ingbar and LE Braverman (eds), The Thyroid, JP Lippincott, Philadelphia, 1985.
2. Auf'mkolk M, Koehrle J, Gumbinger H, et al. Horm Metabol Res 16: 136, 1984.
3. Koehrle J and Hesch RD. Horm Metabol Res 14 Suppl: 42, 1984.
4. Cody V. Endocrine Rev 1: 140, 1980.
5. Okabe N, Fujiwara T, Yamagata Y, et al. Biochim Biophys Acta 717: 179, 1982.
6. Blake CCF and Oatley SJ. Nature 268: 115, 1977.
7. Koehrle J. Schilddruesenhormonstoffwechsel 1983. Ferdinand Enke Verlag, Stuttgart, 1983.
8. Koehrle J, Auf'mkolk M, Spanka M, et al. In M Gabor (ed), Proceedings International Bioflavonoid Symposium, Szeged, 1985, in press.
9. Koehrle J, Auf'mkolk M, Spanka M, et al. In V Cody, E Middleton, and JB Harborne (eds), Plant Flavonoids in Biology and Medicine, Alan Liss, New York, 1985, in press.
10. Somack R, Andrea TA, and Jorgensen EC. Biochemistry 21: 163, 1982.

GENERATED DIHYDROLIPOAMIDE AND CYTOSOLIC COMPONENTS STIMULATE HEPATIC MICROSOMAL THYROXINE 5'-DEIODINATION: SIMILAR EFFECTS OF CYTOSOLIC COMPONENTS ON OTHER SULFHYDRYL COMPOUNDS*

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INTRODUCTION

5'-deiodinase (5'-DI) is a thiol-dependent enzyme (1,2) and sulfhydryl compounds, such as dithiothreitol (DTT), 2-mercaptoethanol (2-ME), and reduced glutathione (GSH, molecular weight [M.W.] 307 Da.) have been shown to function as activators by acting as direct reductants of the enzyme SH groups oxidized during deiodination. While several investigators have reported that isolated hepatic microsomes have very little 5'-DI activity in the absence of added thiols, reconstitution with cytosol has been reported to enhance 5'-DI activity, suggesting that cytosol contains one or more unidentified factors which are involved in 5'-deiodination (3). Recently, we have reported that cytosolic components of approximate M.W. 13,000 Da. (fraction B), which we have assigned an equivalent abbreviation of IMCC, might play a role as a direct reductant in an NADPH-dependent non-glutathione cytosolic reductase system (4). Hence, while sulfhydryl compounds may act directly with the 5'-DI, it is also possible that endogenous cytosolic factors may function as regulatory intermediaries between the enzyme and sulfydryl compounds, and that the overall influence of such compounds on 5'-DI may be mediated by such cytosolic cofactors.

In the present report, we provide evidence that such cytosolic components of intermediate molecular weight (IMCC) may operate as a highly efficient intermediary between the microsomal 5'-DI and a variety of sulfhydryl compounds such as dihydrolipoamide (DHL), DTT, 2-ME, and GSH.

MATERIALS AND METHODS

Hepatic cytosol and microsomes were prepared by standard differential centrifugation methods using 0.125 M phosphate buffer, pH 7.4, containing 1 mM EDTA (PB-EDTA) by procedures previously described from our laboratory (5). Young male Sprague-Dawley rats maintained on regular chow ad libitum were used. Using Sephadex G-50 or Sephacryl S-200 column chromatography, IMCC were prepared by methods similar to those reported from our laboratory (5). 5'-DI activity was determined using either nonradioactive T_4 or outer

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ring (3' or 5') labeled ^{125}I-rT_3 as a substrate. In some experiments, a coupled system with NADH, lipoamide, and endogenous or 0.125 U pig heart lipoamide dehydrogenase was used to generate DHL from lipoamide. In a typical experiment, using T_4 (final concentration 6.4 μM) as a substrate, incubations were conducted with various combinations of DHL-generating system, IMCC, washed microsomes, and PB-EDTA in a final volume of 1 ml. After 60 minutes, 2 ml of ethanol (98%) was added. 5'-DI was determined by measuring T_3 generated in the reaction mixture using a T_3 radioimmunoassay (RIA), and results were expressed as ng of T_3 generated per gm equivalent net weight of microsomes per hour. In similar experiments using ^{125}I-rT_3 as a substrate, incubation mixtures contained ^{125}I-rT_3 (100,000 cpm, final concentration 0.21 nM), washed microsomes, IMCC or buffer, and DHL, DTT, 2-ME, or GSH in a final volume of 300 μl as described previously (5). Incubation was for 15 minutes at 30°C. Reactions were stopped by the addition of 500 μl human serum followed by 500 μl 10% trichloroacetic acid (TCA). Calculations were based on counts of $^{125}I^-$ in 500 μl supernatant, and since 50% of the iodide released was nonradioactive and $^{125}I^-$ in 500 μl supernatant is 2.4 2.4 times less than that in the total volume, our measured activities represent 4.8 times less than the true rate of deiodination after correction for non-specific free iodide release. Hence, the correction for our illustrated results will be 1% $^{125}I^-$ released = 3.0×10^{-3} pmoles rT_3 deiodinated.

RESULTS

Using T_4 as a substrate, measurements of 5'-DI activity were made with various reaction mixtures to determine the effects of added cytosol, NADH, and lipoamide on unwashed microsomal 5'-DI activity. While cytosol alone had no stimulative activity, activation of 5'-DI of unwashed microsomes was maximized when all constituents were present, including NADH, lipoamide, and endogenous lipoamide dehydrogenase (the latter demonstrated to be present in both microsomes and cytosol). In reconstitution experiments of fractions from gel column chromatography of cytosol with microsomes, IMCC was shown to be stimulatory in the 5'-deiodination in the presence of DHL or DHL-generating system.

Using ^{125}I-rT_3 as substrate, directly synthesized DHL, DTT, 2-ME, and GSH were studied alone or in the presence of added 100 μl of IMCC obtained from gel chromatography of cytosol. The maximal stimulatory effect of DHL alone on 5'-DI was produced at a concentration as low as 15 μM, which could be further augmented by three to fourfold in the presence of added 100 μl of IMCC (data not shown). An effect of DTT alone on microsomal 5'-DI could be seen in concentrations as low as 40 μM, which could be further augmented by a factor of 3-4 fold by the addition of IMCC. Similar but less marked effects were observed for 2-ME and GSH at 1 mM concentrations, with a preservation of the known relative activation potency of these thiols towards 5'-DI both in the presence and absence of IMCC (i.e, DHL > DTT > 2-ME > GSH; Table 1).

IMCC in the absence of microsomes, either with or without added DTT, did not exhibit 5'-DI activity.

DISCUSSION

The present data is consistent with previous reports which demonstrated stimulation of hepatic 5'-DI by DHL (6) or its generating system with further augmentation of a maximal stimulatory effect of DHL by cytosolic cofactors (5), which appear to be required for the full activation of 5'-DI.

Table 1. Effects of Various Thiols on Microsomal 5'-deiodination in the Presence and Absence of IMCC

Thiol	Concentration (mM)	5'-DI Activity (mean % ^{125}I-released/15 min) IMCC Absent Result	IMCC Absent Rel. Act.**	IMCC Present Result	IMCC Present Rel. Act.**
GSH	1.000	0.10	7	0.23	15
2-ME	1.000	0.57	38	2.4*	160
DHL	0.062	1.60	107	4.10*	273
DTT	1.000	1.50	100	4.40*	293

IMCC = Intermediate molecular weight cytosolic components. *$p<0.01$ vs IMCC absent; **Relative to DTT activity with IMCC absent=100.

The potentiating influence of IMCC on the maximal stimulating action of other sulfhydryl compounds such as DTT, 2-ME, and GSH has been demonstrated, indicating that the augmenting effect of IMCC is not specific for any particular thiol we have studied to date. Furthermore, the combined effect of IMCC with any thiol was governed by the pre-existing potency of that thiol in the absence of IMCC which failed to alter the relative maximal potency order of each thiol (Table 1). Moreover, IMCC in the presence of DTT was not observed to have any intrinsic 5'-DI activity. Nevertheless, the maximal microsomal 5'-DI activity attainable by any thiol could be further augmented by the addition of IMCC.

These observations suggest the possibility that the 5'-DI may have an absolute requirement for IMCC and that the relative potencies of various thiols as activators of 5'-DI may be a function of their oxidation-reduction potential to reduce oxidized IMCC followed by the reduction of the oxidized enzyme. However, the precise mechanisms involved in the augmentation of sulfhydryl-induced stimulation of microsomal 5'-DI is unknown. Of interest is that the cytosol fraction designated as B, which has similar M.W. to IMCC, is also required for the activation of 5'-DI by a non-glutathione NADPH-dependent cytosolic reductase system (4). Thus, thiols might be generated in a particular constituent of IMCC by a thiol-disulfide interchange resulting in a highly efficient intermediary, which could be the immediate reductant of the oxidized enzyme. Goswami and Rosenberg (7) recently reported the augmentation of 5'-DI activity by a glutaredoxin-like cytosolic component of intermediate (11,000 Da.) molecular weight in the presence of GSH, which could be present in our IMCC. However, it is not known which cofactor (the glutaredoxin-like protein or that described by us [4]) is responsible for the potentiating effects on thiols such as we report.

An additional consideration is the possibility that small amounts of IMCC are adsorbed to or admixed with microsomes, thus explaining the apparent direct reactivation of the oxidized enzyme (ESI) by thiols in the absence of added IMCC. However, the role of IMCC as a regulator of microsomal 5'-DI enzyme activity, possibly involving the induction of a conformational change in 5'-DI site to one more favorable to catalysis rather than by reducing ESI, is also a possibility requiring further study. Assays of 5'-DI activity in tissues are frequently performed in the presence of DTT. Since our observations suggest the possibility that the presence or absence of IMCC in subcellular fractions or tissue preparations could substantially alter the apparent maximal 5'-DI activity, which may also change its apparent sensitivity to propylthiouracil (PTU) as determined by the concentration

of DTT required for maximizing tissue 5'-DI activity, the interpretation of such previously published data requires caution and may be subject to further re-evaluation. Hence, measurements of IMCC concentrations in various tissues, as well as inhibition studies using microsomes in the presence of several concentrations of IMCC, thiouracils, and DTT could be required for a complete understanding of the mechanism of 5'-deiodination and the factors controlling its rate of activation.

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REFERENCES

1. Visser TJ. Biochem Biophys Acta 569: 302, 1979.
2. Leonard JL and Rosenberg IN. Endocrinology 106: 444, 1980.
3. Visser TJ, van der Does-Tobe I, Docter R, et al. Biochem J 157: 479, 1976.
4. Sawada K, Hummel BCW, and Walfish PG. Prog, and Absts. of 57th Annual Meeting of the Endocrine Society, Baltimore, MD, 1985, p 278.
5. Sawada K, Hummel BCW, and Walfish PG. Endocrinology 117: 1259, 1985.
6. Goswami A and Rosenberg IN. Endocrinology 112: 1180, 1983.
7. Goswami A and Rosenberg IN. J Biol Chem 260: 6012, 1985.

CHARACTERISTICS OF T_4 INNER RING DEIODINASE IN THE LIVER, KIDNEY, AND CEREBRAL CORTEX OF RAT: TWO KINDS OF ISOENZYMES

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INTRODUCTION

The major pathway of the peripheral metabolism of thyroid hormone is serial deiodination by enzymes. The first step of the deiodination of thyroxine is the removal of iodine at 5 or 5' position, and these two activities are known to be comparable in in vivo kinetics studies.

The nature of thyroxine 5' deiodinase has been well verified in many tissues by various in vitro studies. However, it was difficult to study in vitro the activity of thyroxine 5 deiodinase, the inner ring deiodinase of the liver or kidney, because the generated reverse T_3 (rT_3) degrades very rapidly during incubation. In the present investigation, by protecting the generated reverse T_3 from degradation by means of addition of anti-rT_3 antibody to the incubation media, quantitative analysis of T_4 inner ring deiodinase was possible.

MATERIALS AND METHODS

Tissue Preparation

Wistar strain rats were sacrificed by ether anesthesia and bleeding from the carotid artery. The liver and kidney were removed, chopped, and homogenized in Tris-HCl buffer, pH 7.4, with a Polytron PT 10 for 2 minutes. The homongenate was centrifuged at 600 x g for five minutes. The pellet was again homogenized by Polytron for 2 minutes, and the supernatants were combined and centrifuged at 15,000 x g for 20 min. This supernatant was then centrifuged at 100,000 x g for 60 min. The pellet was designated as the microsomal fraction. The microsomal fraction of cerebral cortex was prepared by the method of Visser at al. (1).

T_4 Inner Ring Deiodinase Activity

Thyroxine, purchased from Sigma Co., was incubated with total homogenates or microsomes in Tris-HCl buffer, pH 9.0, for 10 min at 37°C. Two volumes of absolute ethanol were added to terminate the reaction. Reverse T_3 in the supernatant was measured by specific radioimmunoassay. The apparent generation of reverse T_3 by the incubation was calculated by subtraction of amount of reverse T_3 in identical tubes prior to incubation

(background) from the total amount of reverse T_3. In the studies with liver and kidneys, anti-rT_3 antiserum or IgG was added to the incubation media, in order to minimize degradation of the generated reverse T_3. When cerebral cortex was studied, antibody was not added because preliminary studies showed that the degradation of reverse T_3 by cerebral cortex was negligible. In the case of the liver and kidney, the degradation of reverse T_3 was still substantial in the presence of the antibody, and the amount of reverse T_3 generated after 10 min incubation was, therefore, further corrected by the degradation rate, estimated by computer simulation of the degradation curve of cold reverse T_3.

Effect of DTT, PTU, Ipodate, and T_3 on T_4 Inner Ring Deiodinase Activities

T_4 inner ring deiodinase activities in fresh homogenates or microsomal fractions of these organs were studied in the presence of various amounts of DTT, PTU, ipodate, and T_3. The "background" and degradation rate were studied in each concentration of these reagents.

RESULTS

Effect of Anti-reverse T_3 Antibody on the Degradation of Reverse T_3

When cold reverse T_3 was incubated with liver or kidney homogenate, the amount of reverse T_3 was decreased rapidly. Fig. 1a shows the degradation curve of reverse T_3, when incubated with liver homogenate at pH 9.0. When antibody was added to the incubation media, the degradation was slowed markedly. The degradation was faster in acidic media (data not shown).

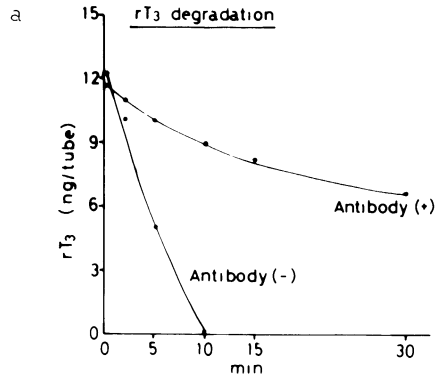
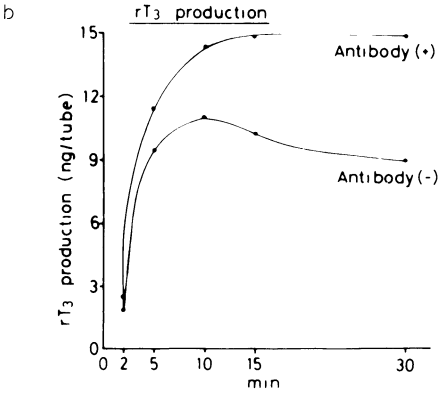


Fig. 1a. Effect of anti reverse T_3 antibody on the degradation of reverse T_3. Reverse T_3 was incubated with rat liver homogenates.



1b. Apparent production of reverse T_3 from T_4 by the incubation of T_4 with rat liver homogenates.

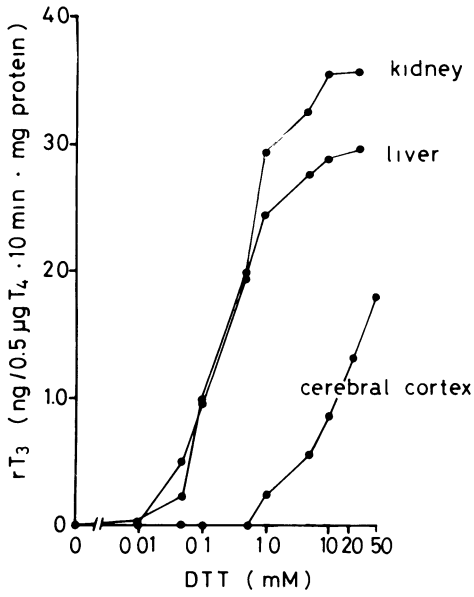


Fig. 2. Effect of DTT on T_4 inner ring deiodinase of microsomes from liver, kidney, and cerebral cortex.

Fig. 1b shows the apparent generation of reverse T_3 from T_4 by the incubation with the same preparation as above.

Effect of DTT on T_4 Inner Ring Deiodinase

T_4 inner ring deiodinase activities were not detected without DTT in microsomes of the liver and kidney. When the concentration of DTT exceeded 0.05 mM, T_4 inner ring deiodinase activities increased in a dose-dependent manner (Fig. 2). T_4 inner ring deiodinase activities in cerebral cortex increased by DTT in a dose-dependent manner from 1 mM to 50 mM.

Effect of PTU on T_4 Inner Ring Deiodinase

T_4 inner ring deiodinase in homogenates of liver and kidney were inhibited by PTU in a dose-dependent manner from 0.5 mM to 10 mM. In contrast, the activities were not inhibited at all by PTU up to 50 mM in cerebral cortex (Fig. 3).

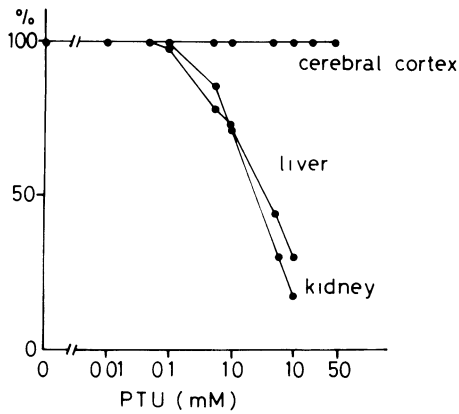


Fig. 3. Effect of PTU on T_4 inner ring deiodinase of homogenates of the liver, kidney and cerebral cortex.

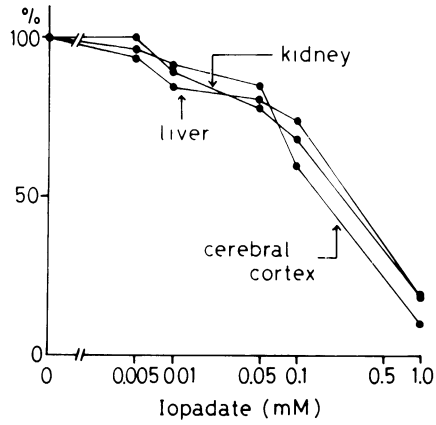


Fig. 4. Effect of ipodate on T_4 inner ring deiodinase of microsomes from liver, kidney, and cerebral cortex.

Effect of Ipodate on T_4 Inner Ring Deiodinase

As shown in Fig. 4, ipodate inhibited the enzyme activities in a dose-dependent manner from 0.01 mM to 1.0 mM in all three organs equally.

Effect of T_3 on T_4 Inner Ring Deiodinase

As shown in Fig. 5, T_3 inhibited the T_4 inner ring deiodinase activities of cerebral cortex in concentration > 0.3 μM. In contrast, no inhibition was observed in liver and kidney.

DISCUSSION

In the present investigation, anti-rT_3 antibody was used to protect reverse T_3 from its degradation. Because preliminary experiments revealed that the optimum pH of T_4 inner ring deiodinase was about pH 8-9, and that the degradation of reverse T_3 was less in alkaline medium, buffers at pH 9.0 were used for the study of the nature of T_4 inner ring deiodinase in liver and kidney preparations. At pH 7.4, the degradation of reverse T_3 was rapid even in the presence of antibody, and accurate measurement of T_4 inner ring deiodinase was impossible. In contrast to liver and kidney, reverse T_3 degradation activity was very low in brain. Therefore, the study of T_4 inner ring deiodinase in cerebral cortex did not require the addition of anti-rT_3 antibody.

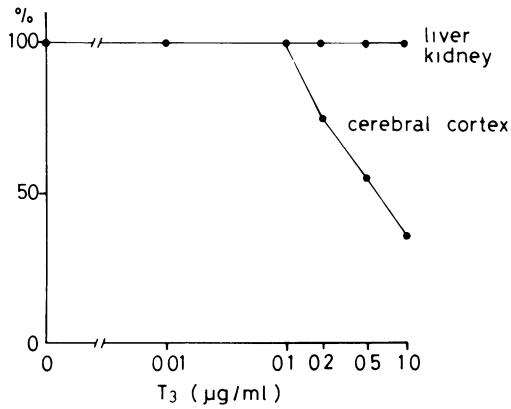


Fig. 5. Effect of T_3 on T_4 inner ring deiodinase of microsomes from liver, kidney, and cerebral cortex.

The microsomes of those organs did not exhibit T_4 inner ring deiodinase activity without DTT. T_4 inner ring deiodinase activities of the liver and kidney were increased by DTT in a dose-dependent manner from 0.05 to 10 mM. The dose of DTT which produced half maximum increase was about 0.2 mM and 0.3 mM in the liver and kidney, respectively. However, in the cerebral cortex, it was more than 10 mM.

T_4 inner ring deiodinase in the liver and kidney was inhibited also by PTU in a dose-dependent manner. In contrast, enzyme activity in the cerebral cortex was not inhibited by PTU in concentrations up to 50 mM. Resistance to PTU and DTT inhibition in T_4 outer ring deiodinase of cerebral cortex was known previously (2).

T_4 inner ring deiodinase in cerebral cortex was inhibited by T_3 at concentrations > 0.3 μM. In contrast, T_3 did not inhibit enzyme activity in the liver and kidney at concentrations up to 1.5 μM. This finding contrasts with the report of the inhibition of outer ring deiodinase by reverse T_3 (3).

In summary, T_4 inner ring deiodinase of cerebral cortex differs markedly from that of the liver and kidney in terms of the sensitivity to DTT, PTU, and T_3. No differences were observed in the nature of T_4 inner ring deiodinase between the liver and kidney. This suggests the presence of two isoenzymes of T_4 inner ring deiodinase with different tissue distribution.

REFERENCES

1. Visser TJ, Leonard JL, Kaplan MM, et al. Proc Nat Acad Sci USA 79: 5080, 1982.
2. Silva JE, Leonard JL, Crantz FR, et al. J Clin Invest 69: 1176, 1982.
3. Kaplan MM and Utiger RD. J Clin Invest 61: 459, 1978.

INHIBITION OF T_4 5'-DEIODINASE IN HUMAN PLACENTAL CELLS BY CYCLIC-AMP, BUTYRATE, AND CALCIUM*

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INTRODUCTION

Conversion of T_4 to T_3 (T_4 5'-deiodination) by human placental cells (1-3), which occurs via the type II iodothyronine pathway (4), is inhibited by physiological concentrations of iodothyronines in the potency order T_4 = rT_3 > T_3 (3). Type II deiodination is responsible for much of the tissue T_3 content in the rat brain and anterior pituitary gland (5), and may, under some circumstances, contribute significantly to the circulating T_3 pool in both rats and humans (5,6). Since type II T_4 5'-deiodination could thus constitute an important step in the expression of thyroid hormone effects, we wished to obtain a more complete understanding of the regulation of this process in a human system.

METHODS

Placentas were obtained within 1 h of delivery of healthy 38-42 week infants. The chorionic membrane, stripped from the amnion, was minced and digested in collagenase-trypsin-deoxyribonuclease in Hanks' balanced salt solution. Cells released between 30 and 90 min were washed and plated in medium 199 containing 10% calf serum and 1% penicillin-streptomycin (complete M-199) for 48 h. Medium was then changed to complete M-199 with or without test agents. At the end of test incubations, the cells were washed, scraped from the dishes in 2 ml phosphosaline buffer, and centrifuged. The pellet was sonicated in 500 µl of Hepes, pH 7.0-0.32 M sucrose -10 mM DTT. Sonicates were kept frozen at -10°C until they were assayed. Other details have been previously described (3).

T_4 5'-deiodination was measured in sonicates from triplicate wells, each assayed in duplicate, by quantitating release of $^{125}I^-$ from [^{125}I]T_4 as described (3). The incubation mixture contained 1 nM T_4, 10 mM DTT, 1 µM nonradioactive T_3, and 25-75 µg cell protein in a volume of 100 µl. Incubations were for 2 h at 37°C in a shaking water bath. Under these conditions, equal amounts of $^{125}I^-$ and T_3 were produced (3). Protein was measured by the method of Bradford (7) with bovine IgG as standard.

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Table 1. Effects of Butyrate and Agents Related to cAMP on Placental T_4 5'-deiodination

Effector	Concentration mM	Time h	N	T_4 5'-deiodination rate % of control ± SD
dbcAMP	0.1	24	2	100 ± 6
"	1.0	24	3	16 ± 1[b]
Butyrate	0.1	24	2	100 ± 11
"	1.0	24	3	68 ± 3[b]
"	2.0	24	3	20 ± 1[b]
"	1.0	24 + wash[a]	2	98 ± 7
8-bromo-cAMP	1.0	24	3	53 ± 5[b]
"	1.0	48	2	57 ± 3[b]
Cholera toxin	0.01	24	2	63 ± 5[b]
Theophylline	1.0	24	4	73 ± 10[c]

Cells were incubated in complete M-199 for 48 h. Medium was then changed to complete M-199 with or without the effector to be tested, and cells were harvested after the indicated times. N is the number of experiments in which each agent was tested. In each experiment, incubations were in triplicate wells, and data from similar experiments were pooled. [a]After a 24 h incubation in the presence of butyrate, cells were washed and incubated in M-199 without butyrate, and compared to control wells that had the same medium-change schedule. [b]$p<0.01$ vs controls in the same experiments by Tukey's test. [c]$p<0.05$ vs control.

RESULTS

Effects of Cyclic AMP (cAMP) Analogs and Related Compounds (Table 1)

Sonicates of dispersed human placental cells incubated for 24 h with 1 mM dibutyryl-cyclic AMP (dbcAMP) had 16% of the T_4 5'-deiodination rate of control sonicates, whereas 0.1 mM dbcAMP had no significant effect. Since this inhibition could have been due to butyrate released from the dbcAMP, butyrate alone was tested, and showed a dose-related inhibition, with 2 mM butyrate having an effect similar to 1 mM dbcAMP. The effects of butyrate were reversible. When wells exposed to butyrate for 24 h had medium changed to control medium for another 24 h, T_4 5'-deiodination rates in cell sonicates did not differ from rates in sonicates of cells never exposed to butyrate.

Because an effect of the cAMP moiety could still have been present, 8-bromo-cAMP was tested. At 1 mM, it caused a reduction in the T_4 5'-deiodination rate to about 55% of control after a 24 or 48 h exposure. To further verify that changes in intracellular cAMP were inhibitory to deiodinase activity, incubations were performed with cholera toxin and theophylline. Both agents reduced T_4 5'-deiodination after 24 h.

Effects of Agents that Alter Intracellular Calcium

Sonicates of cells incubated with 3.4 mM Ca^{++} showed a 30% decrease in T_4 5'-deiodination rate compared to controls, which had 1.7 mM Ca^{++} in the

Table 2. Effect of Calcium-active Agents on Placental T_4 5'-deiodination

Effector	N	T_4 5'-deiodination rate % of control ± SD
Ca^{++} 3.4 mM	3	73 ± 9[a]
EGTA 1.7 mM	3	77 ± 8[a]
Ca 3.4 mM + EGTA 1.7 mM	3	70 ± 6[b]
A-23187 0.1 μM	2	95 ± 9
A-23187 1.0 μM	3	34 ± 3[b]
A-23187 10 μM	3	<5[b]
Verapamil 1.0 μM	2	99 ± 7
Verapamil 10 μM	2	92 ± 6
Verapamil 50 μM	2	65 ± 6[b]
Trifluoperazine 1.0 μM	2	106 ± 12
Trifluoperazine 10 μM	2	93 ± 9

Cells were incubated in triplicate wells for 48 h in complete M-199 with or without the test agent. Sonicates prepared thereafter were assayed for T_4 5'-deiodination in duplicate. N is the number of experiments in which each agent was tested; data from these experiments were pooled. The Ca^{++} concentration in the medium was 1.7 mM except in those wells for which 3.4 mM is specified. [a]$p<0.05$ vs control by Tukey's test; [b]$p<0.01$ vs control.

culture medium (Table 2). Addition of 1.7 mM EGTA to the culture medium reduced the T_4 5'-deiodination rate in cell sonicates to 77% of control. However, increasing the culture medium Ca^{++} concentration to 3.5 mM, to restore the non-chelated Ca^{++} concentration back to the original 1.7 mM, failed to reverse the inhibition of EGTA. This suggests that inhibition by EGTA occurs by EGTA occurs by a mechanism other than chelation of extracellular calcium.

Addition of the calcium ionophore A-23187 to the culture medium resulted in a dose-dependent inhibition of T_4 5'-deiodination in cell sonicates, with near-total inhibition at 10 μM (Table 2). Addition of equal amounts of the ionophore vehicle, ethanol, had no effect. The effects of A-23187 were reversible; changing the medium to M-199 alone after a 48 h exposure restored T_4 5'-deiodination to levels in sonicates of control cells subjected to the same schedule of medium changes. Verapamil was inhibitory to T_4 5'-deiodination at 50 μM, whereas trifluoperazine had no effect at 1 or 10 μM (Table 2).

DISCUSSION

Our data indicate that increased intracellular cAMP is inhibitory to human placental type II iodothyronine deiodination. Results were consistent for the three agents, 8-bromo-cAMP, a non-hydrolyzable cAMP analog and agonist, cholera toxin, which activates adenylate cyclase by ADP ribosylation of the guanine nucleotide subunit of the cyclase complex, and theophylline, which inhibits degradation of endogenous cAMP. These results are opposite

to the marked stimulatory effect of cAMP on this reaction in cultured fetal rat glial precursor cells (8). Although dbcAMP also resulted in decreased T_4 5'-deiodinase activity, this can be attributed in large part to release of butyrate into the culture medium, because addition of butyrate itself caused a similar degree of reversible inhibition. Butyrate alters many functions of cultured cells, acting through changes in acetylation and phosphorylation of histones and in DNA synthesis. It induces placental alkaline phosphatase biosynthesis in cultured JEG-3 choriocarcinoma cells (9). The mechanism by which butyrate alters placental type II deiodinase activity remains to be elucidated. Changes in intracellular Ca^{++} also modulate many intracellular functions. Our results indicate that raising the intracellular calcium, either by increasing the medium Ca^{++} or, presumptively, by adding A-23187, is inhibitory of T_4 5'-deiodination. The absence of effect of trifluoperazine strongly suggests that this effect of calcium is not mediated by calmodulin. It is possible that reducing intracellular calcium is also inhibitory, as judged by the effects of verapamil. However, verapamil has effects besides inhibition of membrane calcium transport channels, and our attempt at reduction of intracellular Ca^{++} by chelation of medium Ca^{++} with EGTA gave ambiguous results.

REFERENCES

1. Banovac K, Bzik L, Tislaric D, et al. Horm Res 12: 253, 1980.
2. Kaplan MM and Shaw EA. J Clin Endocrinol Metab 59: 253, 1984.
3. Hidal JT and Kaplan MM. J Clin Invest 76: (in press), 1985.
4. Kaplan MM. Neuroendocrinology 38: 254, 1984.
5. Silva JE and Matthews P. Endocrinology 115: 2394, 1984.
6. Lum SMC, Nicoloff JT, Spencer CA, et al. J Clin Invest 73: 570, 1984.
7. Bradford M. Analyt Biochem 72: 248, 1976.
8. Leonard JL and Gogolin PJ. Ann d'endocrinologie 45: 61, 1984.
9. Ito F and Chou JY. J Biol Chem 259: 2526, 1984.