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Molecular Basis of Thyroid Hormone Action

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This book is dedicated to our
wives, Ann Oppenheimer and Emily
Samuels, in appreciation of their
patience and understanding

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Preface

Studies in the past five to ten years have provided new insights into the action of thyroid hormones in eukaryotic cells. It has been recognized for decades that the thyroid hormones have profound effects on the growth, development, and metabolism of virtually all tissues of higher organisms. Studies from a number of laboratories using models as diverse as amphibian metamorphosis, mammalian cells in culture, and intact animals have provided evidence that the diverse effects of the thyroid hormones are modulated by a chromatin-associated receptor. Evidence from a number of systems indicates that the thyroid hormone nuclear receptor controls the accumulation of specific messenger RNA molecules that code for the synthesis of specific proteins regulated by the hormone. These proteins may be destined for export and may act to regulate other tissues (e.g., growth hormone) or are enzymes (e.g., malic enzyme) that can further act to regulate specific metabolic events in the cell. In addition, several of the proteins regulated by thyroid hormone are membrane bound (e.g., Na^+,K^+-ATPase, catecholamine receptors), which can further act to modify cell functions or alter the sensitivity of the cell to other humoral factors.

Therefore, like steroid hormones, the thyroid hormones appear to influence processes in cells by acting to regulate the expression of specific genes. Due to rapid developments in the field, we felt that it was timely to review the significant developments and progress in this field; the chapters contained in this book have been contributed by investigators who have made important contributions to this area. The intent of this book is to place this field in perspective and review not only past work but also indicate the questions that need to be answered in future research. The chapter contents describe basic methodology and the use of diverse model systems to explore the molecular basis of thyroid hormone action and are aimed at a broad spectrum of readers who are interested in cell and molecular biology as well as the endocrinologist who is interested in relating molecular mechanisms to clinical thyroidology.

Jack H. Oppenheimer
Herbert H. Samuels

1

The Nuclear Receptor–Triiodothyronine Complex: Relationship to Thyroid Hormone Distribution, Metabolism, and Biological Action

JACK H. OPPENHEIMER

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I. INTRODUCTION

An understanding of the kinetic interrelationships of hormone bound to specific receptors and hormone associated with other tissue and plasma pools in the

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THYROID HORMONE ACTION

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living animal is important in developing a comprehensive understanding of endocrine mechanisms. A definition of the kinetic model allows an assessment of the influence of variation in plasma hormones on the concentration of hormone bound to the specific receptor site and should provide an insight into the relative importance of hormone metabolism at a local and distal tissue site. The use of *in vivo* kinetic analysis can also provide a relatively reliable index of the binding properties of the hormone receptors under physiological settings and thus avoid potential artifacts which may occur when such measurements are carried out exclusively under *in vitro* conditions. The usefulness of *in vivo* kinetic analysis is perhaps best illustrated by the fact that an analysis of iodothyronine equilibration in the intact rat first led to the recognition of high affinity, limited capacity sites in unfractionated pituitaries (Schadlow *et al.* 1972) and to the description of specific nuclear receptor sites in thyroid responsive tissues (Oppenheimer *et al.* 1972a).

Nevertheless, even an exhaustive description of hormone metabolism and distribution still results in an inadequate description of the hormone system. Of critical importance from a biological and a practical clinical point of view is an understanding of the relationship between nuclear hormonal occupancy and specific biological response parameters. Thus, an effort should be made to bridge the gap between the input function, which is conventionally treated by an analysis of tracer kinetics of hormone, and the output function, which is represented by the multiple biological alterations induced by receptor occupancy. Clearly, our knowledge in the latter area is woefully fragmentary. Nevertheless, even a highly provisional effort to synthesize available data could be useful since such attempts could focus attention on specific problems with further experimental studies. The objectives of this chapter, thus, are both to review current concepts relating to the distribution and metabolism of thyroid hormones and to describe, insofar as possible, the quantitative relationship between hormonal action and occupancy at specific nuclear receptor sites.

II. DISTRIBUTION AND METABOLISM OF THE THYROID HORMONES

A. General Principles

The principal secretory products of the thyroid gland are L-thyroxine (T_4) and L-triiodothyronine (T_3). Current estimates suggest that in normal man approximately 15% of circulating T_3 is derived as the result of direct thyroidal secretion, whereas the remaining 85% is the product of conversion from T_4 in peripheral tissues (Surks *et al.*, 1973). In the rat, estimates based on the average plasma concentration of T_4 (40 ng/dl) and T_3 (0.6 ng/dl) and the metabolic clearance rates of T_3 and T_4 (Oppenheimer *et al.*, 1970; Goslings *et al.*, 1976) suggest that the direct thyroidal secretion of T_3 and peripheral conversion contribute approximately equally to the total exchangeable T_3 pool in this species. Although multiple tissues have been shown to be capable of converting T_4 to T_3, quantitative contributions by individual tissues to the total T_3 generation have not been accurately established. Since phenobarbital has been shown to stimulate deiodination by a selective stimulation of the hepatic microsomal system, it is possible to calculate that in the rat approximately 40% of outer ring T_4 deiodination occurs within the liver (Oppenheimer *et al.*, 1968). The rate of total T_4 deiodination and specific 5′ deiodination may not be proportional under all circumstances, and it is impossible, however, to be certain whether the liver is also responsible for 40% of the peripheral conversion of T_4 and T_3. More recently, DiStefano *et al.* (1982) have estimated from a kinetic model based entirely on plasma measurements that approximately 50% of the total T_3 production rate of the rat is derived from the conversion of T_4 to T_3 in the slowly equilibrating tissue pools, such as muscle, with the remainder originating from T_4 conversion in rapidly exchangeable pools as typified by liver and kidney. Additional studies, especially those exploiting direct tissue measurement, are required to assign with precision the derivation of circulating T_3 in the rat.

An important principle governing the peripheral distribution of thyroid hormone is the rapid exchange of T_3 to T_4 between plasma protein and cellular pools (Oppenheimer *et al.*, 1969). The fractional rate of entrance and exit of both iodothyronines into and from tissue generally far exceeds the fractional rate of irreversible metabolism from the animal. The bulk of iodothyronine that enters the cell returns to the plasma before being metabolized by deiodination, deamination, conjugation, sulfation, or simple biliary excretion. The exchange characteristics between plasma and cellular pools, however, vary from tissue to tissue. Thus, liver and kidney equilibrate rapidly, slowly with plasma of brain and muscle, and at an intermediate rate with pituitary and heart. Among the factors that determine the fractional exchange rate are the strength of plasma and intracellular tissue binding as well as the permeability of the endothelial structure in the vessels bathing each tissue (Oppenheimer *et al.*, 1969). Thus, in the liver, relatively large gaps in the endothelial lining allow virtually direct contact between plasma protein and the plasma membrane of the hepatocytes. In contrast, the vascular endothelium in muscle is much less permeable to protein, a fact that probably accounts for the relatively slow rate of exchange of T_3 between plasma and muscle cells. Since over 99% of extrathyroidal iodothyronine is bound to plasma proteins, whether in the circulating plasma compartment, the interstitial fluid, or in the cellular compartment, the movement of hormone from one compartment to another is determined in large part by the mobility of the binding protein. The tight binding of iodothyronines to proteins is related both to existence of stereospecific binding proteins and to the intrinsic adhesiveness of iodothyronines to a variety of surfaces.

B. "Free" Hormone in Plasma and Cells

An important concept is that the free or non-protein-bound hormone is the "active" moiety most directly involved in the stimulation of the molecular processes leading to the expression of thyroid hormone action (Robbins and Rall, 1960). An alternative interpretation of available data is the "collisional hypothesis," which contends that under certain circumstances a direct transfer of iodothyronine from one binding molecule to another may occur without an intervening transition into a discreetly separable free hormone (Oppenheimer *et al.*, 1969). The distance separating the T_3 molecule from the binding molecule is considered to be represented by a probability function characteristic of that binding protein. The transfer of T_3 from one protein binding domain to another at a given distance will then be provided by yet a third probability function related in complex fashion to the other two. The closer the two binding molecules approach each other the higher the probability of transfer. The equilibrium distribution between two species of binding proteins would be precisely what the free T_4 formulation would predict. The theoretical advantage of the collisional hypothesis is that it could explain the extremely rapid mass transfers that have been observed to occur between one compartment and another, as, for example, the interchange of iodothyronines between plasma and liver (Oppenheimer *et al.*, 1967). Adjudication between the free T_4 and the collisional hypothesis must await careful experimental evaluation of the fractional dissociation and association rates of iodothyronine for individual binding proteins. It is necessary to determine whether the rate of debinding of hormone from protein to the free state is sufficiently rapid to account for the observed unidirectional fluxes. Regardless of the mechanistic details of transfer of hormone from one binding site to another, the concept of free hormone remains operationally useful and a convenient way of predicting phase partition of hormones under equilibrium conditions, and the validity of this hypothesis will be assumed in our analysis.

More recently, Partridge and Mietus (1980) have challenged the conventional interpretation of the significance of free hormone concentrations in plasma. These investigators assessed the effect of individual binding proteins (thyroxine-binding prealbumin, albumin, and thyroxine-binding globulin) to retard the unidirectional clearance of labeled iodothyronine by rat liver. They concluded that such retardation did not correlate with what would be expected from determination of the strength of plasma binding by equilibrium dialysis. The interpretation of these experiments, however, can be challenged. Thus, since the rate of dis-

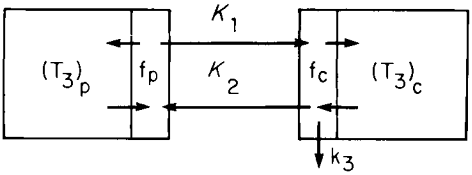

Fig. 1. Schematic relationship between plasma and cellular T_3. $(T_3)_p$, T_3 bound to plasma proteins; f_p, free T_3 in plasma; f_c free T_3 in cells; $(T_3)_p$, T_3 bound to cellular proteins; k_1 and k_2 are the fractional transfer constants from the designated compartments; k_3, the fractional exit rate for the free intracellular compartment. See text for discussion.

sociation from individual plasma binding proteins probably differs widely and since the rate of return from liver to plasma also requires a finite time, the relationship between liver and plasma T_3 cannot be surmised by one-way clearance studies. Moreover, there are no physiological data to support the concepts favored by the authors, namely that individual binding proteins subserve a specific biological function by facilitating the transport of hormone to particular tissues.

An important problem in hormone action is the assessment of the relative concentration of free hormone in plasma and cells. The relationship between plasma and cellular pools of hormone is illustrated in Fig. 1. It is apparent that under steady-state conditions

$$\frac{f_c}{f_b} = \frac{k_1}{k_2 + k_3} \tag{1}$$

where f_c is the concentration of free hormone in tissue c and f_p is the concentration of free hormone in plasma, k_1 the fractional transfer rate of free hormone from plasma to tissue, k_2 the fractional transfer in the opposite direction, and k_3 the irreversible rate of exit of free hormone from the tissue compartment. The tacit assumption is frequently made that free hormone concentration in plasma is the same as that in tissues ($f_c = f_b$). This can be true, however, only when k_2 is much larger than k_3 and when k_1 will therefore approximate k_2. Since it is generally held that the fractional rate of metabolism is much slower than the rate of exchange, the assumption that k_2 is larger than k_3 probably is true for most tissues. Nevertheless, rigorous proof of this proposition for individual tissue compartments has not been provided and exceptions for specific cellular domains may yet be demonstrated. If iodothyronine is transported into and out of cells by simple diffusional processes as has frequently been assumed in the past, k_1 and k_2 would be identical. Nevertheless this prediction has not been tested experimentally, and more recently the view has been advanced that iodothyronine transfer involves facilitated transport or even possibly active transport (Cheng *et al.* 1980; Rao, 1981; Halperin and Hinkel, 1982).

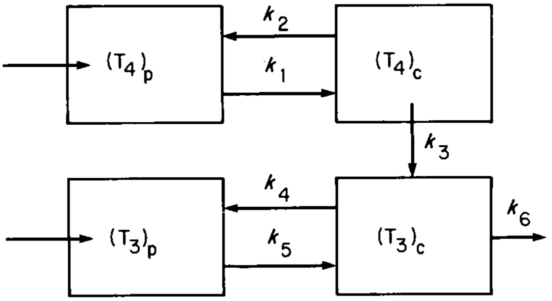

Fig. 2. Schematic representation of the relationship of intracellular total T_3, $(T_3)_c$, to intracellular T_4, $(T_4)_c$, and plasma T_3, $(T_3)_p$. $(T_4)_p$ represents plasma T_4; k_1, k_2, k_4, k_5, the fractional transfer constants from the compartments indicated k_3 represents the fractional rate of conversion of intracellular T_4 to T_3, and k_6, the fractional exit rate of T_3 from the intracellular compartment. See text for discussion.

C. Source of Cellular T_3: Plasma versus Local Deiodination

A related process that has recently received considerable attention (Crantz and Larsen, 1980; Obregon *et al.*, 1979), pertains to the factors that determine the relative contribution to cellular T_3 from T_3 generated locally from T_4 within the cell and T_3 transported into the cell from plasma. In earlier studies, the concept was advanced that T_3 generated from intracellular T_4 is rapidly ejected into the plasma compartment with the implication that no significant gradients of free T_3 between plasma and cells were established (Surks *et al.*, 1973; Goslings *et al.*, 1976; Schwartz *et al.*, 1971; Oppenheimer *et al.*, 1974b). This appears to have been an oversimplification. Larsen and Silva (Silva and Larsen, 1978; Silva *et al.*, 1978; Larsen *et al.*, 1979) showed that in the pituitary, nearly one-half of the cellular T_3 is of local origin. More recently, Crantz and Larsen (1980) and Obregon *et al.* (1979), have also raised the possibility that local monodeiodination may generate such gradients in brain. On the other hand, Surks and Oppenheimer (1977) showed that in liver and kidney exchangeable T_3 is essentially completely derived from plasma, a finding confirmed by Larsen and Silva (Silva and Larsen, 1978; Silva *et al.*, 1978).

The general relationship defining the plasma and cellular origin of T_3 is illustrated in Fig. 2. Under equilibrium conditions the ratio of total cellular T_3 (free plus bound) derived from plasma, $(T_3)_{cp}$, to the total cellular T_3 in plasma derived from all sources, $(T_3)_c$, is provided by the following equation:

$$\frac{(T_3)_{cp}}{(T_3)_c} = \frac{k_5(T_3)_p}{k_3(T_4)_c + k_5(T_3)_p} \tag{2}$$

where the fractional transfer constants are defined in Fig. 2 and $(T_4)_c$ refers to the total cellular T_4 in tissue c. It is apparent that in tissues where $k_5(T_3)_p \gg k_3(T_4)_c$

local deiodination makes a negligible contribution to the total cellular content. In tissues such as liver, k_5 is exceedingly high, a reflection of the rapid exchange of T_3 between plasma tissues. In contrast, in tissues with a slow exchange rate and, therefore, a smaller k_5, the contribution of a given rate of local deiodination to the formation of local T_3 will be significantly higher. It is therefore apparent that the source of T_3 in other slowly equilibriating tissues requires further documentation.

III. KINETIC RELATIONSHIP OF NUCLEAR T_3 TO OTHER T_3 POOLS

A. Fractional Transfer of Hormone: Identification of Nuclear Sites

For most tissues, the fractional transfer of iodothyronine between plasma and cellular pools is largely independent of the concentration of iodothyronine. This is a reflection of the fact that the number of available binding sites in these compartments is very large in relationship to the T_3 content and the fact that the transfer process itself does not behave as a limited capacity system. Even in the case of serum proteins, the binding capacity far exceeds the ambient concentration of thyroid hormone. In man, TBG has the most restricted capacity, 20 μ/dl, which is approximately three times greater than the normal T_4 concentration (Robbins and Rall, 1960; Oppenheimer, 1968). Although a large concentration of T_4 increases the fractional transfer of T_4 from plasma to cells somewhat, the extent of the increase is buffered by the large binding capacities of albumin and TBPA.

From a kinetic standpoint, nuclear sites differ from most other binding sites for iodothyronine inasmuch as their affinity for T_3 is substantially higher and their capacity very much smaller than those exhibited by other binding proteins. Thus, even small perturbations in the mass of equilibrating T_3 will result in a major alteration in the fraction of nuclear sites occupied. The existence of nuclear sites was first suspected in studies designed to define the equilibration of labeled iodothyronine between plasma and various tissues (Schadlow *et al.*, 1972). Tracer doses of T_3 and T_4 were injected intravenously together with increasing doses of the corresponding unlabeled hormone. Three hours afterward the tissue:plasma trichloracetic precipitable radioactive ratio (T:P ratio) was determined (counts per g/counts per ml). In liver, kidney, pituitary, and brain, the T:P ratios of T_4 were independent of the total dose of T_4 injected. Similarly, the T:P ratio for T_3 was independent of dose in liver, kidney, and brain. The adenohypophysis, however, constituted a conspicuous exception (Fig. 3). With increasing T_3, the T:P ratio fell precipitously until a constant value was ap-

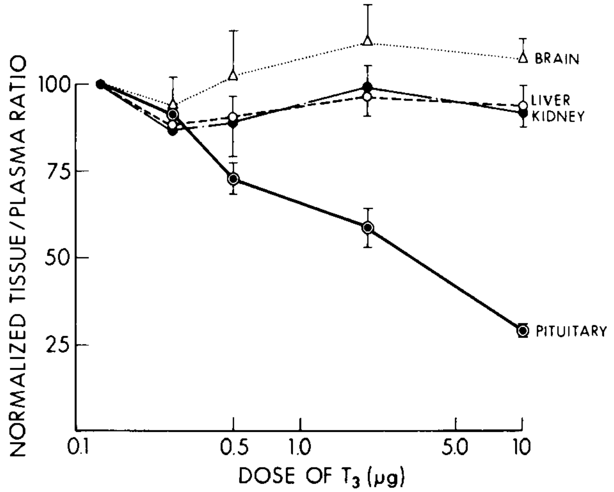

Fig. 3. Increasing doses of T_3 are injected i.v. into male Sprague-Dawley rats 3 hours before killing together with a constant tracer dose of $[^{125}I]T_3$. The tissue/plasma radioactivity ratio are determined and normalized to the value obtained under tracer conditions only. Of the four tissues examined only pituitary shows limited capacity sites without the use of subcellular fractionation. From Schadlow *et al.* (1972).

proached. Chromatographic analysis showed that the pituitary and the TCA precipitable plasma activity were in the form of T_3. These results thus constituted evidence that the pituitary harbored a set of limited capacity binding sites.

These experiments prompted additional studies to identify the subcellular organelle containing the specific T_3 receptor sites. The possibility was also considered that cells other than the pituitary might also contain such sites but that their number might be insufficient to allow detection in unfractionated tissue. Accordingly, liver and kidney were subjected to subcellular fractionation after the injection of combined doses of tracer and nonradioactive T_3 (Oppenheimer *et al.*, 1972a). Animals were killed ½ or 1 hour after injection. Since the $t_{1/2}$ of T_3 is 6 hours, the metabolic transformation of T_3 to other compounds during this period could be effectively ignored. The results of these experiments are illustrated in Fig. 4 and clearly show the nuclear fraction in liver and kidneys contains a set of limited capacity binding sites. In these tissues, the percent of labeled cellular T_3 associated with the purified nuclear fraction declined as the dose of T_3 was increased. In contrast, the other subcellular fractions studied including mitochondria, microsomes, and the cytosol did not give any indications of similar specific binding sites. Thus, the percentage of total hepatic activity associated with the extranuclear fraction could be predicted on the basis of multiple equilibria relationships and the displacement of specifically labeled T_3 from the nucleus to the other subcellular fractions (Fig. 5). For a large range of injected

T_3, extranuclear fractions behave as though they exhibited an unlimited binding capacity. Strictly speaking, however, the binding capacity of cytosolic sites is not truly "infinite" since, under *in vitro* conditions with sufficiently large concentrations of T_3 and T_4, a limited capacity can be demonstrated (Dillmann *et al.*, 1974). Sites defined in this fashion have a capacity two orders greater and an affinity two orders less than that displayed by the nuclear sites. Similarly, a large capacity, low affinity system has also been reported in plasma membranes (Segal and Ingbar, 1980).

The possibility should be considered that the *in vivo* displacement experiments cited above do not have the requisite sensitivity for the detection of small num-

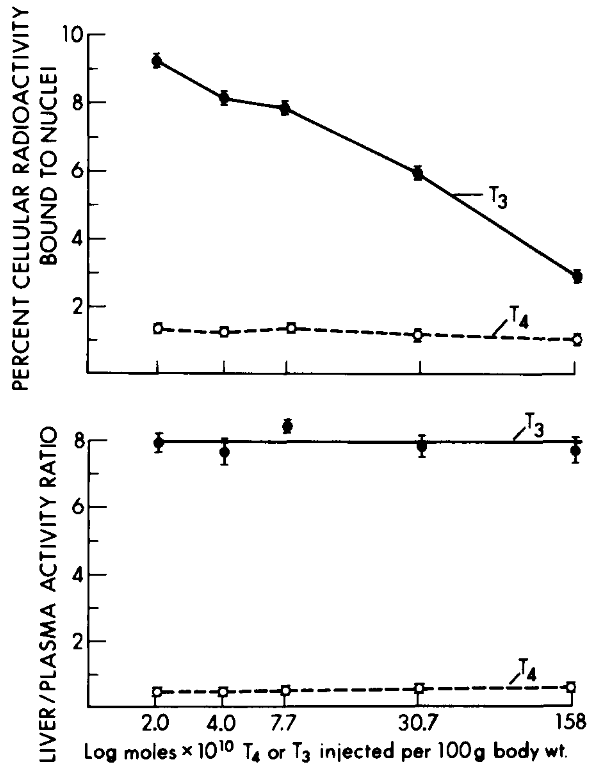

Fig. 4. Demonstration of specific nuclear sites in liver by *in vivo* techniques. Increasing doses of T_3, together with tracer concentrations of $[^{125}I]T_3$, are injected ½ hour prior to killing the rats. Liver homogenates are prepared and subjected to standard subcellular fractionation. With increasing doses of T_3 there is a progressive reduction in the fraction of cellular $[^{125}I]T_3$ associated with the nuclear fraction. Analogous experiments with nonradioactive T_4 and $[^{125}I]T_4$ showed only an imperceptible decrease in the percentage of labeled hormone bound to the nuclei. As indicated in Fig. 3, no specificity could be demonstrated when only crude homogenate was analyzed, either for T_3 or T_4. From Oppenheimer *et al.* (1972a).

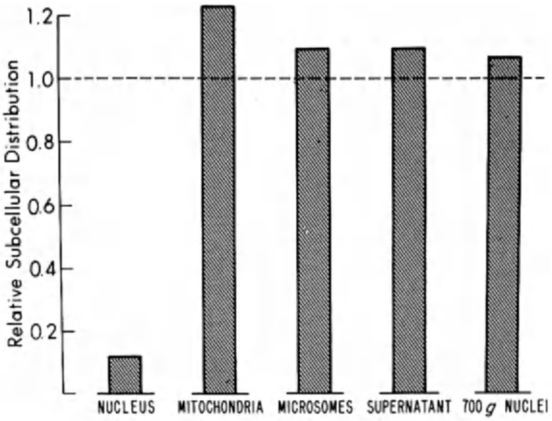

Fig. 5. Subcellular fractionation of liver homogenates is carried out ½ hour after the intravenous injection of tracer doses of $[^{125}I]T_3$ (70 ng/100 g bw) into five rats and saturating doses (10,000 ng/100 g bw) into another group of five rats. Subcellular distribution was carried out with standard techniques. Relative subcellular distribution represents the ratio of the fraction of $[^{125}I]T_3$ associated with a given subcellular fraction after the injection of the saturating dose to the fraction of $[^{125}I]T_3$ associated with that fraction after administration of tracer T_3 only. The results indicate that radioactivity displaced from nuclear fraction is redistributed to the other subcellular components in proportion to their binding avidity for T_3. 700 *g* nuclei represents the supernatant of the crude 700 *g* after separation of nuclear component. These studies fail to demonstrate evidence for specific displaceable T_3 sites in subcellular fractions other than nuclei. From data of Oppenheimer *et al.* (1972a).

bers of limited capacity, high affinity sites against the background of a large number of nonspecific sites. Sterling and co-workers have reported the existence of specific sites in mitochondrial subfractions (Sterling and Milch, 1975; Sterling, 1979). Nevertheless, if these authors are correct in their estimate that there are approximately 2000 sites per cell (Sterling and Milch, 1975), about one-half the number estimated for the nuclear sites, it is somewhat surprising that mitochondrial sites could not be readily demonstrated *in vivo* displacement experiments. Although Greif and Sloan (1978) reported their inability to reproduce the original *in vitro* findings, Gaglia *et al.* (1981) confirmed the presence of specific binding sites in a submitochondrial fraction.

Nevertheless, these investigators noted that 3,3′diiodo-L-thyronine, a substance with relatively minor biological activity and affinity for nuclear sites, was the analog most avidly bound to the inner mitochondrial membrane sites. Perhaps because of their inherent instability, these remain poorly characterized, and their physiological role remains undefined. In contrast to the high capacity system reported by Segal and Ingbar (1980), Pliam and Goldfine (1977) have published Scatchard plots suggesting a set of limited capacity sites on plasma membranes of hepatocytes. These sites appear to differ from the large capacity system reported by Segal and Ingbar (1980) and mentioned above. Additional data are needed to assess the functional significance of these findings.

In this connection, some caution should be introduced with respect to the use of two commonly used terms in this area, ''receptor'' and ''specific.'' ''Receptor'' should imply biological function and in the case of a hormone, the point of initiation of biological effect. Too often the term receptor is loosely used to designate any site where tracer hormone can be displaced with excess unlabeled ligand. As discussed in Section IV, the criteria for accepting a binding site as a receptor are stringent. Similarly, the term ''specific'' is often misunderstood and misused. Specificity refers only to displaceability of labeled ligand to a set of sites and should have no biological functional connotation. It is generally recognized that iodothyronines tend to be extremely ''sticky,'' possibly a reflection of the tendency to hydrophobic interactions with a variety of surfaces. Because of the heterogeneity of most protein surfaces, one would anticipate that certain areas of protein might be more suitable for ligand binding than others. Given reversibility in hormone binding to such surfaces, it is easy to understand how displaceability and ''specificity'' could be an entirely fortuitous property. Moreover, the possibility of the *de novo* generation of specific sites during the course of *in vitro* isolation should also be considered at least as a theoretical possibility. In essence, simple displaceability, though by definition denoting a ''specificity,'' does not constitute sufficient evidence to attribute biological function to such a site.

B. Assessment of Kinetic Nuclear Parameters by *in Vivo* Kinetic Techniques

The interrelationships of T_3 bound to nuclear, cytoplasmic, and plasma pools are of interest (Oppenheimer *et al.*, 1974). Consonant with the rapid exchange between plasma and cytoplasmic T_3 discussed above, T_3 enters the hepatocyte almost immediately after injection of tracer (Fig. 6). Thereafter, the concentration of radioactive T_3 in plasma and cytoplasm falls in a parallel fashion. The function describing nuclear T_3, however, is characterized by a delayed upstroke, with maximal levels of nuclear T_3 being attained about ½ hour after injection. Thereafter, the concentration of nuclear T_3 declines and after several hours approaches the rate of decline exhibited by plasma and cytoplasmic T_3. A kinetic analysis of these data suggests that the nucleus does not contribute significantly to the metabolism of the iodothyronines (Surks *et al.*, 1975). Thus, the unidirectional delivery of T_3 to the hepatic nuclear pool far exceeds the rate of irreversible degradation of T_3 from the animal as a whole. The great bulk of T_3 delivered to the nucleus, therefore, returns to the cytoplasm chemically unchanged. Direct biochemical studies of isolated nuclei have also failed to demonstrate any evidence of T_3 metabolism even after the addition of multiple cofactors (Surks *et al.*, 1975).

The kinetic relationship between the plasma concentrations of T_3 and T_3 specifically bound to nuclei can be effectively exploited to estimate the nuclear T_3 binding capacity of individual tissues, the proportion of such sites occupied under physiological conditions, and the fraction of the total exchangeable cellular T_3 associated with the specific sites (Oppenheimer *et al.*, 1974a,b). Such data can be obtained with only minor modifications of the experimental design described above. Tracer doses of T_3 (10–60 ng/100 g bw) alone and together with increasing doses of unlabeled T_3 (150, 300, 400, and 600 ng/100 g bw) are injected into separate groups, each consisting of four animals. In addition, a saturating dose of 10,000 ng/100 g bw is also administered to a group for the purpose of measuring nonspecific nuclear binding.

The time at which the animals are killed after the single injection is critical in the assessment of binding capacity in euthyroid animals. Theoretically, there is only a single time point at which the specific activity of nuclear T_3 equals that of plasma T_3. This is the "equilibrium time point" of Veall and Vetter, a concept developed in connection with studies of the albumin distribution space in man (Pearson *et al.*, 1958). The specific activity in two exchanging compartments will be identical only when the rate of entrance of tracer to the distal compartment is the same as the rate of exit from that compartment. This point can be

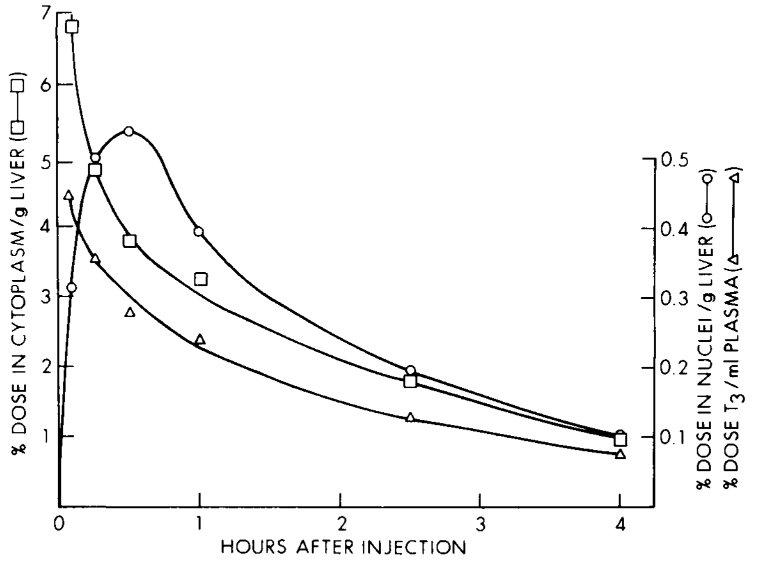

Fig. 6. Time course of concentration of $[^{125}I]T_3$ in plasma, cytoplasm, and nuclei after intravenous injection of a single dose of tracer T_3. From Oppenheimer *et al.* (1974a).

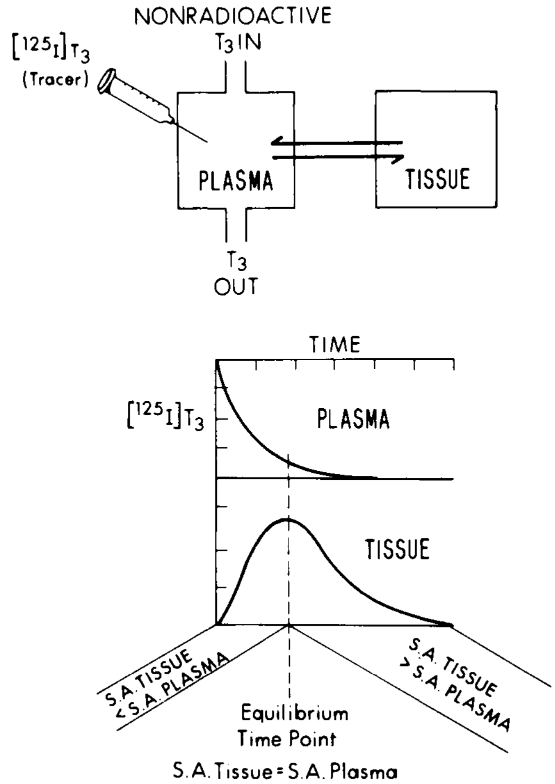
NONRADIOACTIVE
T3 IN
[125I]T3
(Tracer)
PLASMA
TISSUE
T3
OUT
TIME
[125I]T3
PLASMA
TISSUE
S.A. TISSUE < S.A. PLASMA
Equilibrium
Time Point
S.A. TISSUE > S.A. PLASMA
S.A. Tissue = S.A. Plasma

Fig. 7. Principle of the equilibrium time point in the measurement of nuclear binding sites from the nuclear/plasma (*N*/*P*) isotopic [^{125}I]T$_3$ ratio and the radioimmunoassayable plasma T$_3$, concentration. It can be shown that when the first derivative of tissue accumulating is zero and the tissue [^{125}I]T$_3$ concentration is maximal, the specific activity of intracellular T$_3$ is equal to the specific activity of plasma T$_3$. Thus, the product of the (*N*/*P*) ratio determined at the equilibrium time point and plasma T$_3$ yields the endogenous nuclear T$_3$.

identified when the rate of change of tracer in the distal compartment is zero (Fig. 7). For the sake of simplicity, we have described the exchange between plasma and individual cellular T$_3$ pools in terms of a two-compartmental system. Inspection of Fig. 6 reveals that for the liver, the equilibrium time point occurs approximately 30 minutes after injection. The equilibrium time point for each tissue is characteristic for that tissue and is contingent on those factors that determine the plasma-tissue equilibration kinetics discussed previously (Oppenheimer *et al.*, 1974b). The equilibrium time point for rapidly equilibrating tissues such as liver and kidney occurs at 30 minutes. The equilibrium time point for brain is delayed until 3 hours, a probable reflection of the slower rate of equilibration of this tissue with the plasma and T$_3$ pools. Pituitary and heart exhibit intermediate equilibrium time points, 1 and 1.5 hours, respectively. With the intermediate and slowly equilibrating tissues, the tissue uptake of tracer as a function of time shows a relatively broad plateau region rather than sharply defined maximal points. The equilibrium time point is, therefore, defined at the midportion of the plateau. For the slowly equilibrating tissues, the assumption is also made that the intracellular equilibration between cytoplasmic and nuclear T_3 occurs relatively rapidly in comparison to the equilibration between plasma and total cellular hormone. This assumption has been verified for brain (Oppenheimer *et al.*, 1974a).

In order to evaluate the nuclear binding parameters, the following measurements are made at the equilibrium time point: (1) The total $[^{125}I]T_3$ content per gram body weight, (2) the nuclear content of $[^{125}I]T_3$ per gram tissue, (3) the $[^{125}I]T_3$ content per ml plasma, and (4) the concentration of radioimmunoassayable T_3 in plasma. In general, the radioactivity in tissue can be assumed to be in the form of T_3 since ^{125}I, the major product of degradation, is almost immediately ejected from the cell. The concentration of $[^{125}I]T_3$ in plasma can be approximated by simple TCA precipitation, although more accurate quantitation of T_3 requires the use of immunoadsorbents. Nuclear receptor losses can be estimated from the recovery of total homogenate DNA in the separated nuclei. The specific nuclear:plasma ratio $(N/P)_S$ can be determined from the following expression.

$$(N/P)_S = (N/P)_T - (N/P)_\infty \qquad (3)$$

where $(N/P)_T$ is the observed total nuclear:plasma ratio under tracer conditions and $(N/P)_\infty$ is the ratio determined after injection of a saturating dose of T_3 and considered to represent nonspecific T_3 binding. The product of $(N/P)_S$ and the T_3 by radioimmunoassay thus will indicate the mass of T_3 specifically bound to the nuclear fraction. When plotted as a function of the terminal plasma T_3 concentration, this value achieves a well-defined plateau representing the nuclear T_3 binding capacity M. Since the plateau values are subject to the greatest experimental error, it is advantageous to use all of the available points in estimating the binding capacity. To this end, the function $(P/N)_S$ can be plotted as a function of P, the plasma T_3 concentration (Oppenheimer *et al.*, 1975). Simple rearrangement of the law of mass action will show that for a single class of noncooperative sites this plot will yield a straight line with a slope of $1/M$ and an intercept on the ordinate of $(1/M\mathrm{k})$ where k is the equilibrium constant governing the exchange of T_3 between nuclear and plasma pools. When tracer concentrations of T_3 are injected the product of the $(N/P)_S$ ratio and the plasma T_3 concentration will provide an estimate of the content of the endogenous T_3 specifically bound to nuclear receptors. The fraction of total specific sites occupied by T_3 under physiological circumstances can, therefore, be determined. Moreover, since the total content of labeled T_3 per gram tissue is also known, it is possible to calculate the proportion of total exchangeable tissue T_3 sites that are stereospecific.

The results of these studies are summarized in Table I. It is apparent that the number of specific binding sites per milligram DNA varies widely from one tissue to another. Of the tissues studied, the anterior pituitary contains the highest concentration of receptor sites per milligram of DNA and the testis the lowest. If we assume each nucleus contains 8 pg of DNA (Leuchtenberger *et al.*, 1951), the binding capacity data indicate that there are approximately 6000 binding sites per nucleus in the anterior pituitary, 4000 in the liver, and 16 in the testis. The number of binding sites in spleen is also small, approximately 400 per nucleus, comparable to the number observed in circulating white cells (Tsai and Samuels, 1974). The relatively low number of specific binding sites in spleen and testis accords with the apparent lack of responsiveness of these tissues to T_3, at least using the conventional criterion of enhanced oxygen consumption (Barker, 1955). On the other hand, brain, another "nonresponsive" tissue, contains about 2000 specific binding sites per cell. The lack of response of this tissue to oxygen consumption is well documented (Barker, 1955), and has recently been confirmed both in adult and neonatal tissue (Schwartz and Oppenheimer, 1978a). Moreover, in neonatal brain, the nuclear binding capacity exceeds that of adult brain and is equivalent to that of adult liver (Schwartz and Oppenheimer, 1978b). Furthermore, solubilized nuclear binding sites in liver and brain exhibit identical physicochemical characteristics, with equivalent chromatographic mobility on DEAE-Sephadex, similar binding affinities, and the same analog binding spectrum (Schwartz and Oppenheimer, 1978a). The critical role of thyroid hormones in the ontogeny of the central nervous system has been well documented and is reviewed elsewhere in this volume (see Chapter 14). Either the nuclear binding sites in brain have no functional significance in the adult or the appropriate indices of biochemical action remain to be identified.

Among the various tissues examined, approximately 50% of the binding sites of each tissue appear to be occupied in the euthyroid state. This is precisely what one would anticipate on the basis of qualitatively identical nuclear sites and the same intracellular free T_3 concentration in each tissue. Although the concentration of nuclear sites varies widely from tissue to tissue, the sites themselves are probably identical. Of parenthetic interest is that using the criterion of binding capacity per milligram DNA, binding affinity, chromatographic mobility on DEAE-Sephadex and analog binding spectrum, T_3 nuclear binding sites from human kidney and liver are indistinguishable from sites derived from the corresponding tissues of the rat (Schuster *et al.*, 1979). Additional studies are needed to confirm this suspicion of evolutionary conservation.

Our analysis, however, was based on the implicit assumption that T_3 available for nuclear binding is derived exclusively from plasma. In other words, all the T_3

TABLE I

Characteristics of Nuclear T_3 Binding in Different Rat Tissues[a,b]

Tissue	Binding capacity: ng/mg DNA	Binding capacity: Normalized to liver (=1)	Binding capacity: ng/g tissue	Binding capacity: Normalized to liver (=1)	Total DNA/g tissue (mg)	Nuclear[c] T_3 tissue (% total)	Saturation at endogenous T_3 levels (%)
Liver	0.61	1.0	1.77	1.00	2.90	12.9	47
Brain	0.27	0.44	0.42	0.24	1.55	13.5	39
Heart	0.40	0.65	0.80	0.45	2.01	15.4	44
Spleen	0.018	0.03	0.31	0.18	17.27	13.0	50
Testis	0.0023	0.004	0.022	0.01	9.56	3.0	
Kidney	0.53	0.87	2.61	1.47	4.93	9.0	35
Anterior pituitary	0.79	1.30	6.58	3.72	8.33	52.6	48

[a] Each tissue was studied at the predetermined equilibrium time. Entries represent the average values from two to eight separate experiments for the different tissues. Corrections were made for losses of DNA.

[b] Data from Oppenheimer *et al.* (1974b).

[c] Specifically bound.

generated in a given tissue was presumed to be totally exported to the tissue before being taken up again by that tissue. The problem of the relative contribution of locally generated and plasma-derived T_3 has already been discussed (Section II,C). As pointed out, local T_4 to T_3 conversion in the pituitary (Silva and Larsen, 1978; Silva *et al.*, 1978; Larsen *et al.*, 1979) and in brain (Crantz and Larsen, 1980) may lead to higher steady-state free intracellular hormone concentration in these tissues than is expected from the plasma free T_3 concentration. The existence of free T_3 gradients in the pituitary, therefore, would suggest that the product of the ambient plasma T_3 concentration and the nuclear:plasma ratio would underestimate the nuclear T_3 content and the percentage saturation of nuclear sites in this tissue. In fact, Silva and Larsen (Silva *et al.*, 1978) have reported that approximately 75% of the pituitary nuclear sites are occupied in contrast to the 50% estimated by Oppenheimer *et al.* (1974b). If Larsen's laboratory is correct in the supposition that local T_4 to T_3 conversion in the brain exerts an even more profound impact on the composition of intracerebral T_3, it follows that CNS nuclei are correspondingly more saturated than is the pituitary. Additional studies, preferably involving direct measurement of nonradioactive nuclear T_3 in conjunction with estimates based on the nuclear:plasma isotopic ratio, are required to resolve these issues. Under any circumstance, the binding capacity determined by *in vivo* displacement techniques should not be influenced by the source of intracellular T_3. In fact, the pituitary nuclear T_3 binding capacity reported by Silva and Larsen corresponds closely with that reported by Oppenheimer *et al.* (1974b).

Morever, there appears to be a general agreement that the principal source of T_3 in liver, kidney, and heart is derived largely from plasma (Silva and Larsen, 1978). Therefore, the original proposal, namely, that these sites are approximately 50% saturated, holds. This conclusion is further strengthened by the report of Surks and Oppenheimer indicating agreement in the estimate of hepatic and renal nuclear T_3 with three independent assays: ^{125}I equilibration, direct radioimmunoassay, and the *in vivo* displacement technique described above (Surks and Oppenheimer, 1977).

These data also allow calculation of the effective "*in vivo*" affinity of nuclear sites in rapidly exchangeable tissues. Thus, the reversible association of T_3 with specific nuclear sites can be represented by the following equation.

$$T_3 + N \rightleftharpoons T_3N \tag{4}$$

where T_3 is the free T_3 concentration as determined by equilibrium dialyses, N is the number of unoccupied nuclear sites, and T_3N is the T_3 nuclear complex. From the law of mass action it follows that

$$k_a = \frac{T_3N}{(M - T_3N)(T_3)} = \left(\frac{F}{1-F}\right)\left(\frac{1}{T_3}\right) \tag{5}$$

where k_a is the association constant, M the total number of binding sites, and F is the fraction of specific sites occupied under physiological conditions (T_3N/M).

The issue of whether or not the effective free hormone concentration in cells is identical to that measured in plasma has been discussed above. Theoretically, the intracellular free hormone concentration could be higher than that in plasma either because the fractional entrance rate into the cell exceeds the fractional exit rate from the cell or because local cellular 5′ deiodination contributes to the establishment of a T_3 gradient. These considerations are of some importance in the interpretation of the numerical values for k_a derived from substitution into Eq. (5). Since the free T_3 concentration in plasma appears to be approximately 6×10^{-12} M and 0.5 of the hepatic sites are occupied, the calculated *in vivo* association constant is 1.6×10^{11} M^{-1}. This figure, however, is substantially greater, at least by an order of magnitude, than the association constant as measured at 37°C in whole nuclei or in solubilized nuclear preparations *in vitro* (Surks *et al.*, 1975; Silva *et al.*, 1977). At the same time, the binding capacities determined *in vitro* and *in vivo* are almost identical.

In order to explain the higher affinity constant estimated *in vivo,* one could postulate that the true free T_3 concentration within the hepatic cell *in vivo* is some 10-fold higher than that in plasma. Sirce the currently available evidence suggests that there is no substantial contribution to hepatocellular T_3 from local T_4 to T_3 conversion, one would have to postulate that a relative diminution in the free T_3 fractional exit rate sustains the intracellular free T_3 gradient. Another possible explanation of the differences between *in vitro* and *in vivo* affinity constants is that undefined factors are responsible for substantially stronger binding *in vivo* than *in vitro*. Thus, the ionic milieu of nuclear binding could differ significantly between *in vivo* and *in vitro* states. Nevertheless, it is difficult to explain why estimates of nuclear affinity in intact GH_1 cells (3.45×10^{10} M^{-1}; Samuels and Tsai, 1974) human lymphocytes (3.24×10^{10} M^{-1}; Tsai and Samuels, 1974) and isolated hepatocytes (1.78×10^{10} M^{-1}; Mariash *et al.*, 1981) are also substantially less than that calculated from *in vivo* data in the intact rat (4.7×10^{11} M^{-1}; Oppenheimer *et al.*, 1974a). It follows either that the effective free hormone concentration in plasma is systematically underestimated by equilibrium dialysis or that the cytoplasmic T_3 concentration is approximately 10-fold higher than the free hormone concentration in plasma. The latter connotes cellular pumping action, which would require the expenditure of metabolic energy. Regardless of the ultimate resolution of these questions, the *in vivo* estimate of nuclear binding provides at least a relative measure of the effective strength of nuclear binding under physiological and pathophysiological conditions.

In vivo displacement techniques also make it possible to assess the fractional exit rate of T_3 from the specific nuclear sites (Oppenheimer *et al.*, 1976). The experimental approach is as follows. Tracer T_3 is injected i.v. This is followed 30 minutes later with an i.v. "loading" dose of T_3, which will prevent tracer reuptake by the nuclear sites. The fractional exit rate of T_3 from the nuclear sites is determined by killing groups of animals at periodic intervals after the second dose. With this technique, the $t_{1/2}$ of exit has been estimated to be approximately 15 minutes, corresponding to a dissociation rate constant of 0.0462/minute. Assuming an *in vivo* equilibrium association constant of $1.6 \times 10^{11}\ M^{-1}$, the calculated forward rate constant (from free plasma hormone to nuclear site) would, therefore, be $2.17 \times 10^{10}\ M^{-1}$ minute^{-1}.

IV. THE INITIATING ROLE OF THE NUCLEAR BINDING SITE

Any consideration of the quantitative interrelationship between nuclear occupation by T_3 and a hormone action is based on the tacit assumption that the nuclear sites measured are the exclusive points of initiation of these biochemical processes resulting in the hormone action under consideration. In other words, the nuclear binding sites should act as true receptors.

The evidence supporting such a role for nuclear sites has been reviewed (Oppenheimer, 1979). As discussed above, the nuclear binding sites exhibit the characteristics of limited capacity and high affinity, criteria that are generally deemed to be necessary but should by no means be considered sufficient to allow them to be identified as receptors. Additional support for the receptor function of nuclear binding sites comes from the close temporal association of nuclear occupancy by T_3 and a generalized increase in RNA formation (Tata *et al.*, 1962; 1963) including poly(A)-containing RNA (Dillmann *et al.*, 1978b). These increases occur within 6 hours after T_3 injection. An even earlier response, within 1–2 hours, is the elevation in specific mRNA sequences coding for proteins known to be stimulated by the thyroid hormones (Martial *et al.*, 1977; Seo *et al.*, 1977; Samuels and Shapiro, 1976; Towle *et al.*, 1981) (see Chapter 6 for a more detailed discussion of the effect of thyroid hormone on RNA synthesis). It is clear that the shorter the gap between occupancy of the sites by T_3 and the observed effects, the more plausible is the casual relationship.

In this connection Seelig *et al.* (1981) have recently reported on the basis of radiofluorograms of *in vitro* translational products of hepatic poly(A^+) RNA that thyroid hormone administration results in a 20-fold increase in the rate of formation of a specific mRNA (spot 14) which achieves maximal values within a few hours after injection. More recent studies by Jump, Seelig, Towle, and Oppenheimer (unpublished) with a specific cDNA to the mRNA of spot 14 prepared by Towle indicates that the lag time spot 14 mRNA is only 20 minutes. Nevertheless, temporal association cannot be used to *prove* causality. Thus, one could postulate for the sake of argument that the true receptors are extranuclear in location and that the occupation of the nuclear sites and postulated extranuclear receptors were simply coincident phenomena.

Theoretically, the causal relationship between nuclear occupancy and response could be tested by perturbing the number or affinity of the putative nuclear receptor sites. Although a number of physiological and pathophysiological stimuli, including starvation, partial hepatectomy, and glucagon administration result in a decrease in hepatic nuclear binding sites (Dillmann *et al.*, 1978a; Dillmann and Oppenheimer, 1979), each of these stimuli probably exerts extranuclear effects on the system and thus limit the potential usefulness of this approach in testing the nuclear hypothesis. In this connection, it is of interest that Samuels has recently reported that enhanced acetylation stimulated by butyrate results in a profound loss of nuclear sites in GH_1 cells (Samuels *et al.*, 1980). Whether this decrease impairs the ability of T_3 to induce growth hormone remains to be determined.

The widespread distribution of nuclear receptor sites among all tissues capable of responding to thyroid hormone has been used as an argument to support the concept that the specific nuclear sites serve an initiating role in thyroid hormone action (Oppenheimer *et al.*, 1974b). As pointed out in Section III,B, however, the correlation between the tissue content of nuclear binding sites and hormonal action is fraught with the inherent problem of determining whether a given tissue is responsive to thyroid hormone. Reference has already been made to the dilemma posed by brain, which contains a substantial number of nuclear binding sites despite the apparent lack of responsiveness of this tissue to T_3 as measured by O_2 consumption, mitochondrial α-GPD activity, or cytosolic ME. Moreover, if oxygen consumption cannot be used as a universal criterion of thyroid hormone action, it is difficult to be absolutely certain that the decrease in nuclear sites in spleen and testis tissues, which also fail to exhibit increased respiration after T_3, connotes a global lack of thyroid hormone responsiveness. These considerations point to the importance of a broad survey of biochemical and physiological responses to hormone administration and deprivation in a given tissue.

A potentially interesting correlation between nuclear binding and biological response was suggested by the studies of Bernal *et al.* (1978) who showed that lymphocytes and fibroblasts in a patient with known resistance to thyroid hormone exhibited an apparent decrease in the affinity of nuclear sites. Both in the laboratories of the author and H. H. Samuels it has been exceedingly difficult to obtain reproducible data on the number and occupancy of leukocyte nuclear sites in euthyroid individuals. The concentration of leukocyte receptor sites appears to be extremely small, probably less than 200 per nucleus. Moreover, normal nuclear binding has been reported in the lymphocytes of affected individuals from a family with documented thyroid hormone resistance (Lamberg *et al.*, 1978), as well as in fibroblasts of other patients with the syndrome of peripheral resistance to thyroid hormone (Chait *et al.*, 1982; Bantle *et al.*, 1982).

Perhaps the most convincing set of findings supporting the concept of the initiating role of the nuclear sites is the striking parallelism between nuclear binding of thyroid hormone analogs and hormonal action as measured by conventional biological response parameters. In such studies, nuclear binding has

TABLE II

Relative Nuclear Binding Affinity of Thyroid Hormone Analogs to T_3[a]

Analog	Relative binding affinity ($T_3 = 1$)	
	In vitro	*In vivo*
L-T_3	1.0	1.0
D-T_3	0.6	0.7
Triiodothyroacetic acid (triac)	1.6	1.0
Isopropyl T_2	1.0	1.0
L-T_4	0.1	0.1
Tetraiodothyroacetic acid (tetrac)	0.16	0.05
3,3′,5′-T_3 (reverse T_3)	0.001	0
Monoiodotyrosine	0	0
Diiodotyrosine	0	0

[a] Methods for determining binding affinity *in vitro* and *in vivo* have been described elsewhere (Koerner *et al.*, 1974, 1975; Oppenheimer *et al.*, 1973).

been assessed by *in vivo* displacement (Oppenheimer *et al.*, 1973), *in vitro* binding of isolated nuclei (Koerner *et al.*, 1974, 1975) and binding by solubilized hepatic nuclear extracts (Torresani and DeGroot, 1975; Latham *et al.*, 1976). Displacement techniques involve determination of the dose of a given thyroid hormone analog required to displace tracer $[^{125}I]T_3$ from the nuclear sites. Considering the potential pitfalls in comparing *in vivo* and *in vitro* techniques, there is a remarkable similarity in the results obtained by these methods (Table II). A prerequisite for thyromimetic activity and action is 3′ substitution in the phenolic ring. Compounds with disubstitution in the 3′ and 5′ position are bound less firmly to nuclear sites and exhibit less biological activity than the corresponding monosubstituted compounds. Analogs constrained in a distal conformation are more active by both criteria than compounds fixed in the proximal conformation. Biological and nuclear binding activity can be maintained by substituting sulfur or a methylene groups for oxygen in the ether linkage. Weak but definite binding and biological activity is shown by compounds in which a methyl group is substituted for the iodine in T_4 and T_3 (Koerner *et al.*, 1974).

Apparent discrepancies between the thyromimetic and nuclear binding activities of several analogs can be resolved if the metabolic and distributive characteristics are taken into consideration. For example, compounds lacking the 4′ phenolic group fail to displace $[^{125}I]T_3$ from nuclear sites under *in vitro* conditions, but show considerable biological displacement and thyromimetic activity when injected *in vivo* (Koerner *et al.*, 1974). The activation is explained by 4′ hydroxylation, which occurs in the intact animal (Barker and Shimada, 1964). Special attention has also been directed to the effects of triac, the acetic analog of T_3. This compound has been reported to have ⅓ to ⅙ of the thy-

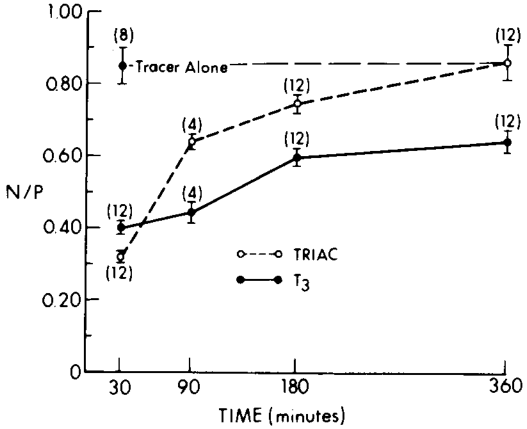

Fig. 8. Nuclear/plasma of $[^{125}I]T_3$ (*N/P*) as a function of time after the injection of 6.8×10^{-10} moles of T_3 and triiodothyroacetic acid (triac) per 100 g bw. Numbers in parentheses indicate the number of animals averaged at each point. Bars indicate SEM. *N/P* returns more rapidly after injecting triac than T_3. From Oppenheimer *et al.* (1973).

romimetic activity of T_3 (Money *et al.*, 1960; Westerfield *et al.*, 1965); yet both by *in vitro* as well as by *in vivo* assay techniques, the binding of triac to specific nuclear receptor either equals or exceeds that of T_3 (Oppenheimer *et al.*, 1973; DeGroot and Strausser, 1974). The failure of the biological activity to match nuclear binding can again be explained by biological transformations of the compounds *in vivo*. Triac is metabolized substantially more rapidly than is T_3. Thus, when equimolar doses of T_3 and triac are administered as daily pulse doses, the net residence time of triac on the nuclear sites is considerably shorter than that of T_3. In addition, the greater intensity of plasma protein binding of triac may contribute significantly to the lower effective mass of triac bound to the nuclei (Goslings *et al.*, 1976). Experimental verification of the shorter nuclear occupancy by triac is provided in experiments in which the nuclear:plasma (*N/P*) ratio of tracer $[^{125}I]T_3$ is determined as a function of time following the administration of equimolar doses of T_3 and triac (Fig. 8) (Oppenheimer *et al.*, 1973). The *N/P* ratio returns substantially more rapidly after the injection of triac than T_3, a reflection of the shorter occupancy of the nuclear sites by triac. Peripheral distribution and metabolism of triac have also been compared by isotopic techniques (Goslings *et al.*, 1976). Another example of an apparent dissociation between biological activity and nuclear binding is T_4, which is bound to nuclear sites with an avidity only 1/10 to 1/20 that of T_3, but exhibits a biological activity approximately 1/3 that of T_3. The concept that T_4 derives most of its biological activity through transformation to T_3 has already been discussed (Section II,A) and has assumed a central role in current thinking in this area.

Since the distribution and metabolism of thyroid hormone analogs are only incompletely understood, it is actually somewhat surprising that the correlation between nuclear binding and biological activity is as high as reported. This probably is a reflection of the fact that there is inherently a lesser degree of variation in the metabolism of thyroid hormone analogs than the binding of such compounds to nuclear sites. Since GH_1 cells grown in tissue culture do not metabolize iodothyronines, the problems posed by distribution and metabolism are obviated in this preparation. Samuels and co-workers (1979) have taken advantage of this by correlating nuclear binding and growth hormone response to a number of thyroid hormone analogs. These investigators have also shown an excellent correlation between nuclear occupancy and the rate of production of growth hormone by these cells.

On the basis of these considerations there appears to be adequate, if indirect, evidence for assuming that the nuclear sites under discussion do function as true receptors. A conclusive demonstration of an initiating role probably must await the development of a reconstituted *in vitro* transcriptional system in which the addition of isolated nuclear–T_3 receptor complex can be shown to result in the formation of mRNA sequences coding for proteins known to be stimulated by thyroid hormone.

V. QUANTITATIVE RELATIONSHIP BETWEEN NUCLEAR OCCUPANCY AND RESPONSE

Efforts to describe the relationship between nuclear occupancy and a given response necessarily takes the form of an input–output problem. Given the state of ignorance about the precise molecular events following occupation of the nuclear sites, the intermediate steps between input and output may be regarded as constituents of a "black box." We must also make the assumption in our analysis that the function representing occupancy of the sites is in fact the *only* point of initiation. If extranuclear factors participate in the initiating mechanisms, the relationship between nuclear occupancy and response could be quite misleading. Although considerable interest has been expressed by several investigators about the possibility of extranuclear initiating events (Sterling, 1979), currently available evidence favoring the physiological role of such processes is inconclusive. Such T_3-augmented processes as enhanced transport of deoxyglucose (Segal and Gordon, 1977) and cycloleucine (Goldfine *et al.*, 1976) require high concentrations *in vitro*. Additional studies, however, are required to settle this important question. Should the physiological relevance of a specific extranuclear action be established it would then be necessary to determine whether such actions would influence those protein synthetic processes considered in the following discussion.

The relationship between nuclear occupancy and response in the intact animal has been examined rigorously only for a few indices of thyroid hormone effects. Nevertheless, it is of interest to note the high degree of variability in the functions describing this relationship. For the purposes of this discussion, it is possible to classify responses according to the following patterns: (1) linear, (2) amplified, and (3) attenuated. The criteria for this classification are as follows. Consider the ideal state of a set of completely thyroidectomized animals, each maintained under steady-state conditions at specific constant rates of T_3 infusion and evaluate the effect of the rate of infusion on the response. Let ΔR be the increment in response over that exhibited in the thyroidectomized animal and ΔR_{max} the maximal response that can be elicited by T_3. If q_{n} represents the fraction of nuclear sites occupied at a given rate of infusion, then an ideal linear system should show a direct proportionality between $\Delta R/\Delta R_{\mathrm{max}}$ and q_{n} (Fig. 9). If a response is amplified, the slope of the occupancy–response curve rises progressively with increasing occupation. Thus, the occupancy–response curve becomes convex upward and the second derivative of the function assumes a positive value at every level of occupancy. If the response is attenuated, then the rate of change of increment will progressively decrease and the shape of the occupancy–response curve will be convex downward. The second derivative of the function will assume a negative value at all points. A convenient way of quantitating the response characteristic is with the use of an amplication factor, f, which is defined as $\Delta R_{\mathrm{max}}/\Delta R_{\mathrm{eu}}$ where ΔR_{eu} is the response above hypothyroid levels in the euthyroid animal. Since approximately ½ of the nuclear sites are occupied, in an ideal linear system, $f \approx 2$. Any value greater than 2 will be a

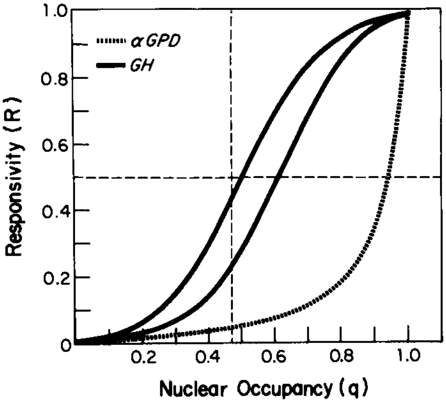

Fig. 9. Relationship between fractional nuclear occupancy of by T_3 and the responsivity of the parameter measured [rate of appearance of pituitary growth hormone and hepatic α-glycerophosphate dehydrogenase (α-GPD)]. Note that the occupancy relationship in the case of α-GPD is highly nonlinear and amplified whereas pituitary accumulation appears to be more linearly linked to nuclear occupancy in the two sets of experiments illustrated. From Coulombe *et al.* (1978).

measure of the degree of amplication, and any value less than 2 will be an expression of the degree of attenuation.

In the intact rat the rate of formation both of α-GPD and malic enzyme appears to be highly amplified with *f* values ranging from 10 to 15 (Oppenheimer *et al.*, 1978). Lipogenic enzymes other than malic enzyme also appear to be subject to amplified response, including fatty acid synthetase and the hexose monophosphate shunt enzymes glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Mariash *et al.*, 1980a). On the other hand, a number of response parameters appear to be generally linear. For example, the relationship between the pituitary content of rat growth hormone and nuclear occupancy is linear from zero occupancy to half occupancy (Coulombe *et al.*, 1978). Nevertheless, full occupancy of the pituitary nuclear receptors does not lead to a steady-state increase in growth hormone content above normal. Whether this is due to an intrinsic "down regulation" of nuclear receptor sites or to an enhanced fractional removal rate of the pituitary growth hormone has not been determined. The relationship between α_{2u}-globulin in liver and nuclear occupancy also appears to exhibit a generally linear relationship (Dillmann *et al.*, 1977b). The absolute decrease in serum cholesterol and creatine phosphokinase can be considered as attenuated responses, but as will be pointed out below, another interpretation will be offered.

Although it is by no means clear that the amplified response is typical of the thyroid hormone response system, the molecular basis for the amplification presents an interesting problem. The mechanism responsible for amplification must occur beyond the step of receptor binding since there is no evidence for a cooperative interaction in the nuclear T_3 receptors themselves (Surks *et al.*, 1975). More recent studies by Towle *et al.* (1980, 1981) have shown the amplification process is reflected at the level of messenger RNA. The steady-state level of cytosolic malic enzyme was shown to be proportional both to the concentration of the specific mRNA for this enzyme as measured in a translational assay and the relative rate of malic enzyme synthesis as determined in pulse labeling studies.

Some insight into the quantitative relationship between T_3 nuclear occupancy and biological effect may be obtained from a study of the interaction of thyroid hormone and dietary factors in the induction of malic enzyme and other lipogenic enzymes. The experimental basis for these studies is described in detail in Chapter 9. Briefly, it has been shown both that a high carbohydrate diet and thyroid hormone induce lipogenic enzymes. For malic enzyme, there is again a direct correlation between the steady-state level of ME and the concentration of mRNA coding for this enzyme as determined in the translational assay (Towle *et al.*, 1980a). An analysis of the combined effects of a high carbohydrate diet and variable doses of T_3 has shown that there is a synergistic interaction between T_3 and some as yet unidentified factor generated by the high carbohydrate diet

(Mariash *et al.*, 1980b). This factor does not appear to be insulin since a synergistic interaction between fructose and T_3 can be demonstrated in streptozotocin-induced diabetic animals (Kaiser *et al.*, 1980). Moreover, since glucose by itself can induce malic enzyme formation in primary hepatocyte cultures, it is clear that altered levels of hormone, in response to a high carbohydrate diet, are not necessary for the induction of this enzyme (Mariash *et al.*, 1981). These findings, therefore, raise the possibility that a glycolytic intermediate or an intracellular product of carbohydrate metabolism may be directly responsible for the enhanced synthesis of lipogenic enzyme mRNA.

These studies have also strongly suggested that there appears to be a multiplicative relationship between the signals originating from the high carbohydrate diet and from the T_3 nuclear complex. Thus, with severe hypothyroidism, induction of lipogenic enzymes by carbohydrate is severely impaired. Conversely, diminished carbohydrate availability is probably also responsible for the marked diminution in the ability of T_3 to induce malic enzyme (Westerfield *et al.*, 1965). Starvation may be accomplished both by a deprivation of carbohydrate as well as an elevation in the level of circulating glucagon (Aguilar-Parada *et al.*, 1969; Grey *et al.*, 1970) which itself has been shown to result in diminished malic enzyme responsiveness to T_3 (Goodridge and Adelman, 1976).

The relationship between T_3 and carbohydrate may be typical of other interactions of this hormone. T_3, cortisol and dihydrotestosterone must interact in the induction of the specific messenger RNA for α_{2u}-globulin, the exportable hepatic protein found in rat liver (Roy and Dowbenko, 1977; Kurtz and Feigelson, 1976). Moreover, there also appears to be an interaction between cortisol and thyroid hormone in the induction of a messenger RNA for pituitary growth hormone (Samuels *et al.*, 1977; Martial *et al.*, 1977).

In man, recent efforts have been made to relate various commonly used tissue indices of thyroid hormone to the calculated nuclear occupancy (Bantle *et al.*, 1980). In this study, it was assumed that nuclear occupancy in any given tissue could be predicted from the estimation of the free T_3 concentration and the affinity of nuclear sites determined in studies with human liver and kidney nuclei (Schuster *et al.*, 1979). The relationship between heart rate and nuclear occupancy appears relatively linear (Figs. 10–13), whereas the relationship between nuclear occupancy and the activity of creatinine phosphokinase and the concentration of serum cholesterol suggests an inverse correlation.The latter raises the possibility that the serum cholesterol and CPK levels are actually determined by the activity of a clearance mechanism, which in turn is directly related to nuclear occupancy. Such a model would lead to the observed parabolic relationships. Experimental data support the concept that the metabolism of LDL lipoprotein is enchanced by thyroid hormone (Walton *et al.*, 1965), possibly through a stimulation in the generation of LDL receptors (Chait *et al.*, 1979). Moreover, recent studies in the dog provide a direct demonstration that thyroid hormone status influences the level

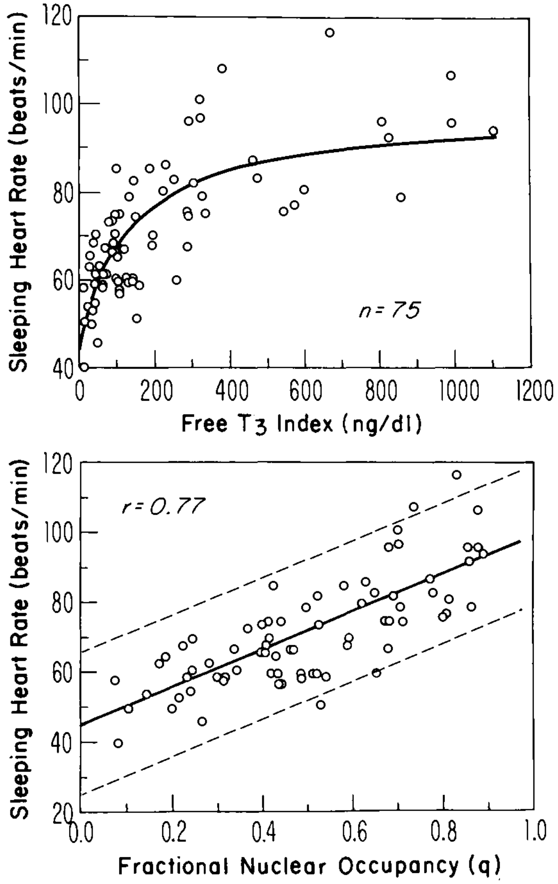

Fig. 10. Relationship between calculated nuclear occupancy and common clinical indices of thyroid hormone action. Nuclear occupancy is estimated for the affinity of human liver nuclei determined in other experiments (Schuster *et al.*, 1979) and the plasma T_3 concentrations. Serial measurement of the clinical parameters and the free T_3 indices were made in 16 hypothyroid patients treated with progressively increasing doses of L-T_4 as well as in 19 hyperthyroid patients treated with surgical ablation, ^{131}I therapy, or antithyroid drugs. Mean heart rate determined by prolonged Holter monitoring. (Upper panel) Relationship between heart rate and serum free T_3 index. (Lower panel) Relationship between fractional nuclear occupancy and heart rate. Dashed lines indicate 95% confidence limits. From Bantle *et al.* (1980).

of serum creatine phosphokinase exclusively by altering the clearance rate of this enzyme (Karlsberg and Roberts, 1975). If the clearance mechanism is then assumed to be directly proportional to nuclear occupancy, the reciprocal of the cholesterol concentration and CPK activity should be linearly related. As shown in Fig. 11, this appears to be the case. Of additional interest is that the duration of the

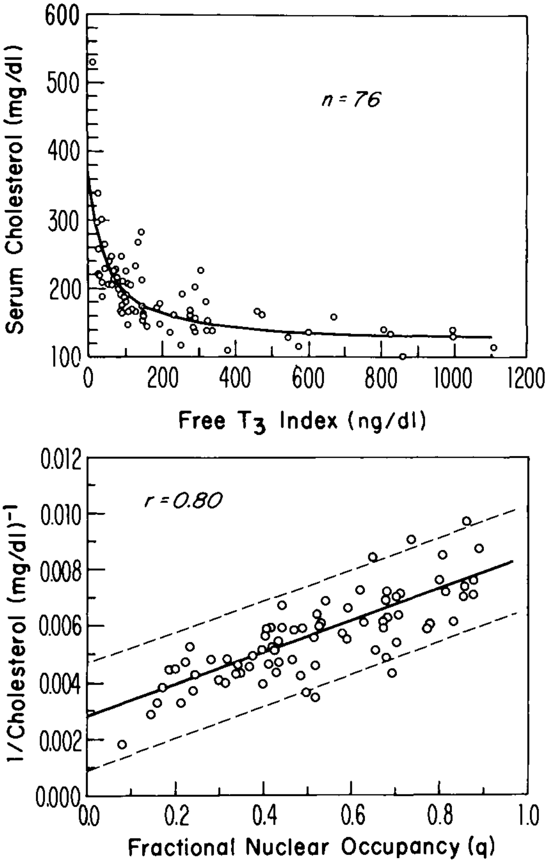

Fig. 11. (Upper panel) Relationship between free T_3 index and serum total cholesterol. (Lower panel) Relationship between the reciprocal of the serum cholesterol and the calculated nuclear occupancy. If T_3 acted by stimulating a process responsible for accelerating the fractional removal of cholesterol from serum one might anticipate a direct relationship between nuclear occupancy and the inverse of the serum cholesterol. From Bantle *et al.* (1980).

relaxation phase of the Achilles tendon reflex, another important clinical parameter of thyroid hormone action, also is inversely related to nuclear occupancy. This is compatible with the speculation that thyroid hormone may govern the removal of some substance accumulating in nerve or muscle during the hypothyroid state which may be responsible for the observed delay in the reflex relaxation phase. Other interpretations are, of course, possible.

In all likelihood, there are multiple steps between the point of initiation of thyroid hormone action at the receptor and its final expression at the cellular level. Such complexity undoubtedly accounts for the diversity of observed relationships between hormonal response to occupancy. The possibility should also be considered that the apparent linear relationships observed are simply the fortuitous summation of many component nonlinear steps.

VI. LAG TIME OF THYROID HORMONE EFFECTS

The long delay between thyroid hormone administration and the onset of response has attracted considerable attention of investigators and clinicians. A previous proposal made by the author and his colleagues was that the slow onset and decay of thyroid hormone was determined by a single "long-lived intermediate" (Oppenheimer *et al.*, 1972b; Dillmann *et al.*, 1977a). This inference was

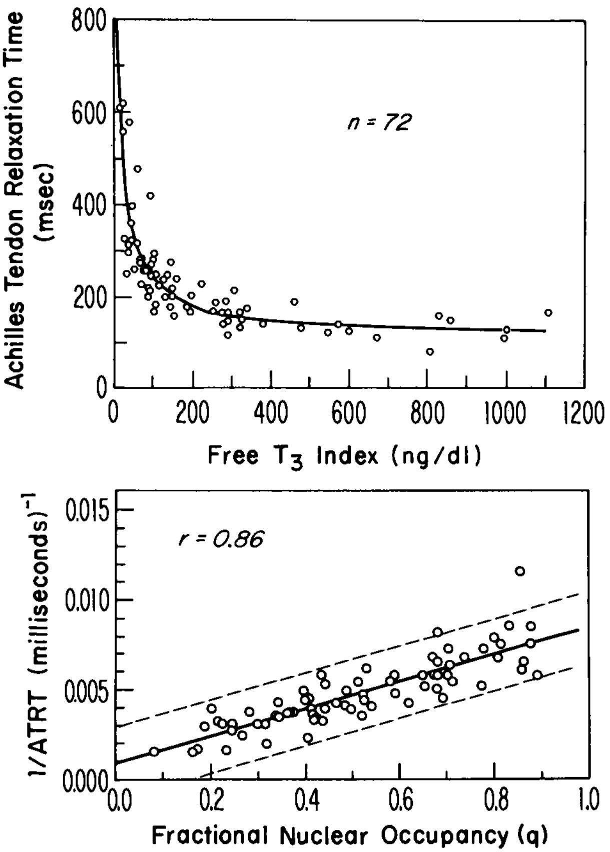

Fig. 12. Relationship (upper panel) between free T_3 index and the Achilles tendon relaxation time and (lower panel) the reciprocal of the Achilles tendon relaxation time and the calculated nuclear occupancy. From Bantle *et al.* (1980).

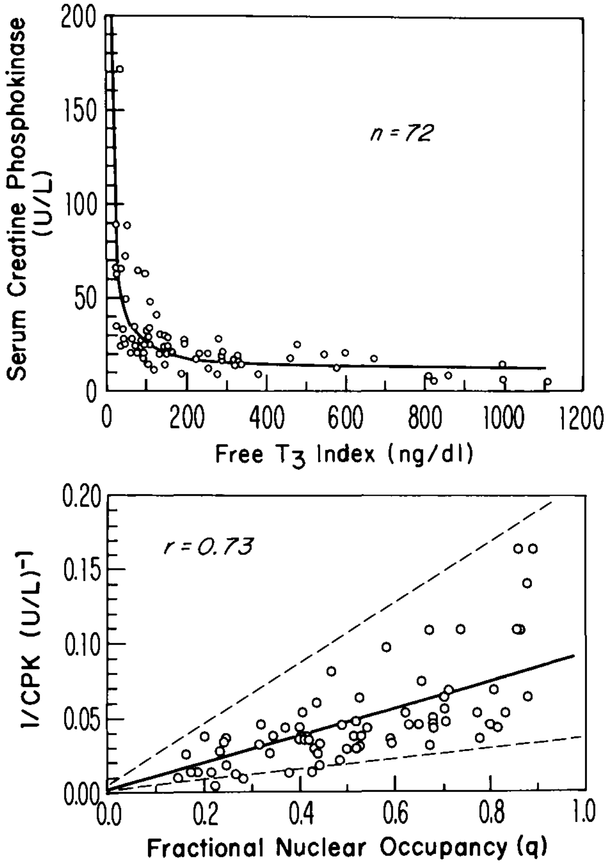

Fig. 13. Relationship (upper panel) between serum creatinine phosphokinase level and the free T_3 index and (lower panel) the reciprocal of the creatinine phosphokinase level and the calculated nuclear occupancy. From Bantle *et al.* (1980).

based on what was perceived to be a common decay rate for multiple thyroid hormone response parameters (Oppenheimer *et al.*, 1972b). In other studies, the administration of α-amanitin, a substance presumed to be a general inhibitor of polymerase II, was shown to inhibit induction both of ME and α-GPD by a large dose of T_3 (Dillmann *et al.*, 1977a). In such experiments, we observed that following the metabolism of α-amanitin, the delayed effect of the injected T_3 became apparent with a surge in the appearance of new enzyme. This was attributed to the long-lived "imprint" at the nuclear level.

More recent experiments, however, have cast considerable doubt on the construct of the long-lived intermediate. Thus, the decay of lipogenic and hexomonophosphate shunt enzymes does not proceed with a common $t_{1/2}$ of 3–4 days as originally suspected. Although most enzymes decrease with a $t_{1/2}$ of 3 days, fatty acid synthetase declines much more rapidly with a $t_{1/2}$ of 1.5 days (Mariash *et al.*, 1980a). This finding is incompatible with the hypothesis and points to the more likely alternative that the decline in thyroid hormone effects is determined by the intrinsic $t_{1/2}$ of the induced protein. Further support for this conclusion is the recent finding that the $t_{1/2}$ of mRNA for malic enzyme is approximately 10–12 hours (Towle *et al.*, 1981). The lag period between the occupation of the nuclear receptor and the appearance of the messenger RNA for malic enzyme appears to be relatively short, perhaps less than 2 hours. Thus, entrance into and exit from the stimulated state are a function of the stability of the malic enzyme molecule, and not that of the messenger RNA. Our original postulation of the long-lived intermediate, therefore, was based in large part on an incomplete knowledge of the $t_{1/2}$ of decline of individual thyroid hormone stimulated processes. Our results with α-amanitin, however, are not as readily explained. It appears very likely from the experimental results that α-amanitin cannot uniformly inhibit RNA polymerase activity under these conditions. One potential explanation is that generation of mRNA for ME and α-GPD proceed even in the presence of α-amanitin and that the synthesis of specific protein cofactors required for translation is inhibited. After α-amanitin has been metabolized substantial quantities of the specific mRNA for ME and α-GPD would have accumulated. With renewed synthesis of the putative translational cofactor, a surge in α-GPD and ME synthesis is therefore observed. In order to verify this hypothesis, additional experiments are required to assess the concentration of specific mRNA for ME during the period of α-amanitin inhibition.

VII. CONCLUDING REMARKS

Perhaps it would be most appropriate to view the biological functions of thyroid hormone as a result of an evolutionary exploitation of a simple molecule that has the potential for accelerating the rate of formation of mRNA species coding for a variety of key proteins. Induction of specific proteins, perhaps as a result of an interaction of T_3 with locally produced growth factors, could account for the developmental actions of thyroid hormone. Similarly, in the adult, stimulation of the synthesis of mRNA sequences coding for several enzymes could explain the simultaneous increase in the rates of formation and degradation of carbohydrates, lipids, and proteins, which is so characteristic of thyroid hormone action. The potential value of such "futile cycles" has recently been discussed by Newsholme (1980). It is conceivable, moreover, that the stimulation of these cycles by T_3 is also related to the thermogenesis that is such a characteristic feature of thyroid hormone action at the cellular level.

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Identification and Characterization of Thyroid Hormone Receptors and Action Using Cell Culture Techniques

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MOLECULAR BASIS OF
THYROID HORMONE ACTION

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I. INTRODUCTION

The thyroid hormones have a profound effect on the growth, development, and metabolism of essentially all tissues of higher organisms (Wolff and Wolff, 1964). L-Thyroxine (L-T_4) and L-triiodothyronine (L-T_3) have been shown to stimulate cell replication (Samuels *et al.*, 1973), cell differentiation and development (Frieden and Just, 1970), calorigenesis and oxygen consumption (Ismail-Beigi and Edelman, 1971), regulation of enzymic activity (Goodridge and Adelman, 1976; Oppenheimer *et al.*, 1977; Weinberg and Utter, 1979), α_{2u}-globulin production (Kurtz *et al.*, 1976; 1978), induction of catecholamine receptors (Williams *et al.*, 1977; Tsai and Chen, 1978), mammary gland function (Vonderhaar, 1977), stimulation of growth hormone production in somatotrophic cells (Solomon and Greep, 1959; Tsai and Samuels, 1974), and inhibition of the secretion and/or production of thyrotoropin (Florsheim, 1974; Gershengorn, 1978) and prolactin (Snyder *et al.*, 1973; Tsai and Samuels, 1974). In addition thyroid hormones have also been shown to interact with steroid hormones in specific tissues in a synergistic fashion to regulate specific biological responses (Samuels *et al.*, 1977a; Martial *et al.*, 1977; Kurtz *et al.*, 1976; Sassa and Kappas, 1977; Vonderhaar, 1977; Gardner *et al.*, 1978).

Several of these processes have been studied in detail both in intact animals *in vivo* and in cultured cells, and in each case thyroid hormone action appears to involve a process that requires both RNA and protein synthesis. Furthermore, in three mammalian systems [the induction of growth hormone in cultured rat pituitary cells (Seo *et al.*, 1977; Martial *et al.*, 1977; Shapiro *et al.*, 1978); the induction of α_{2u}-globulin in the rat (Roy *et al.*, 1976); the stimulation of malic enzyme (Towle *et al.*, 1980)], the hormone-stimulated response appears to result from an increase in the accumulation of specific mRNA molecules coding for the synthesis of these proteins. In addition, abundant evidence supports the concept that most if not all of the significant cellular responses to thyroid hormone in mammalian cells is mediated by a cellular receptor localized to the cell nucleus which regulates the accumulation of specific mRNA molecules (Samuels, 1978; Oppenheimer and Dillmann, 1978; Latham *et al.*, 1978).

Evidence to support these concepts have been derived both from *in vivo* studies in intact animals (Oppenheimer and Dillmann, 1978; Oppenheimer, 1979) as well as with cultured cells (Samuels, 1978). Cell culture techniques have provided significant information regarding the mechanism of thyroid hormone action in eukaryotic cells, and have certain inherent characteristics that allow for the construction of specific studies that cannot be carried out in intact animals. The advantages of a cell culture system include that of precise control of hormonal concentrations, the ability to observe very early biochemical events controlled by the thyroid hormones, and permit an assessment of thyroid hormone receptor synthesis and degradation using density labeling techniques

(Raaka and Samuels, 1981). Furthermore, cultured cells can be used to examine the organization of the receptor in chromatin and to assess whether the total nuclear bound receptor exists in different populations with regard to the affinity for chromatin or the distribution to specific chromatin fractions which may be enriched for transcriptionally active genes (Samuels *et al.*, 1980; Raaka and Samuels, 1981).

II. BIOLOGICAL EFFECTS OF THYROID HORMONE IN CELL CULTURE

GH_1 cells, a growth hormone and prolactin-producing rat pituitary cell line, was the first cell culture system reported to be responsive to physiological concentrations of L-T_3 and L-T_4 (Samuels *et al.*, 1973). An essential aspect of the experimental design was the use of hypothyroid calf serum as a component of cell culture media which promotes the development of a thyroid hormone depleted state under cell culture conditions. This was necessary since commercially obtained calf serum contains physiological concentrations of L-T_3 and L-T_4 that would not permit a precise analysis of thyroid hormone action in cell culture.

The concentration of L-T_3 present in euthyroid calf serum is approximately 140 ng/dl or 2 n*M* (Samuels *et al.*, 1973). L-T_4 concentrations are 8 μg/dl or approximately 100 n*M* (Samuels *et al.*, 1973). Both L-T_3 and L-T_4 exist in bound and free forms and only a very small fraction exists in the free form which appears to correlate best with biological activity. Theoretical considerations as well as experimental studies indicate that as serum is diluted a reequilibration occurs, and the free hormone concentration remains relatively constant in spite of dilution (Oppenheimer and Surks, 1964). Therefore, dilution of euthyroid calf or fetal calf serum 5- to 20-fold in the preparation of tissue culture media would lower the total hormone concentration, but the free hormone concentrations would be expected to remain relatively close to the physiological level.

Thyroid hormone depleted serum for cell culture studies can be obtained from various species after complete thyroidectomy and studies using GH_1 cells have utilized serum from a thyroidectomized calf. As previously discussed (Samuels, 1978) it is essential to monitor all serum samples obtained after thyroidectomy for L-T_3 and L-T_4 levels. Even if only small residual thyroid tissue remains after thyroidectomy, L-T_3 and L-T_4 levels may increase with time resulting in serum that contains hormone levels not sufficiently low for use in cell culture studies. Alternatively, serum can be depleted of both L-T_3 and L-T_4 using an anion exchange resin (AG-1-X-10; Bio-Rad) (Samuels *et al.*, 1979c). This resin effectively depletes L-T_3 from calf serum without significantly altering the amino acid or the total protein content, or the electrophoretic pattern of serum proteins. In GH_1 cells, calf serum depleted of thyroid hormone using this procedure yields serum that when used as a medium supplement results in biological responses identical to those obtained with media supplemented with thyroidectomized calf serum. In addition, resin treatment of euthyroid calf serum does not alter the growth of GH_1 cells if the thyroid hormone concentration is restored. AG-1-X-10 resin is useful in preparing thyroid hormone depleted serum in situations where thyroidectomy is not feasible (e.g., thyroid hormone depleted fetal calf serum) or where thyroidectomy would be required on a large number of small animals (e.g., mice). Currently in our laboratory thyroid hormone depleted serum is obtained using the resin technique rather than by thyroidectomy since cultured GH_1 cells behave identically with both types of serum preparations. It should be pointed out, however, that the resin may remove certain growth promoting factors required for other cell types. Under these circumstances depletion of thyroid hormone by using resin techniques may not be useful. Although the resin AG-1-X-10 was initially used to deplete L-T_3 and L-T_4 from fetal calf and calf serum, this resin is no longer commercially available. However, virtually identical results can be obtained using AG-1-X-8 resin obtained from Bio-Rad.

A. Effect of Thyroid Hormone in Cultured GH_1 Cells

We have identified a minimum of five cellular responses to physiological concentrations of the thyroid hormones in GH_1 cells. These include stimulation of cell division (Samuels *et al.*, 1973; Tsai and Samuels, 1974), enhancement of glucose metabolism (Samuels *et al.*, 1973), and increased uridine transport (Samuels and Tsai, 1972). Furthermore, thyroid hormone inhibits the basal as well as the thyrotropin releasing hormone (TRH) stimulated increase in prolactin secretion (Tsai and Samuels, 1974). In addition to the above, L-T_3, L-T_4, and other thyroid hormone analogs stimulate a 4- to 6-fold increase in the rate of growth hormone synthesis which appears to be independent of changes in cell replication or total cell protein synthetic rates (Tsai and Samuels, 1974; Samuels and Shapiro, 1976). The regulation of the growth hormone response by thyroid hormone has been the parameter most extensively examined and provides some insights into the molecular mechanisms by which thyroid hormone controls cell processes in mammalian cells.

GH_1 cells synthesize growth hormone and rapidly release the peptide into the medium (Tsai and Samuels, 1974; Samuels and Shapiro, 1976). The growth hormone is not degraded intracellularly or after release into the medium and the intracellular half-life (30 minutes) appears to totally reflect rates of hormone release (Samuels and Shapiro, 1976). Therefore, quantitation of the level of medium growth hormone as a function of time gives an estimation of the synthetic rate of the polypeptide hormone. Evidence to support this comes from studies in which the growth hormone synthetic rate was directly measured after incubating cells with L-[^{35}S]methionine for 5–12 minutes followed by selective immunoprecipitation of the intracellular radiolabeled growth hormone using highly specific antigrowth hormone antibody raised in baboons (Samuels and Shapiro, 1976; Shapiro *et al.*, 1978; Samuels *et al.*, 1979a,b). In GH_1 cells thyroid hormone rapidly stimulates growth hormone production rates solely by inducing an increase in the *de novo* synthesis of the polypeptide hormone. L-T_3 stimulated synthetic rates of growth hormone by 1.5-fold in 1.25 hours, 2-fold in 2.5 hours, 3- to 4-fold after 8.5 hours and 6- to 8-fold after 24 hours of incubation (Samuels and Shapiro, 1976).

Quantitation of growth hormone mRNA levels in GH_1 cells using the *in vitro* wheat germ translation system documented that the stimulation of growth hormone synthetic rates by L-T_3 resulted from an increase in the abundance of growth hormone mRNA (Shapiro *et al.*, 1978; Samuels *et al.*, 1979a). Similar observations have been made using cultured GH_3 cells (Seo *et al.*, 1977) and cultured GC cells (Martial *et al.*, 1977), two closely related pituitary cell lines. The physiological relevance of the induction of growth hormone synthesis using GH_1 cells and related cell lines in culture is underscored by the observation that thyroid hormone plays an important role in controlling the production of growth hormone in the rat pituitary *in vivo* (Hervas *et al.*, 1975). Furthermore, thyroid hormone stimulates an increase in the pituitary content of growth hormone in the thyroidectomized rat (Solomon and Greep, 1959; Hervas *et al.*, 1975) with the same onset and time course as in cultured GH_1 cells (Samuels, 1978).

Although thyroid hormone clearly plays an important role in regulating certain cell processes independent of other hormones, it has become increasingly clear that thyroid hormone also interacts synergistically with other hormones to regulate specific cellular responses. For example, the regulation of α_{2u}-globulin synthesis and mRNA levels in rat liver is modulated by thyroid hormone, androgen, growth hormone, and glucocorticoid (Kurtz *et al.*, 1976; Roy et al., 1976; Roy, Chapter 7, this volume). Cultured GH_1 cells and related cell lines also provide an interesting model to study multihormonal control by thyroid and other hormones. As described above in cultured GH_1 cells, thyroid hormone induces an increase in growth hormone synthesis and growth hormone mRNA levels. This occurs in both serum-containing and serum-free media which is devoid of glucocorticoid hormones (Samuels *et al.*, 1977a; 1979a; Shapiro *et al.*, 1978). In the absence of thyroid hormone, however, glucocorticoid hormones induce virtually no change in growth hormone synthesis or growth hormone mRNA (Shapiro *et al.*, 1978). In the presence of thyroid hormone, however, glucocorticoids further increase the response induced by thyroid hormone approximately 2- to 5-fold. Changes in the rates of growth hormone synthesis parallel changes in growth hormone mRNA levels (Shapiro *et al.*, 1978), suggesting that both hormones act to regulate the level of growth hormone mRNA in a synergistic fashion. Furthermore, 17α-methyltestosterone, a glucocorticoid antagonist, inhibited the rate of growth hormone synthesis and the accumulation of growth hormone mRNA in cells incubated with thyroid hormone plus glucocorticoid to that of thyroid hormone alone (Samuels *et al.*, 1979a). This effect of thyroid hormone on the glucocorticoid induction of the growth hormone response does not reflect a general influence of thyroid hormone on glucocorticoid action in GH_1 cells, since the glucocorticoid induction of glutamine synthetase in the same cells is not influenced by thyroid hormone (Samuels *et al.*, 1978). Furthermore, thyroid hormone does not influence the total cell level or nuclear translocation of the glucocorticoid receptor and glucocorticoid does not influence the level of nuclear associated thyroid hormone receptors (Samuels *et al.*, 1977a,1978). (Evidence documenting that the cellular receptor mediating the action of thyroid hormone in GH_1 cells is a chromatin associated protein is discussed below.) These studies indicate that the thyroid hormone control of glucocorticoid induction of growth hormone may be a selective process, and that both nuclear associated receptors influence the expression of the growth hormone response in a synergistic fashion (Shapiro *et al.*, 1978; Samuels *et al.*, 1979a).

B. Regulation of Other Responses by Thyroid Hormone in Isolated Cells in Culture

In addition to the modulation of growth hormone by thyroid hormone, other cell culture systems have been recently described in which the *in vitro* response to thyroid hormone mirrors that observed *in vivo* in the intact animal. One of the predominant cellular responses controlled by the thyroid hormones is the regulation of calorigenesis and oxygen consumption. Because of the stimulation of oxygen consumption changes in mitochondrial enzymatic activity after thyroid hormone adminstration was initially explored. As a result of these studies one of the first enzymatic activities shown to be regulated by thyroid hormone was α-glycerophosphate dehydrogenase which is associated with the inner mitochondrial membrane (Lee and Miller, 1967). In addition thyroid hormone stimulates an increase of malic enzyme in rat liver (Oppenheimer *et al.*, 1977) as well as in avian liver (Goodridge, 1975). The induction of these enzymes requires both RNA and protein synthesis (Lee and Miller, 1967; Goodridge, 1975). Although the induction of α-glycerophophate dehydrogenase may play a role in the increase in oxygen consumption of the cell, Ismail-Beigi and Edelman and their co-workers (Ismail-Beigi and Edelman, 1971; Asano *et al.*, 1976; Lo and Edelman, 1976; Ismail-Beigi *et al.*, 1979) have presented evidence that the calorigenic response stimulated by thyroid hormone occurs as a result of an increase in energy expenditure related to sodium and potassium transport across the plasma membrane. The biochemical membrane unit that appears to regulate sodium and potassium transport is the sodium- and potassium-activated ATPase (Na^+,K^+-ATPase). Thyroid hormone administration increases Na^+,K^+-ATPase activity, which achieves a maximal value 48 hours after injection of L-T_3.

Using radioisotopic labeling techniques, Lo and Edelman (1976) demonstrated that the induction of the enzyme by L-T_3 appears to be related to an increase in the synthetic rate of a component of the membrane-bound enzyme.

Recently, several cell culture approaches have documented that the regulation of malic enzyme and α-glycerophosphate dehydrogenase appear to be under primary control by thyroid hormone and do not reflect a secondary effect of thyroid hormone due to the L-T_3 release of other factors that might occur *in vivo.* Ismail-Beigi *et al.* (1979) examined the effect of L-T_3 on oxygen consumption, Na^+,K^+-ATPase, and α-glycerophosphate dehydrogenase in adult rat hepatocytes during 4 days of primary culture. The dose–response relationship for each of these parameters was identical, suggesting common control by the same rate-limiting process modulated by thyroid hormone in the cell. Furthermore, the time course of induction of each of these responses in isolated rat liver cells in short-term culture was identical to that observed after L-T_3 injection *in vivo.* Therefore, short-term adult rat liver cell culture may provide a useful model to clarify how L-T_3 stimulates oxygen consumption in the cell and its relationship to sodium and potassium membrane transport as well as mitochondrial enzymic activity. Hepatic malic enzyme appears to be under multifactorial control and is stimulated by thyroid hormone and carbohydrate administration (Towle *et al.*, 1980). Using short-term culture, Goodridge demonstrated that thyroid hormone stimulated malic enzyme in chick liver cells and this response could be potentiated by insulin and inhibited by glucagon (Goodridge and Adelman, 1976; Goodridge, Chapter 8). The regulation of malic enzyme by L-T_3 and carbohydrate has been recently examined by Mariash *et al.* (1981) using short-term rat hepatic cell culture. Malic enzyme activity is stimulated by L-T_3 and independently by the level of glucose in the culture medium. This study documents that the L-T_3 induction of malic enzyme appears to reflect an effect of L-T_3 on liver cells and not a secondary change due to the secretion of other factors (e.g., insulin). Furthermore, this study suggests that glucose concentrations can regulate malic enzyme activity and therefore the induction of malic enzyme by carbohydrate administration does not absolutely require the secretion of other factors resulting from carbohydrate administration.

The feedback regulation of thyrotropin (TSH) secretion by thyroid hormone plays an essential role in maintaining a normal thyroidal economy. Inhibition of TSH secretion by thyroid hormone is one of the most sensitive indicators of thyroid function. In addition long-term thyroid hormone administration results in a decrease in the pituitary content of TSH (Obregon *et al.*, 1979). Thyroid hormone not only inhibits basal TSH secretion, but can also inhibit TSH secretion mediated by thyrotropin releasing hormone (TRH) administration *in vivo.* Recently, Gershengorn and co-workers (1979; Gershengorn, 1978) have utilized mouse thyrotropic tumor cells to study the regulation of TSH secretion and production by thyroid hormone and TRH. In this system thyroid hormone inhibits the secretion and production of mouse TSH and decreases the TRH stimulation of TSH secretion. The decrease of the TRH effect in the cell appears to reflect in part an effect of L-T_3 on decreasing TRH receptors as well as altering the biological action of TRH which occurs distal to the TRH-receptor complex (Gershengorn, Chapter 13).

In addition to inhibiting the TSH response in mouse thyrotropic cells, thyroid hormone also inhibits both basal and TRH stimulated prolactin production in GH_1 cells (Tsai and Samuels, 1974) as well as in GH_3 cells (Perrone and Hinkle, 1978). The inhibition of prolactin production in GH_1 cells by L-T_3 occurs with the same dose–response relationship as the induction of growth hormone synthesis (Tsai and Samuels, 1974) indicating that the modulation of both responses are controlled by the same rate-limiting process in these cells. More recently, Perrone and Hinkle (1978), examined the effect of L-T_3 and TRH on prolactin synthesis in GH_3 cells. L-T_3 inhibited both prolactin secretion as well as prolactin synthesis and influenced the TRH mediated stimulation of release and synthesis to a greater degree than the basal response. Furthermore L-T_3 decreased the steady-state level of TRH receptors in GH_3 cells cultured with serum containing media with a half-maximal reduction occurring at approximately 0.2 n*M* L-T_3 which is identical to the L-T_3 concentration that induces a half-maximal increase in the rate of synthesis of growth hormone in GH_1 cells (Samuels *et al.*, 1976). This suggests that the decrease in TRH receptor number is also controlled by the same rate-limiting event that modulates growth hormone synthesis in cultured somatotropic cell lines. As described below, the thyroid hormone induction of growth hormone synthesis, inhibition of prolactin and TSH production, and inhibition of TRH receptor levels parallel the occupancy of the thyroid hormone nuclear receptor in these cell lines, suggesting that each of these events is controlled by the interaction of thyroid hormone with the same class of nuclear associated receptors.

Cell culture has also been used to investigate the possible mechanisms by which thyroid hormone increases the sensitivity of the cardiovascular system to catecholamines. It has been recognized for many years that thyroid hormone sensitizes the myocardium to catecholamines (Harrison, 1964) and a number of the cardiovascular manifestations of hyperthyroidism can be partially reversed with β-adrenergic blocking agents (Riddle and Schwartz, 1970). Since β-adrenergic catecholamine receptors act to increase cAMP production, thyroid hormone may influence catecholamine response by modulating the catecholamine receptor or altering the rate of cAMP generation or intracellular breakdown. Recently, evidence has been presented to suggest that thyroid hormone may influence myocardial sensitivity to β-adrenergic catecholamine agonists by stimulating an increase in the level of the plasma membrane β-adrenergic receptor. This has been demonstrated in the rate heart *in vivo* by Williams and co-workers (1977) and is reviewed in Chapter 11 by Williams and Lefkowitz. Similar responses have been observed in cultured myocardial cells by Tsai and Chen (1978). The stimulation of cellular cAMP by epinephrine after thyroid hormone incubation in myocardial cell cultures in paralleled by a thyroid hormone-mediated increase in the cellular β-adrenergic receptor level. The thyroid hormone concentration-dependent stimulation of β-adrenergic levels occurs over the same L-T_3 dose–response as other mediated functions described above in cultured cells and shows an excellent correlation with the occupancy of the thyroid hormone nuclear receptor by L-T_3; findings suggesting that thyroid hormone regulates β-adrenergic levels by a mechanism modulated at the level of the cell nucleus.

Other isolated cell or cell culture systems that show responses to thyroid hormones which mirror responses observed after *in vivo* injection of hormone in intact animals are (1) thyroid and glucocorticoid hormone regulation of δ-aminolevulinic acid synthetase production, which is a rate-limiting enzyme in the pathway of heme synthesis in chick liver cells (Sassa and Kappas, 1977); (2) stimulation of chondrotin sulfate synthesis in isolated cartilage cells by thyroid hormone and somatomedian (Audhya *et al.*, 1976); and (3) regulation of milk production in mammary gland explants by thyroid hormone, prolactin, glucocorticoid, and insulin (Vonderhaar, 1977). It is clear from the above discussion that many of the *in vivo* actions of thyroid hormone in intact animals can be reproduced using cell culture systems. Biological responses to thyroid hormone in intact animals may reflect a primary direct action of thyroid hormone on the cell or a secondary response due to alterations in secretion of other hormones or factors. As discussed, a number of biological responses appear to be under multifactorial control in which thyroid hormone may influence the cellular response to other regulatory components. Since the cellular environment can be more precisely controlled in cell culture, isolated cell systems should contribute significant information regarding the detailed mechanisms by which thyroid hormone can independently as well as synergistically regulate physiologically relevant events in mammalian cells.

III. IDENTIFICATION AND CHARACTERIZATION OF THYROID HORMONE RECEPTORS IN CULTURED CELLS

Cellular receptors that mediate the actions of thyroid hormones can be expected to have specific properties that might be predicted by the characteristics of the induced biological response, and the relative affinity of such a receptor for L-T_3, L-T_4, and synthetic hormone analogs. If the association of thyroid hormone with such a receptor regulates a rate-limiting step in thyroid hormone action, the characteristics of hormone binding might be expected to demonstrate a good correlation with the observed biological properties of the system. Because the maximal biological response is limited, the putative receptor might be expected to exhibit limited capacity or saturable binding for the thyroid hormones. Furthermore, one might expect to observe a good correlation between the relative affinity of L-T_3, L-T_4, and hormonal analogs for the receptor and the relative biological activity of these compounds. A binding component that fulfills these criteria would likely function as a receptor that initiates the biological actions of thyroid hormone. Such a binding component would have to be considered, however, as a putative receptor until its specific biochemical role can be identified in intact cells and until this action can be reproduced under completely *in vitro* conditions with purified receptor and other cellular components.

A. Identification of Thyroid Hormone Nuclear Receptors in Cell Culture

In 1972, Schadlow and co-workers reported that increasing concentrations of L-T_3 administered to rats showed a progressive decrease in the pituitary to plasma concentration ratio of L-T_3, indicating that the pituitary demonstrated limited capacity to bind thyroid hormone. Cell fractionation studies demonstrated that the limited capacity binding sites for L-T_3 were restricted to the cell nucleus. Using cultured GH_1 cells, Samuels and Tsai (1973) demonstrated that these cells contain high affinity, limited capacity binding sites for L-T_3 and LT_4 that were localized to the nucleus and not to the extranuclear fraction. Figure 1 illustrates the kinetics of binding of 30 p*M* L-[^{125}I]T_3 to nuclei, mitochondria, and cytosol which were isolated after L-T_3 incubation in intact cells. The magnitude of limited capacity or saturable binding was quantitated by carrying out a simultaneous incubation of L-[^{125}I]T_3 in the presence of a 200-fold molar excess of nonradioactive L-T_3. Binding of L-[^{125}I]T_3 to mitochondria and cytosol in intact cells occurred very rapidly and attained equilibrium with the hormone

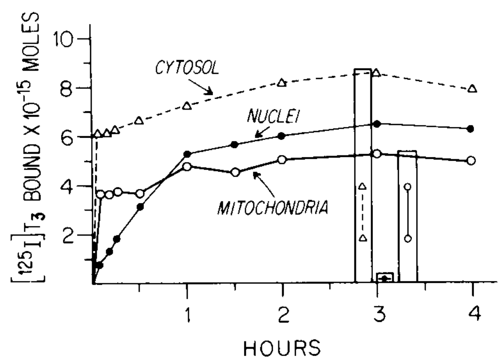

Fig. 1. Time course of binding of L[^{125}I]T_3 (30 p*M*) after incubation of intact GH_1 cells with serum-free media. The bar graphs illustrate the magnitude of L-[^{125}I]T_3 binding in the presence of a 200-fold molar excess of nonradioactive L-T_3. From Samuels and Tsai (1973).

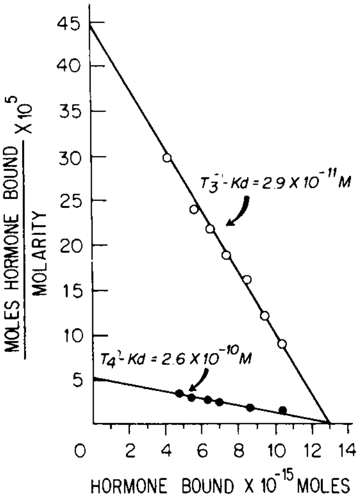

Fig. 2. Scatchard plot of L-T_3 and L-T_4 nuclear binding after incubation with intact cells and serum free media. Redrawn from Samuels and Tsai (1973).

concentration in the medium within 10–15 minutes. At this concentration of L-[^{125}I]T_3 binding to nuclei occurred more slowly and attained the maximal value after 1 hour of incubation. As shown in Fig. 1, nonradioactive T_3 inhibited the binding of L-[^{125}I]T_3 to nuclei by greater than 95% but did not alter the binding to the mitochondria or cytosol fractions.

The association of L-T_3 and L-T_4 with nuclear binding components in the intact cell was analyzed by the method of Scatchard (1949). Figure 2 illustrates the binding of L-T_3 and L-T_4 to nuclei using intact cells under completely serum-free conditions (Samuels and Tsai, 1973). Both ligands yield linear Scatchard plots indicating a simple noncooperative interaction between hormone and the nuclear binding component. The estimated equilibrium dissociation constant (K_d) for L-T_3 was 0.029 n*M* and the estimated K_d for L-T_4 was 0.26 n*M*. Therefore a 10-fold higher concentration of L-T_4 resulted in half-maximal binding indicating that the affinity for L-T_4 was one-tenth that of L-T_3. Analysis of the ^{125}I radioactivity bound to nuclei indicated that the L-[^{125}I]T_4 remained intact, and did not represent L-[^{125}I]T_3 formed as a result of L-T_4 to L-T_3 conversion by the cells. In addition the Scatchard plot for each hormone extrapolated to the same point indicating that an idential number of binding sites for L-T_3 and L-T_4 existed in GH_1 cell nuclei. This was estimated in this study to be 8000 binding sites per GH_1 cell nucleus. In GH_1 cells the level of receptor varies between 8000 and 20,000 binding sites per cell nucleus (Samuels *et al.*, 1976, 1977b). As discussed below, the level of the nuclear binding component in GH_1 cells is regulated by the ambient thyroid hormone concentration.

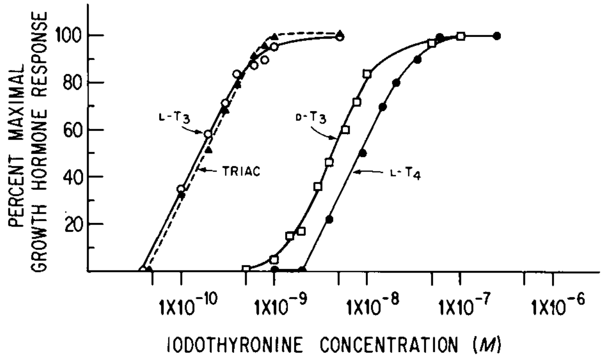

Fig. 3. Dose–response induction of growth hormone synthesis by iodothyronines. GH_1 cells were incubated with different concentrations of L-triiodothyronine (L-T_3), triiodothyroacetic acid (triac), D-triiodothyronine (D-T_3), or L-thyroxine (L-T_4). The growth hormone that accumulated in the serum-containing medium between 24 to 48 hours of incubation was determined by radioimmunoassay and is a direct measurement of growth hormone synthetic rates (Samuels and Shapiro, 1976). From Samuels *et al.* (1979b).

B. Dose–Response Induction of Growth Hormone by Hormone Analogs: Relation to the Relative Affinity for the Thyroid Hormone Nuclear Binding Component

Figure 3 illustrates the dose–response stimulation of growth hormone synthesis induced by different concentrations of L-T_3, triiodothyroacetic acid (triac), D-T_3, and L-T_4 using medium supplemented with 10% hypothyroid calf serum (Samuels *et al.*, 1979b). L-T_3 and triac showed virtually identical dose–response relationships with half-maximal responses occurring at 0.17 n*M* for L-T_3 and 0.19 n*M* for triac. D-T_3 induced a half-maximal growth hormone response at 4 n*M* and L-T_4 induced a half-maximal response at 9 n*M*. Therefore, under conditions in which medium is supplemented with 10% hypothyroid calf serum, L-T_3 and triac showed identical response curves, while D-T_3 had 1/20 and L-T_4 had 1/50 of the activity of L-T_3. An estimate of the relative affinity of L-T_3, triac, D-T_3, and L-T_4 for the thyroid hormone nuclear receptor was performed in intact cells in medium that contained thyroid hormone depleted calf serum to allow for a relative comparison to the growth hormone dose response curves (Samuels *et al.*, 1979b). Figure 4 illustrates these results in which cells were incubated with 2 n*M* L-[^{125}I]T_3 plus different concentrations of nonradioactive L-T_3, triac, D-T_3, and L-T_4 for 3 hours. Inhibition of binding of L-[^{125}I]T_3 to the nuclear binding component in serum-containing medium in cells is identical to the relative growth hormone response induced by each iodothyronine (Fig. 3). This observation supports the concept that the thyroid hormone nuclear binding component functions as a biologically relevant receptor that controls the accumulation of growth hormone mRNA in this system. To make a direct comparison between the relative affinity for the receptor and the induction of growth hormone response, the cellular responses were studied using medium supplemented with 10% thyroid hormone depleted calf serum.

Although this allows a valid relative comparison between iodothyronine analog binding characteristics and growth hormone induction, it does not allow a direct measurement of the intrinsic differences in biological activity as a result of differences in serum binding of the analogs. For example, based on the 10-fold differences in the relative affinity of L-T_4 and L-T_3 for the nuclear binding component using serum-free medium (Fig. 2), L-T_3 would be expected to have a 10-fold greater intrinsic biological activity than L-T_4. This value differs from the approximate 50-fold difference in the L-T_4 effect (Figs. 3 and 4) relative to that of L-T_3 using serum-containing medium. To document that this relative difference between serum-free and serum-containing media was due to differences in the serum binding of L-T_3 and L-T_4, free hormone concentrations were quantitated by equilibrium dialysis using medium that contained 10% thyroid hormone depleted calf serum (Samuels *et al.*, 1979b). Between 0.01–5 n*M* L-T_3 and 0.1–50 n*M* L-T_4 (Fig. 5) the percentage of total media hormone concentration in the free fraction was estimated to be 5% for L-T_3 and 1% for L-T_4. Therefore, by correction for relative differences in serum binding, L-T_4 appears to have one-tenth of the relative biological activity of L-T_3 and one-tenth of the relative affinity for the thyroid hormone nuclear binding protein. Based on the excellent

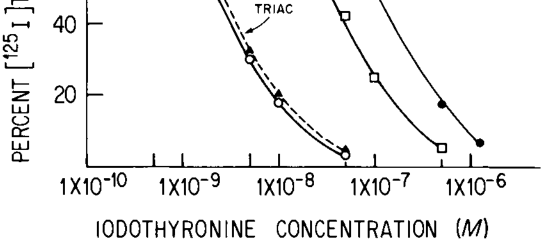

Fig. 4. Relative affinity of iodothyronines for the nuclear receptor in intact cells in serum-containing medium. GH_1 cells were incubated with 2 n*M* *L*-[^{125}I]T_3 and the indicated concentrations of nonradioactive L-T_3, triac, D-T_3, and L-T_4 for 3 hours in medium containing 10% thyroid hormone depleted calf serum. From Samuels *et al.* (1979b).

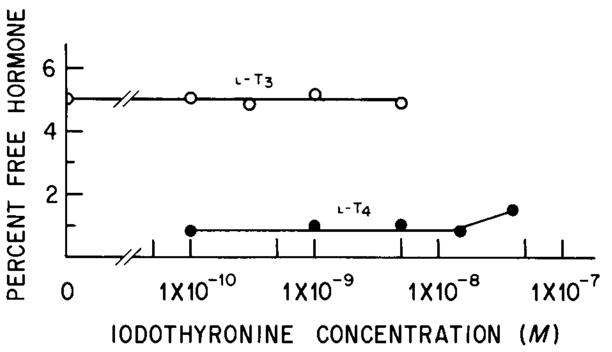

Fig. 5. Percent free L-T_3 and L-T_4 in medium containing 10% calf serum at different hormone concentrations. These results were determined by dialysis at 37°C of serum supplemented medium containing tracer L-[^{125}I]T_3 or L-[^{125}I]T_4 along with nonradioactive L-T_3 or L-T_4 against serum-free media.

agreement between the relative affinity for the nuclear binding component and the relative biological activity of hormonal analogs in GH_1 cells (Figs. 3 and 4), the thyroid hormone nuclear component appears to function as a biologically functional receptor that mediates the action of thyroid hormone in these cells.

C. Binding of Thyroid Hormone to Isolated Nuclei *in Vitro*

Although thyroid hormone rapidly binds to receptor in intact cells, several factors appear to be critical for the association of thyroid hormone with isolated nuclei *in vitro* (Samuels and Tsai, 1973, 1974; Spindler *et al.*, 1975; DeGroot and Torresani, 1975). Optimal *in vitro* binding occurs between pH 7.3 and 7.9 with dithiothreitol or 2-mercaptoethanol at concentrations above 0.1 m*M*. With these conditions and 0.5 n*M* L-[^{125}I]T_3, at least 90% of the total nuclear bound L-[^{125}I]T_3 associates with the receptor. Using these conditions, saturable binding of L-[^{125}I]T_3 was identified in isolated GH_1 cell nuclei as well as isolated rat liver nuclei *in vitro* (Samuels and Tsai, 1973, 1974). Scatchard analysis of isolated GH_1 cell and rat liver nuclei documented identical number of copies of nuclear receptor for L-T_3 as in intact cells, suggesting that the binding component detected with isolated nuclei *in vitro* is identical to that determined with cells (Samuels and Tsai, 1974). However, the estimated affinity for the L-T_3 receptor interaction using isolated nuclei was approximately 5- to 6-fold lower than that determined with intact cells, which likely reflects differences in cell entry of iodothyronines or to the nuclear environmental conditions in the cell compared to the isolated nuclear binding assay.

Recently, Cheng *et al.* (1980), using a fluorescent derivative of L-T_3, presented evidence that thyroid hormone may accumulate in cells by an active process involving ligand mediated endocytosis after association with a plasma membrane binding component. Therefore, it remains possible that the 5- to 6-fold lower affinity for L-T_3 and L-T_4 observed with isolated nuclei *in vitro* may result from the ability of the intact cell to concentrate hormone yielding higher intracellular concentrations compared to that present in the media. Evidence to support the possibility that the cells may selectively concentrate iodothyronines comes from a comparison of the relative affinity of the receptor for thyroid hormone analogs using intact cells and isolated nuclei. Figure 6 illustrates the binding of iodothyronine analogs to nuclear receptors in intact cells cultured in serum-free media, and to isolated GH_1 cell nuclei *in vitro* (Fig. 7). In intact cells triac demonstrated a 3-fold higher affinity than L-T_3, while D-T_3 and L-T_4 had approximately one-tenth of the L-T_3 affinity. Using isolated nuclei *in vitro* (Fig. 7) the relative affinities of L-T_4 and triac compared to L-T_3 are essentially identical to that observed with intact cells (Fig. 6). In contrast, however, D-T_3 has almost an identical relative affinity for the receptor in isolated nuclei as L-T_3, which significantly contrasts with that observed in the intact cell experiment (Fig. 6).

Studies with isolated liver nuclei by Koerner *et al.* (1975) and DeGroot and Torresani (1975) also reported that D-T_3 had an affinity equal to or slightly less than L-T_3. Since D-T_3 is only weakly active after injection in intact animals it has been suggested that this discrepancy indicates that the cell nucleus is not the only site of action of thyroid hormone in the cell (Goldfine *et al.*, 1976). However, as illustrated in Figs. 3 and 4, using intact cells in serum-containing media, the dose–response induction of growth hormone by D-T_3 parallels the relative af-

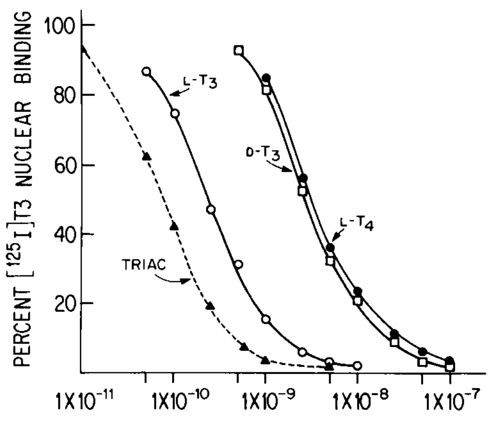

Fig. 6. Relative affinity of iodothyronines for the nuclear receptor in intact cells in serum-free media. The concentration of L-[^{125}I]T_3 was 0.2 n*M* and the concentrations of L-T_3, triac, D-T_3, and L-T_4 are as indicated. (From Samuels *et al.* (1979b).

finity of D-T_3 for the receptor, indicating an excellent relationship between the occupancy of the receptor and the induction of the biological response. Therefore, the significant difference in binding of D-T_3 using isolated cells under serum-free conditions as compared to isolated GH_1 cell or liver nuclei implies a relative decrease in cell entry of D-T_3 compared to the other hormonal analogs. Whether different cells and tissues concentrate thyroid hormone to different extents is currently unknown.

Since the thyroid hormone nuclear receptor appears to have an identical affinity for L-T_3 and L-T_4 in virtually all tissues (Oppenheimer and Dillmann, 1978), if selective differences in tissue uptake of iodothyronine occurred, those tissues that concentrate thyroid hormone would appear more sensitive and demonstrate leftward shifts of the dose–response curve. Biological dose–response studies in other cell culture systems appear to be identical to that observed in GH_1 cells. Gershengorn (1978) reported that 0.2 n*M* L-T_3 resulted in half-maximal inhibition of TSH production in mouse thyrotropic cells and Tsai and Chen (1978), using cultured myocardial cells, reported that a half-maximal increase in β-adrenergic receptor levels was induced by 0.3 n*M* L-T_3. Therefore, the dose–response characteristics for L-T_3 induction of growth hormone synthesis (Fig. 3) appear to be a general response characteristic for L-T_3 inducible functions in cell culture and suggest that thyrotropic cells, somatotropic cells, and myocardial cells accumulate the L-T_3 to the same extent. Whether differences in tissue uptake of thyroid hormone occurs in different organ systems *in vivo* is currently unknown and warrants further investigation.

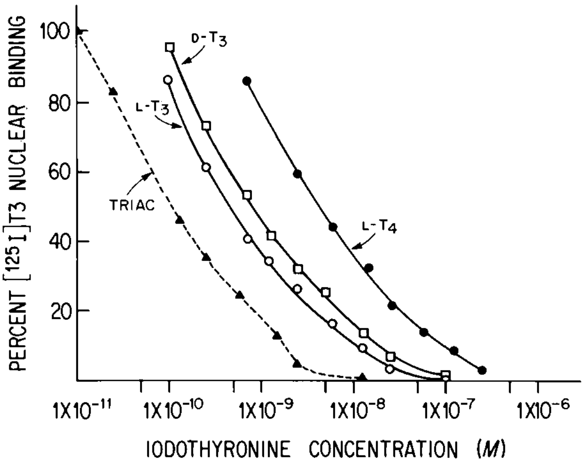

Fig. 7. Relative affinity of iodothyronines for the nuclear receptor with isolated GH_1 cell nuclei *in vitro*. GH_1 cell nuclei were incubated with L-[^{125}I]T_3 at 0.25 n*M* and the concentrations of nonradioactive L-T_3, triac, D-T_3, and L-T_3 indicated. The incubation was at 37°C using buffer conditions previously described (Samuels *et al.*, 1974b). From Samuels *et al.* (1979b).

D. *In Vitro* Characterization of Solubilized Nuclear Receptors

Further characterization of the putative nuclear receptor can be achieved after solubilization using 0.4 *M* KCl. This procedure can extract between 60 and 80% of thyroid hormone receptors from GH_1 cells or rat liver nuclei (Samuels *et al.*, 1974b). The association of thyroid hormone with solubilized nuclear receptors *in vitro* can be achieved using the identical buffer conditions as with the isolated nuclear binding assay (Samuels *et al.*, 1974a) and the bound and free hormone is separated by Sephadex G-25 (fine) chromatography. Alternatively Dowex-1, an ion exchange resin that binds free L-T_3, can also be used to separate the unbound L-T_3 from the L-T_3–receptor complex (Torresani and DeGroot, 1975). The kinetics of binding of L-T_3 with solubilized nuclear receptors in GH_1 cells at 37°C are extremely rapid with full equilibration occurring within 5 minutes of incubation. Scatchard analysis of L-$[^{125}I]T_3$ and L-$[^{125}I]T_4$ binding to solubilized receptor in GH_1 cells demonstrated K_d values of 0.16 and 1.2 n*M*, respectively (Samuels *et al.*, 1974b). These K_d values are essentially identical to the values estimated with isolated rat liver and GH_1 cell nuclei (Samuels and Tsai, 1974), and indicate that the affinity for hormones does not change after the receptor is isolated in soluble form. As discussed in Chapter 4 the solubilized receptor from GH_1 cells has a sedimentation coefficient of 3.8 S, a Stokes radius of 3.3 nm, a particle density of 1.36 g/cm^3 and an estimated molecular weight of 54,000. The frictional ratio related to shape was calculated to be 1.212 indicating a relatively globular structure (Perlman *et al.*, 1982). These values are virtually identical to that reported for receptor solubilized from rat liver nuclei by Latham *et al.* (1976).

E. Binding of L-T_3 and L-T_4 to Cytosol *in Vitro*

In target cells, steroid hormones initially interact with a cytosolic receptor (O'Malley and Means, 1974). The hormone–receptor complex undergoes a temperature-dependent conformational change to generate a form that interacts with components in the cell nucleus. Therefore, in the absence of hormone, steroid receptors are primarily found in the cytosol, while with hormone, the receptor is predominently localized to the cell nucleus. In contrast with steroid hormone receptors, a cytosolic form of the thyroid hormone nuclear receptor has not been identified. Studies with intact GH_1 cells (Fig. 1) do not demonstrate a kinetic transfer of cytosol bound L-T_3 to nuclei. In addition a cytosol binding component in intact cells over a wide concentration range of L-T_3 and L-T_4 was not detected (Samuels and Tsai, 1973). Furthermore, the level of nuclear bound thyroid hormone nuclear receptor in the hypothyroid animal is not reduced compared to that of the euthyroid state (Oppenheimer *et al.*, 1975; Spindler *et al.*, 1975). In GH_1 cells the level of nuclear bound receptor in the hormone-depleted state is actually higher than that in the presence of hormone (the mechanism of receptor regulation by thyroid hormone will be discussed in Chapter 4) (Samuels *et al.*, 1976). Therefore, unlike the steroid hormones, a cytosol to nuclear translocation of receptor is not elicited by the ligand and the level of receptor is likely a reflection of the intrinsic rates of receptor synthesis and degradation. Although a high affinity, limited capacity cytosol binding protein for L-T_3 is not identified in intact cells a binding component can be identified *in vitro* using the same buffer conditions as for the solubilized nuclear receptor (Samuels *et al.*, 1974b). Scatchard analysis of cytosol binding of L-T_3 and L-T_4 demonstrated linear Scatchard plots with K_d values of 1.13 and 0.287 nM, respectively. Therefore, in GH_1 cell cytosol the affinity for L-T_4 is 4-fold greater than that for L-T_3. The cytosol iodothyronine binding component contrasts with the observations of the relative affinities of L-T_3 and L-T_4 with nuclei or intact GH_1 cells (Samuels *et al.*, 1974a,b). In addition the 4-fold greater affinity for L-T_4 compared to L-T_3 in cytosol also contrasts with the observed greater intrinsic biological activity of L-T_3 determined in cultured GH_1 cells and intact rats (Samuels *et al.*, 1973; Money *et al.*, 1960). In addition, reverse L-T_3 (3,3′,5′-triiodo-L-thyronine) has a higher affinity for the cytosol binding component than L-T_3. This is of significance since reverse L-T_3 appears to have little or no biological activity (Samuels *et al.*, 1973), and a very low affinity for the nuclear receptor either with intact cells (Samuels, 1978), isolated nuclei (Koerner *et al.*, 1975), or with solubilized nuclear extracts *in vitro* (Samuels, 1978). These results suggest that the observed cytosol binding component is likely not related to the nuclear binding activity, or to the observed biological effects of the thyroid hormones. Furthermore, evidence has been presented, using a dense amino acid labeling technique to quantitate receptor synthesis and receptor half-life, that the nuclear bound receptor reflects a steady state solely determined by receptor synthesis and receptor degradation and that a significant pool of cytoplasmic binding protein, which functions as a precursor to the nuclear receptor, does not exist (Raaka and Samuels, 1981).

IV. MODULATION OF THYROID HORMONE NUCLEAR RECEPTOR LEVELS

A. L-T_3 Reduces Thyroid Hormone Receptors in GH_1 Cells

Although Figs. 3 and 4 demonstrate an excellent relationship between the relative biological response induced by different iodothyronines and their relative affinity for the thyroid hormone nuclear receptor, these studies do not resolve whether the biological response is a complete linear function of the occupancy of the total receptor population by hormone. GH_1 cells appear to be uniquely suited for a receptor occupancy biological response analysis, since unlike intact ani-

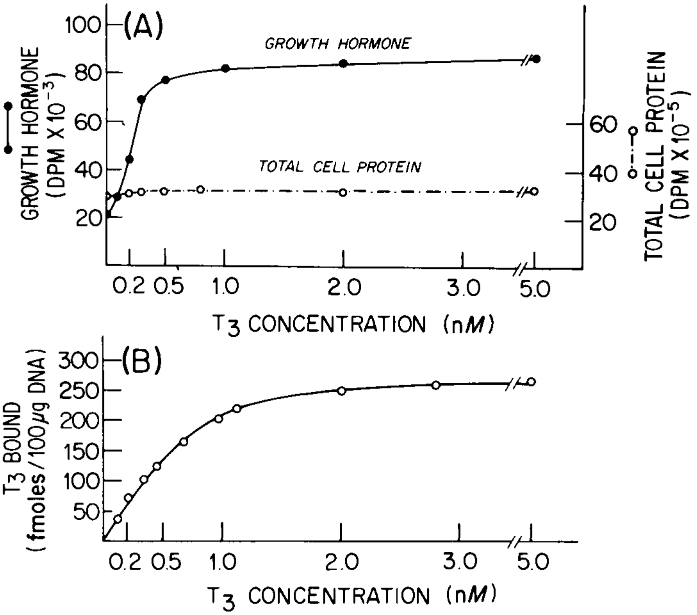

Fig. 8. Relationship of L-T_3 concentrations to growth hormone synthesis (A) and L-T_3 nuclear binding (B). Both studies were carried out with medium supplemented with 10% (v/v) thyroid hormone depleted calf serum. L-T_3 nuclear binding was determined after a 3 hour incubation with L-[^{125}I]T_3. Growth hormone and total cell protein synthetic rates were determined by L-[^{3}H]leucine after 24 hours of L-T_3 incubation. From Samuels *et al.* (1976).

mals, hormone concentrations can be maintained at a constant level and, therefore, the magnitude of L-T_3–receptor binding can be directly related to the output of the biological response of the system. Figure 8 illustrates a comparison of the dose–response relationship of growth hormone synthesis (Fig. 8A) and L-[^{125}I]T_3 nuclear binding (Fig. 8B) in cells cultured in medium containing 10% thyroid hormone depleted calf serum. Growth hormone synthetic rates were estimated after a 24-hour incubation with L-T_3 followed by a 12-minute incubation with L-[^{3}H]leucine to estimate synthetic rates (Samuels and Shapiro, 1976). L-T_3 up to 5 n*M* had no effect on total protein synthetic rates while growth hormone synthetic rates increased four-fold to approximately 3% of total cell protein synthesis with a half-maximal response at 0.22 n*M* L-T_3. The L-T_3 nuclear binding curve was determined by incubating various L-[^{125}I]T_3 concentrations with cells for 3 hours, using identical media conditions. With serum-containing media the L-T_3 concentration that resulted in half-maximal nuclear binding was 0.5 n*M* and therefore the biological response curve is shifted leftward of the L-T_3 nuclear occupancy curve. In addition to this deviation of the biological and binding curves, the shapes of the curves are dissimilar over the

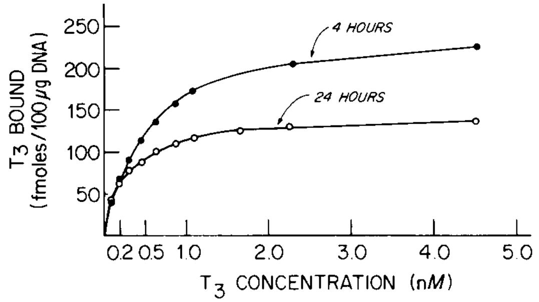

Fig. 9. Binding of L-[^{125}I]T_3 to nuclear receptors after a 4 hour and 24 hour incubation with intact GH_1 cells. From Samuels *et al.* (1976).

range of L-T_3 concentrations and imply that the stoichiometry of the biological response differs from that of the L-T_3 receptor interaction.

Because the growth hormone dose–response curve was determined after a 24-hour L-T_3 incubation, whereas the receptor binding curve was determined after a 3-hour incubation, the possibility was considered that L-T_3 altered the nuclear receptor population during the 24 hour incubation prior to the time that growth hormone synthesis was determined. Therefore, L-[^{125}I]T_3 binding at different concentrations was determined with intact cells for short (4 hour) and long (24 hour) incubation times (Samuels *et al.*, 1976). Figure 9 illustrates that although the binding of L-T_3 after a 24-hour incubation is similar at low L-T_3 concentrations, the maximal amount of L-T_3 bound in the 24-hour incubation was 60% of the 4-hour incubation. Expressed as a function of maximal L-T_3 binding, the concentration of L-T_3 that results in half of the maximal level of L-T_3–receptor complexes was 0.5 n*M* for the 4-hour incubation and 0.3 n*M* for the 24-hour incubation. The value determined for the 24-hour incubation demonstrated a closer agreement with the L-T_3 concentration (0.22 n*M*) that induced a half-maximal growth hormone response during the same period of incubation. These results are consistent with a time- and dose-dependent depletion of L-T_3 nuclear receptor levels. Figure 10 illustrates the kinetics of receptor reduction in GH_1 cells mediated by L-[^{125}I]T_3. Receptor depletion was examined by incubating one group of flasks with 5 n*M* L-[^{125}I]T_3 from the beginning of the study; control flasks initially received no L-[^{125}I]T_3. Both groups of flasks were incubated at 37°C and 1 hour before the cells were harvested 5 n*M* L-[^{125}I]T_3 was added to the control cells to quantitate receptor levels. This concentration of L-T_3 binds to greater than 95% of the nuclear receptor population and permits an indirect estimate of total receptor levels (Samuels *et al.*, 1976). Thyroid hormone receptor levels remain constant in the control cells but rapidly decrease in a time-dependent fashion in the cells that received L-[^{125}I]T_3 from the beginning of the study to 55% of the control cell levels. Scatchard analysis indicated that the reduction of L-T_3 receptor binding reflects a decrease in total receptor and does not reflect an alteration in affinity of receptor for hormone (Samuels *et al.*, 1976). Furthermore, the total reduction of receptor levels was virtually identical whether examined with nuclei or whole cells and, therefore, L-T_3 does not reduce receptor levels by eliciting a shift of receptor from the nuclear to the cytoplasmic compartment.

B. Relationship of Nuclear Receptor Occupancy and Reduction of Receptor by Iodothyronines

Figure 11 illustrates the influence of L-T_3 concentrations on total nuclear receptor levels after a 24-hour incubation in GH_1 cells. In curve 1, the cells were first incubated with the L-[^{125}I]T_3 concentrations indicated for 24 hours. The L-[^{125}I]T_3 concentration was then adjusted to 5 n*M* and the cells were incubated for an additional 3 hours to estimate total receptor levels. As shown by curve 1, nuclear receptor levels decreased approximately 50% with increasing L-T_3 concentrations and half-maximal depletion occurred at 0.17 n*M* L-T_3. To exclude the possibility that the effect of L-[^{125}I]T_3 on receptor depletion reflected a dose-related toxic effect of the ^{125}I radioactivity, cells were first incubated with various concentrations of nonradioactive L-T_3 for 24 hours followed by a 3-hour incubation with 5 n*M* L-[^{125}I]T_3. The femtomoles of total L-T_3 bound was calculated from the resultant L-[^{125}I]T_3 specific activity at each L-T_3 concentration. The results are illustrated by curve 2 of Figure 11 and are identical to the study which utilized only L-[^{125}I]T_3 in curve 1. This excludes the possibility that receptor depletion occurred secondary to ^{125}I radiation damage to the cells during the 24 hour incubation. Furthermore, the concentration of L-T_3 that results in

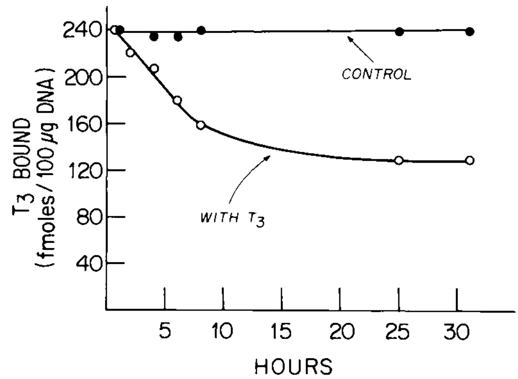

Fig. 10. Kinetics of nuclear receptor depletion by 5 n*M* L-[^{125}I]T_3. To study receptor depletion one group of cells received 5 n*M* L-[^{125}I]T_3 (○) at the beginning of the study, while control cells (●) received 5 n*M* L-[^{125}I]T_3 1 hour prior to nuclear isolation. From Samuels *et al.* (1976).

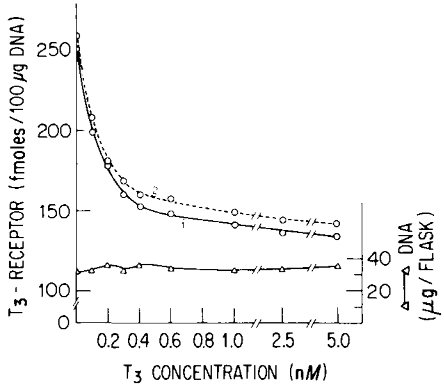

Fig. 11. Dose-dependent depletion of nuclear receptors by L-T_3 in GH_1 cells. Curve 1 received only different concentrations of L-[^{125}I]T_3 while curve 2 received only nonradioactive L-T_3. In each case after 24 hours L-[^{125}I]T_3 was added to adjust the final L-[^{125}I]T_3 concentration to 5 n*M* and the cells were incubated for an additional 3 hours. Total receptor binding was determined in curve 2 after correction of specific activity differences and assumes full equilibration of the system. From Samuels *et al.* (1976).

half of the maximal level of receptor depletion was 0.17 n*M*, which is not identical but in good agreement with the L-T_3 concentration that results in half-maximal occupancy of the receptor (0.5 n*M*). This suggests that L-T_3 mediated receptor depletion occurs secondary to a process that is dependent on the association of L-T_3 with the receptor binding site.

This conclusion is supported from (1) studies of iodothyronine analog concentrations on reducing total nuclear receptor (Samuels *et al.*, 1979b) and (2) the relationship between the initial rates of disappearance of receptor and the fractional occupancy of the receptor population by L-T_3. Figure 12 compares the

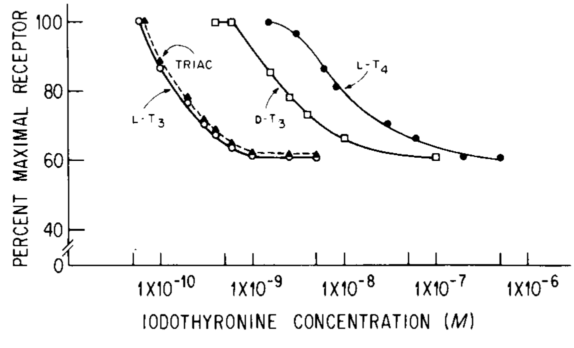

Fig. 12. Relative effect of iodothyronines on receptor depletion in GH_1 cells. From Samuels *et al.* (1979b). For experimental details see this reference.

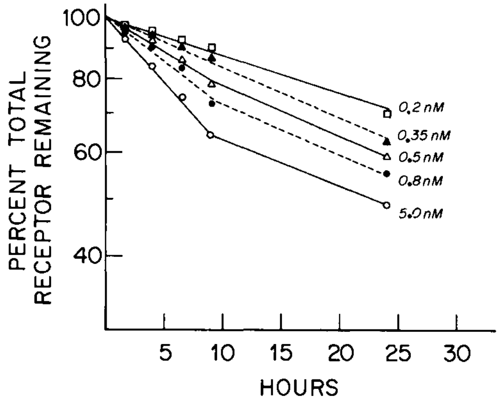

Fig. 13. Kinetics of total receptor depletion at five L-T_3 concentrations. GH_1 cells were incubated with L-[^{125}I]T_3 concentrations of 5, 0.8, 0.5, 0.35, and 0.2 n*M*. One hour prior to the times indicated the L-[^{125}I]T_3 concentrations were each adjusted to 5 n*M*, which gives an estimate of total receptor levels. From Samuels *et al.* (1977b).

dose-dependent reduction of total thyroid hormone nuclear receptor levels by different concentrations of L-T_3, triac, D-T_3, and L-T_4 (Samuels *et al.*, 1979b). Each compound elicited a 40% decrease in total nuclear receptor levels and the dose-dependent reduction of receptor parallels the affinity of each analog for the receptor binding site (see Fig. 4). Figure 13 illustrates the kinetics of receptor depletion examined at five L-T_3 concentrations that are known to occupy a specific percentage of total receptor (0.2 n*M*, 25%; 0.35 n*M*, 40%; 0.5 n*M*, 50%;

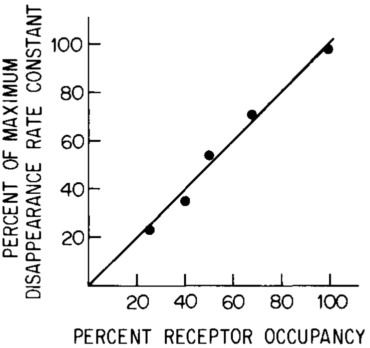

Fig. 14. Relationship of nuclear receptor occupancy to rate of receptor loss. The initial disappearance rate constants of receptor loss was calculated by dividing the $t_{1/2}$ values into 0.693. The value for the highest concentration of L-triiodothyronine, 5 n*M* (0.1155 hour^{-1}), was considered to be 100 and the other rate constants are expressed as a percentage of this value. The percentage of total receptor sites occupied at each of the L-triiodothyronine concentrations was taken from the study by Samuels *et al.* (1976). These are 25% at 0.2 n*M*, 40% at 0.35 n*M*, 50% at 0.5 n*M*, 69% at 0.8 n*M*, and 95% at 5 n*M*. From Samuels *et al.* (1977b).

0.8 n*M*, 69%; and 5 n*M*, 95%) (Samuels *et al.*, 1977b). At each L-T_3 concentration, the initial rates of receptor depletion demonstrate first-order kinetics. A comparison of the disappearance rate constant calculated from the initial rates of receptor disappearance indicates that the kinetics of receptor loss is a perfect linear function of the percentage of total receptor binding sites occupied by L-T_3 (Samuels *et al.*, 1977b). These results are illustrated in Figure 14 in which the disappearance rate constant for receptor loss at 5 n*M* L-T_3 (0.1115 hour^{-1}) was considered to be 100 and the values at lower hormone concentrations a percentage of the 5 n*M* value. The percentage of receptor binding sites occupied at each hormone concentration was determined from the study of Samuels *et al.* (1976) and Fig. 8. The kinetics of receptor depletion appears to be a direct function of the receptor occupancy curve, and 0.5 n*M* L-T_3 results in half-maximal occupancy and rate of depletion (Samuels *et al.*, 1977b).

C. Biological Implications of Receptor Reduction by Thyroid Hormone

Hormone mediated receptor depletion may play a role in influencing the characteristics of the induced biological response in GH_1 cells (Samuels *et al.*, 1976). Figure 15 compares the growth hormone synthetic dose–response curve with the receptor binding and depletion curves. The synthetic curve is from Fig. 8 and the binding and receptor depletion curve are from Figs. 9 and 11. For purpose of comparison the results are expressed as a percent of maximal growth hormone synthesis (curve 1), receptor depletion (curve 2), or nuclear binding determined after a 4-hour (curve 3) or a 24-hour (curve 4) incubation. Compared

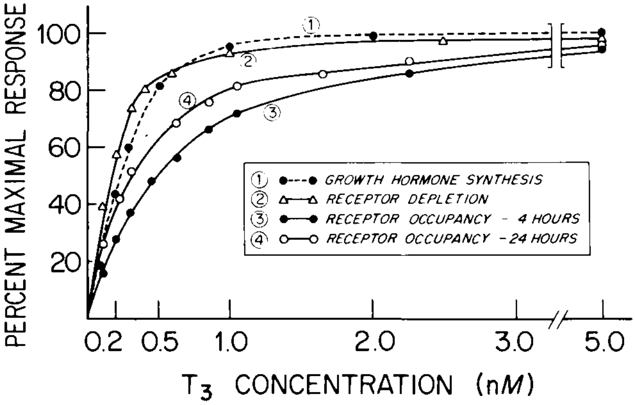

Fig. 15. Relationship of receptor depletion and receptor occupancy to growth hormone synthesis. For comparison the results are expressed as a percent of maximal response or binding. From Samuels *et al.* (1976).

to the 4 hour occupancy curve, the 24-hour receptor occupancy curve shows very good agreement (half-maximal binding, 0.3 n*M*) with the biological response curve (half-maximal response, 0.22 n*M*). Of interest is that the response curve for receptor depletion (curve 2) is virtually identical over the entire range of L-T_3 concentrations to the dose–response curve for the induction of growth hormone synthesis. Therefore, thyroid hormone action could result from the binding of L-T_3 to the remaining nondepleted receptor population which regulates a rate-limiting nuclear event proportional to the number of L-T_3–receptor complexes. Alternatively, hormone-mediated receptor depletion may be the rate-limiting event in thyroid hormone action. By this mechanism the receptor could function as a regulatory repressor, and the action of thyroid hormone would be secondary to hormone-mediated receptor reduction and result in derepression of a specific nuclear response.

These alternative mechanisms predict a difference in cell response to L-T_3 as the receptor is progressively reduced. In the first case, the biological response would be expected to decrease in association with a reduction in receptor levels, whereas in the second case, the biological response would increase as a function of time as the receptor is depleted. Quantitation of the instantaneous rates of growth hormone mRNA synthesis should distinguish between these two possibilities. If hormone-mediated receptor loss initiated the action of thyroid hormone, the instantaneous rate of mRNA synthesis would be expected to increase with time and be inversely related to the level of receptor. Alternatively, if the binding of L-T_3 with the nondepleted remaining receptor population is rate limiting for initiating the action of thyroid hormone, the instantaneous rate of growth hormone mRNA synthesis would be expected to decrease with time as the receptor is progressively reduced. Although this analysis has not yet been carried out, indirect evidence suggests that as the receptor population is reduced the cellular response to L-T_3 decreases in a parallel fashion. In GH_1 cells in the absence of thyroid hormone, nuclear receptor levels decrease with increasing cell density even though the total rate of protein synthesis per cell remains constant (Samuels *et al.*, 1977b). In association with this reduction in nuclear receptor levels is a parallel decrease in the magnitude of induction of growth hormone synthesis by thyroid hormone (Samuels *et al.*, 1977b). This suggests that the induction of the growth hormone response by L-T_3 is proportional to the number of L-T_3–receptor complexes and that the thyroid hormone induced response is not initiated as a result of reduction of receptor levels mediated by thyroid hormone.

It should be pointed out that hormone-modulated receptor depletion has not been reported to occur *in vivo*. Using labeling studies in intact animals, Oppenheimer *et al.* (1975) reported that the concentration of receptor binding sites in rat liver was identical in euthyroid rats and animals rendered hypothyroid by thyroidectomy. In addition in an examination of the binding of L-$[^{125}I]T_3$ to isolated nuclei *in vitro*, Spindler *et al.* (1975) quantitated an identical number of binding sites in nuclei obtained from the livers of hypothyroid and euthyroid rats. A similar assessment of receptor in nuclei of pituitary cells has not been carried out *in vivo*. However, L-T_3 incubation elicits a reduction in nuclear receptor levels in dispersed cells obtained from rat pituitary (Samuels *et al.*, 1979b). Therefore, it remains possible that depletion of receptor by thyroid hormone may be restricted to the somatotroph cell or to only several tissues *in vivo*.

If thyroid hormone controlled the level of nuclear receptors in somatotrophs in vivo, our observations on hormone-mediated reduction of receptor and growth hormone synthesis in GH_1 cells may allow for prediction of the growth response in different thyroidal states *in vivo*. In the hypothyroid rat, receptor levels would be high and the somatotroph would be expected to show a marked increase in the growth hormone response to a single injection of thyroid hormone. This has been reported by Hervas *et al.* (1975). In addition, with chronic thyroid hormone administration sufficient to induce thyrotoxicosis, reduction of the receptor might be expected to lower the magnitude of growth hormone response relative to that of the euthyroid state. Only a limited number of studies, however, have examined the effects of chronic thyroid hormone administration on the growth hormone response in the rat (Solomon and Greep, 1959; Coulombe *et al.*, 1978). In studies relating to thyroid function and the pituitary content of growth hormone, Solomon and Greep (1959) estimated the growth hormone content of the rat pituitary to be 26 μg/mg pituitary ± 36.1% from the hypothyroid rat, and 170 μg/mg pituitary ± 36% in the euthyroid rat. After chronic thyroid hormone administration sufficient to induce thyrotoxicosis, the growth hormone content of the pituitary decreased to 75.2 μg/mg pituitary ± 35.5%. Coulombe *et al.* (1978) reported that daily injection of thyroid hormone in euthyroid rats sufficient to develop thyrotoxicosis decreased the pituitary growth hormone content compared to euthyroid controls. Therefore, the growth hormone response parameters in the rat as a function of the thyroidal state approximates a bell-shaped curve with a reduction in pituitary growth hormone content both in the hypothyroid and thyrotoxic state.

Studies on the influence of thyroid hormone on the physiology of growth hormone in man tend to parallel the observations in the rat although the studies demonstrate some variability in the response (MacGillivary *et al.*, 1968; Iwatsubo *et al.*, 1967). In a series of reported studies between 40 and 80% of hypothyroid patients demonstrated a blunted growth hormone response to insulin induced hypoglycemia and in the majority of these patients the response normalized after restoration of the euthyroid state (MacGillivary *et al.*, 1968; Iwatsubo *et al.*, 1967; Root *et al.*, 1967). In thyrotoxicosis, insulin-induced hypoglycemia tends to result in a subnormal growth hormone response compared with euthyroid controls and this is restored to normal levels after restoration of euthyroidism (Burgess *et al.*, 1966; Finkelstein *et al.*, 1974). Finkelstein *et al.*

(1974) also quantitated the growth hormone plasma half-life and 24-hour secretory rates in hyperthyroid patients and euthyroid controls. The plasma half-lives were unchanged in hyperthyroidism but the 24-hour secretory rates were reduced by 43% and this was restored to normal after restoration of the euthyroid state.

Therefore, although studies in man do not allow estimation of growth hormone synthesis or pituitary growth hormone content, the general pattern of growth hormone response as a function of thyroidal state is qualitatively similar to that determined experimentally in the intact rat. Modulation of the thyroid hormone nuclear receptor levels in the somatotroph provides a plausible explanation for the influence of the thyroidal state on the growth hormone response *in vivo*. Whether receptor regulation by thyroid hormone occurs in different tissues *in vivo* remains open to question and warrants additional investigation. Since modulation of receptor levels by thyroid hormone would tend to sensitize cells in hypothyroid animals to thyroid hormone and partially desensitize the cell in the thyrotoxic state to hormone, this putative autoregulatory mechanism in the cell would result in an additional mechanism that influences tissue response to thyroid hormone over and above the regulation of thyroid hormone levels in serum controlled by the pituitary–thyroid axis.

V. SUMMARY AND CONCLUSIONS

Figure 16 is a schematic representation which summarizes the current concepts of thyroid hormone action in cells and the interaction of hormone with biologically relevant cellular receptors. L-T_3 and L-T_4 enter the cell by yet undefined mechanisms. The hormone likely enters the cell in the free unbound form and whether permeation occurs by passive diffusion or by a transport system that can concentrate hormone has not been clearly defined. Unlike steroid hormones, the thyroid hormones associate with a nuclear receptor directly without any requirement for an initial association with a cytoplasmic form of the receptor. The receptor is a chromatin-associated protein that can be solubilized from nuclei using high salt conditions and has an estimated molecular weight of approximately 50,000–55,000. The level of thyroid hormone nuclear receptor appears to reflect a steady-state value, which in GH_1 cells can be autoregulated by thyroid hormone as a result of an interaction of the hormone with the receptor binding site. Although the nuclear event controlled by the thyroid hormone–receptor complex has not been defined, presumably it involves transcriptional and/or posttranscriptional nuclear events that result in an increase in accumulation of specific mRNA molecules that code for the synthesis of specific proteins regulated by thyroid hormone. These proteins may be destined for export and may act to regulate other tissues (e.g., growth hormone) or are soluble enzymes (malic enzyme) that can further act to regulate specific metabolic events of the

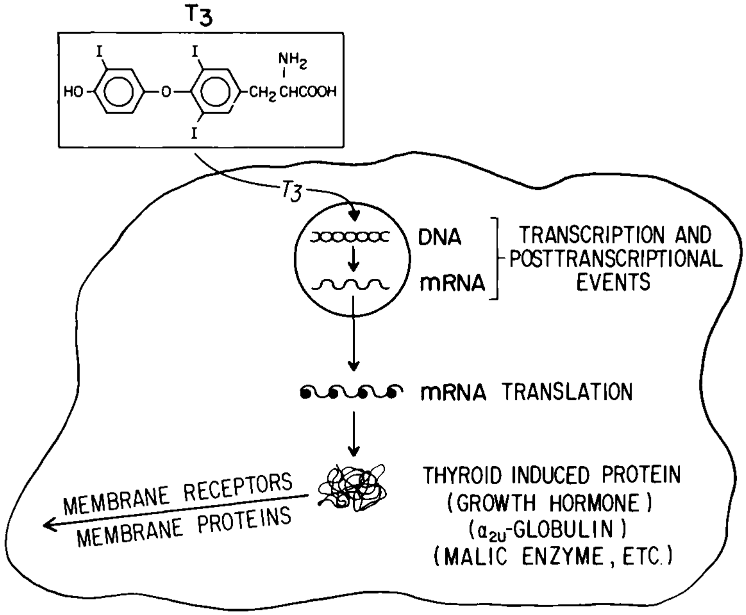
T3
I
I
NH2
HO
O
CH2CHCOOH
I
T3
DNA
mRNA
TRANSCRIPTION AND
POSTTRANSCRIPTIONAL
EVENTS
mRNA TRANSLATION
MEMBRANE RECEPTORS
MEMBRANE PROTEINS
THYROID INDUCED PROTEIN
(GROWTH HORMONE)
(α2u-GLOBULIN)
(MALIC ENZYME, ETC.)

Fig. 16. Summary of the current concepts of thyroid hormone action.

cell. In addition several of the proteins regulated by the thyroid hormones are membrane bound (e.g., Na^+,K^+-ATPase, catecholamine receptors), which can further act to effect modifications in cell function or alter the sensitivity of the cell to other hormonal factors. Although it has not been documented that the stimulation of Na^+,K^+-ATPase or catecholamine receptors results from the stimulation of the accumulation of their mRNA molecules, the induction requires both RNA and protein synthesis, suggesting that the regulation of these membrane bound proteins occurs as a result of stimulation of mRNA production and protein synthesis. In addition to stimulation of the synthesis of plasma membrane bound proteins, thyroid hormone also stimulates the synthesis of enzymes that may be localized in other subcellular organelles (e.g., α-glycerophosphate dehydrogenase in mitochondria). Although α-glycerophosphate dehydrogenase is associated with mitochondria, the enzyme appears to be synthesized in cytoplasmic polysomes and therefore is under control by the eukaryotic and not mitochondrial genes.

Although both cell culture as well as intact animal studies have contributed significantly to the current concepts of thyroid hormone action in cells, the detailed aspects relevant to the mechanism of the nuclear action of the thyroid hormones need to be clarified. These include (1) identification of precise components in the nuclear chromatin with which the thyroid hormone nuclear receptor interacts and clarification of how this interaction is involved in the initiation of transcriptional or posttranscriptional events; (2) identification of the factors that control the synthesis and fractional turnover rate of the nuclear associated receptor and the mechanism of this regulation; (3) detailed clarification of the mechanisms by which thyroid hormone interacts with other hormones and factors to modulate specific cellular responses; and (4) purification of the receptor and reproduction of the nuclear action of the thyroid hormone with reconstituted cellular components *in vitro*. Significant developments have recently occurred in several of these areas and Chapter 4 summarizes studies on the cell biology of the receptor with regard to quantitation of receptor synthesis and receptor degradation and the organization of the receptor in chromatin domains.

ACKNOWLEDGMENTS

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3

Physicochemical Characterization of the Intranuclear Thyroid Hormone Receptor

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I. INTRODUCTION

As discussed in Chapter 2, limited capacity binding sites for thyroid hormones are present in the nuclei of thyroid hormone-responsive tissues (Oppenheimer *et al.*, 1972; Samuels and Tsai, 1973; DeGroot *et al.*, 1974; Kistler *et al.*, 1975; Spindler *et al.*, 1975). Several lines of correlative evidence suggest that these nuclear sites are receptors that mediate certain of the target cells' responses to thyroid hormones (for reviews, see Oppenheimer, 1979; Eberhardt *et al.*, 1980). Thus, these receptors bind thyroid hormones at physiological concentrations (Samuels *et al.*, 1973) and bind analogs of thyroid hormones in direct proportion to the analogs' thyromimetic potency (Koerner *et al.*, 1975). The receptors are

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present in high concentrations in tissues that are known to respond to thyroid hormones and are absent in certain nonresponsive tissues (Oppenheimer *et al.*, 1974). In addition, the intranuclear localization of the receptor is correlated with the hormones' influence on specific mRNA production (Martial *et al.*, 1977; Wegnez *et al.*, 1982). Moreover, recent experiments indicate that thyroid hormones stimulate the rate of transcription of the growth hormone gene in intact rat pituitaries and in cultured rat anterior pituitary cells (GC) (Mellon-Nussbaum *et al.*, 1982). Thus, the thyroid hormone receptor–hormone complex may mediate the transcription of specific genes in target cells. Finally, there is some evidence that these receptors may be absent or functionally altered in fibroblasts from patients who exhibit peripheral resistance to thyroid hormones (Bernal *et al.*, 1978).

In this chapter, we discuss studies of the physicochemical characterization of the receptor and where possible relate such studies to the mechanism of action of thyroid hormones.

II. INTRANUCLEAR LOCALIZATION OF THE THYROID HORMONE RECEPTOR

In studies examining the intranuclear localization of the nuclear receptor sites, Surks *et al.* (1973) isolated rat liver nuclei and treated them with Triton X-100 to remove the outer nuclear membrane and used 0.14 *M* NaCl and 0.1 *M* Tris to extract the soluble nuclear proteins. When a small dose of $[^{125}I]T_3$ had been administered *in vivo* prior to nuclear isolation, 50–70% of the nuclear $[^{125}I]T_3$ remained with the residual nuclear pellet. By contrast, when a large dose of T_3 was injected so that most of the cell-associated hormone was nonspecifically bound (i.e., associated with sites with a much lower affinity and higher capacity for T_3 than the receptors), 70–90% of the nuclear-bound T_3 was removed by Triton X-100. Similarly, when a small dose of $[^{125}I]T_4$ was injected, most of the bound T_4 was nonspecifically associated with the nucleus, since it was removed with the nuclear outer membrane; however, 10–15% of the T_4 was associated with the nuclear pellet. These data suggested that specifically bound T_3 and T_4 are associated with the chromatin, whereas nonspecific T_3 and T_4 binding occur largely at the outer nuclear membrane.

The localization of the receptors in chromatin has been further documented in several other studies. Spindler *et al.* (1975) prepared chromatin from rat liver nuclei containing bound T_3. These studies indicated that the amount of specifically bound T_3 in chromatin was identical to that present in the nuclei from which the chromatin had been prepared. In addition, Charles and co-workers (1975) treated chromatin containing bound T_3 with formaldehyde, which cross-links histones and a small proportion of the acidic proteins to the DNA, but does not

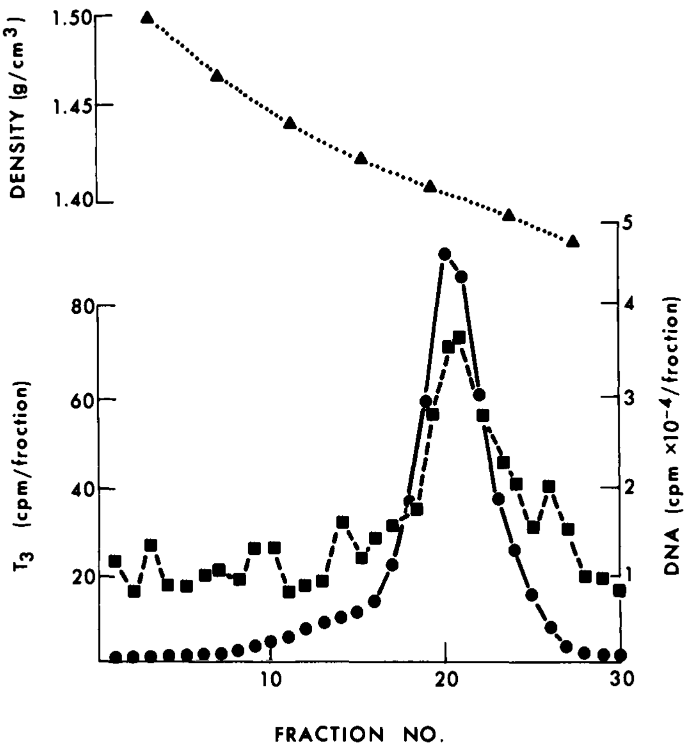

Fig. 1. Cesium chloride (▲) equilibrium density gradient centrifugation of formaldehyde-fixed HeLa chromatin containing specifically bound ^{125}I-labeled triiodothyronine (T_3). DNA content (●) was measured by [^{3}H]thymidine uptake *in vivo*. Specifically bound [^{125}I]T_3 is indicated by the squares (■). Reprinted from Charles *et al.* (1975).

link randomly chosen proteins (Doenecke and McCarthy, 1975). When formaldehyde-linked chromatin preparations were centrifuged in cesium chloride gradients, radiolabeled T_3 sedimented with the DNA (Fig. 1), again indicating that the receptor is bound to chromatin. Defer and colleagues (1974a,b, 1977; Thomopoulos *et al.*, 1974) have shown that T_3 binds to isolated chromatin. All these studies, taken together, strongly imply that the nuclear receptors for thyroid hormone are chromatin-localized proteins.

Additional studies have been performed to further define the site of receptor association within chromatin. Charles and co-workers (1975) subjected HeLa cell chromatin labeled with [^{125}I]T_3 to hydrodynamic shear and fractionated it by sucrose gradient velocity sedimentation. As shown in Figs. 2 and 3, the receptors were found to be distributed throughout the chromatin fractions but were concentrated relative to DNA in the more slowly sedimenting fractions. These fractions also contained a lower ratio of protein to DNA, most of the endogenous RNA polymerase activity (Doenecke and McCarthy, 1975), and all the capacity for binding exogenously added RNA polymerase. These results suggest that thyroid hormone receptors are nonrandomly distributed within chromatin with a possible enrichment in those fractions that contain actively transcribed DNA.

The nature of the localization of receptors in chromatin has also been examined with the use of nucleases. Gardner (1975) treated rat liver chromatin with micrococcal nuclease, an enzyme that liberates the DNA-histone containing nucleosomes and nucleosome oligomers (for review, see Elgin and Weintraub, 1975; Felsenfeld, 1978). In these experiments, the majority of the bound T_3 remained associated with the nuclei after 65% of the DNA had been liberated. Thus, there was a higher concentration of receptors in the DNase-resistant fraction, which is relatively enriched in nucleolar chromatin. He suggested that receptors are concentrated in nucleoli (Gardner, 1975). More recent evidence (Gardner, 1978) indicates that the thyroid hormone receptors are preferentially associated with nucleosome oligomers and not monomers. These latter findings were interpreted as suggesting that the receptors bind to DNA in the internucleosomal spacer region. Further studies indicating that the receptor is localized within DNase but not RNase susceptible regions of chromatin have been

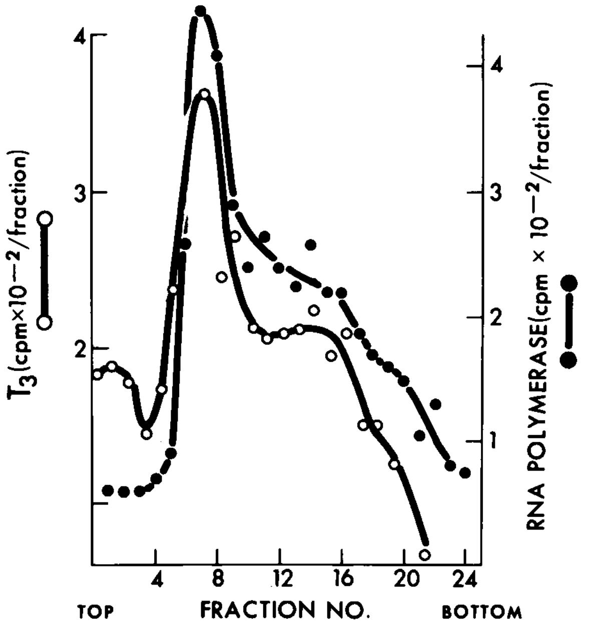

Fig. 2. Distribution of thyroid hormone receptor (○) and endogenous RNA polymerase activity (●) in fractionated HeLa chromatin. HeLa chromatin was sheared hydrodynamically and separated by sucrose gradient sedimentation. Reprinted from Charles *et al.* (1975).

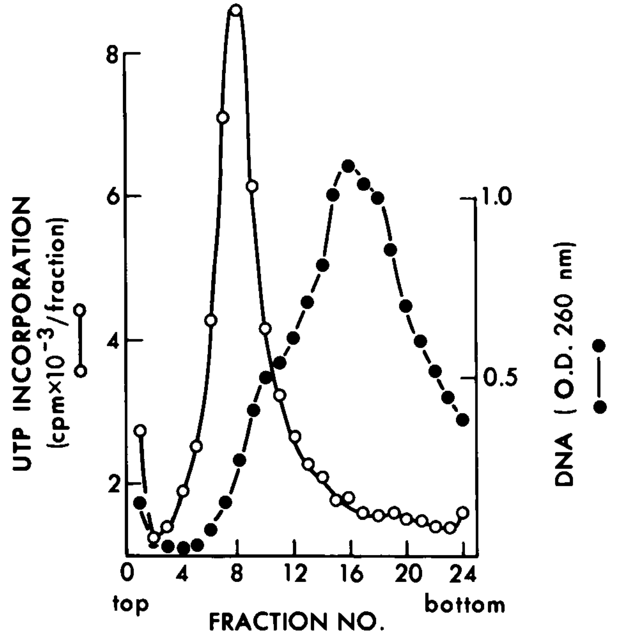

Fig. 3. Distribution of DNA (●) and template activity (○) as measured by [^{3}H]UTP incorporation of fractionated HeLa chromatin. Chromatin was hydrodynamically sheared and fractionated by sucrose gradient sedimentation. Data from Charles *et al.* (1975).

carried out by Yoshizato *et al.* (1977). More recent studies of the characterization of the micrococcal nuclease-released receptor have been carried out by Samuels *et al.* (1980), Perlman *et al.* (1982), Jump and Oppenheimer (1980), and Gruol (1980); these studies are described in Section IV.

Gottesfeld *et al.* (1974, 1976) have reported that DNase II treatment of chromatin releases a fraction enriched in middle and nonrepetitive, tissue-specific sequences transcribed *in vivo*. In addition, the globin gene from Friend leukemia cells is enriched 2.4-fold in this template-active fraction (Gottsfeld and Partington, 1977) when the cells are induced to synthesize globin with dimethyl sulfoxide but not in uninduced cells. By contrast, in another cell line of Friend cells there is a 7-fold enrichment of globin gene sequences in the template-active chromatin fraction from dimethyl sulfoxide-induced or uninduced cells (Wallace *et al.*, 1977). Moreover, Wallace *et al.* (1977) found no globin sequences in the template-active fraction of a cell line that had lost the ability to produce globin but that contained globin gene sequences in the inactive fraction. In another line of Friend leukemia cells, Lau *et al.* (1978) found equal concentrations of globin sequences in template-active and -inactive fractions, although chromatin-associated RNA contained a higher concentration of globin sequences in the template-active fraction. The reason for the above discrepancies are not known but may be partially explained by the differentiated characteristics of the different cell lines. Taken together the data suggest that the nuclease treatment liberates DNA that is being transcribed.

In studies designed to determine whether thyroid hormone receptors were localized in actively transcribed regions of chromatin, Levy and Baxter (1976) applied the Gottesfeld techniques to chromatin from cultured rat anterior pituitary cells (GC). In these studies there was only a small difference in the specific distribution of receptors between the active and inactive chromatin fractions. The receptors were distributed in proportion to the DNA content of each fraction, and most were localized in the "inactive" fraction. More recently, Samuels *et al.* (1977) performed similar experiments with GH_1 cell chromatin. However, in their studies the chromatin released by nuclease digestion (5% of the total) was further precipitated with Mg^{2+}. The chromatin that remained soluble after Mg^{2+} treatment (approximately 20% of the DNA which was released by DNase treatment) was enriched for transcriptionally active material approximately fivefold relative to the chromatin pellets. Compared to all other fractions prepared by their procedure, a small fraction of the receptors was concentrated about 8-fold relative to DNA in the "transcriptionally active," Mg^{2+}-soluble fraction (Samuels *et al.*, 1977). However, as Levy and Baxter (1976) found, most of the receptors were not released under conditions that released most of the pulse-labeled and chromatin-associated [^{3}H]uridine (a possible index of newly synthesized RNA). The reasons for these differences are not known and a definitive answer to the question of whether receptors are relatively enriched in active chromatin must await further studies, including proof that the Gottesfeld technique does release transcriptionally active chromatin. However, both studies suggest that the major proportion of the receptors are not located in transcriptionally active chromatin.

A number of workers have examined the influence of thyroid hormones on the localization of the receptor in the nucleus. In these studies the levels of nuclear receptors in hypothyroid and euthyroid rat liver have been quantitated by Scatchard analysis of [^{125}I]T_3 binding by isolated nuclei. Results from these studies indicate that there is no difference in the levels of receptors in the presence or absence of hormone (Fig. 4; Spindler *et al.*, 1975; DeGroot *et al.*, 1976). In similar experiments quantifying the levels of receptors in hypothyroid rat liver (Valcana and Timiras, 1979) and cerebral hemisphere (Valcana and Timiras, 1978) there was an apparent increase in the number of nuclear receptors when compared to euthyroid or hyperthyroid animals. It cannot be concluded from these studies, however, that the hormone causes a decrease in nuclear T_3-binding activity, since the reduced binding activity observed might be explained by the incomplete exchange of [^{125}I]T_3 with endogenous, unlabeled hormone. Although Samuels *et al.* (1973) earlier reported that preincubation of GH_1 cells

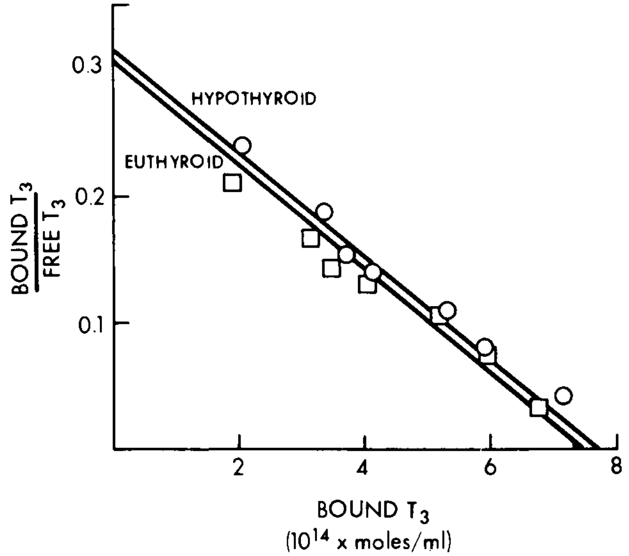

Fig. 4. Scatchard analysis of the binding of $[^{125}I]T_3$ by nuclei from euthyroid (○) and hypothyroid (☐) rat liver. The data illustrate that the concentration of nuclear receptors in the liver is independent of the concentrations of thyroid hormones in the animal. Data reprinted from Spindler *et al.* (1975).

with T_3 resulted in increased levels of intranuclear receptors as measured by $[^{125}I]T_3$ binding, more recent data indicate that T_3 actually produces a reduction (ca. 50%) in the level of receptors in GH_1 cell nuclei (Samuels *et al.*, 1977). The predominant view derived from these experiments is that the thyroid hormone receptor is an intrinsic chromosomal protein, whose localization in the nucleus is not dependent on the hormone. In this respect thyroid hormone receptors differ from the steroid receptors, whose tight association with the chromatin is dependent on the presence of the steroid.

III. DNA BINDING PROPERTIES OF THE RECEPTOR

To test possible mechanisms of the localization of receptors within chromatin, MacLeod and Baxter (1975, 1976) examined the ability of partially purified, solubilized receptors to bind to purified DNA. They found that such solubilized receptors bind to DNA avidly at physiological ionic strengths (e.g., in the 0.05–0.15 *M* NaCl range). This property differs from the steroid receptors, whose binding to DNA at these ionic strengths is markedly reduced (Rousseau *et al.*, 1975). The solubilized receptors were bound equally well by native and denatured DNA and by DNA from different eukaryotic species or from prokaryotes. Receptor–DNA interactions were stronger than interactions with cytoplasmic RNA, nuclear RNA, poly(dA·dT)·poly(dA·dT), or poly(dG·dC)·poly(dG·dC). In addition, there was no significant binding of the receptor to tRNA, poly(dA), ATP, or GTP. Thus, the receptor has a marked preference for binding to DNA as compared to RNA; however, the DNA binding detected under these conditions may be relatively nonspecific.

When increasing concentrations of receptors are incubated with DNA, many more receptors can be bound by DNA than are associated with the nucleus (MacLeod and Baxter, 1976). Thus, the capacity of the DNA for binding the receptor is very large. This finding and the observation that prokaryotic and eukaryotic double-stranded DNA's bind receptors with equal avidity could imply that there is extensive nonspecific association of the receptors with DNA. In these studies no evidence for sequence-specific DNA binding was obtained, although this cannot be excluded. It is possible that most of the receptors are located at sites where biological responses to thyroid hormone are not elicited and that a subpopulation of receptors bound at sites not yet clearly defined is responsible for the responses that do occur. Whereas these data indicate that the receptor or some receptor-associated protein is a DNA-binding protein, it is not clear from these studies with crude receptor-containing preparations whether the receptor itself or a protein associated with it is binding to DNA.

An examination of the X-ray crystallographic structure of thyroid hormone-binding prealbumin (TBPA) suggested the presence of a DNA binding site on this protein. This observation suggested that the receptor and TBPA could be related. Thus, a β-pleated sheet structure was found in TBPA which resembled a DNA binding site (Fig. 5). This structure was enriched in amino acids with ionic side chains which could bind to the phosphate groups of DNA, and also contained tryptophan molecules which could intercalate between the DNA base pairs. Although TBPA does not appear to be a DNA binding protein (MacLeod and Baxter, 1975), the possibility that the receptor (which, as discussed previously, may be a DNA binding protein) and TBPA might be related deserves further consideration. Two possibilities can be envisioned to account for these results. First, TBPA and the receptor might contain common subunits. TBPA is composed of four identical polypeptide subunits, has a molecular weight of 55,000, and has two hormone binding sites (Kanda *et al.*, 1974). Using affinity labeling, Nikodem *et al.* (1980) found that the nuclear thyroid hormone receptor consists of a single polypeptide with a molecular weight of 56,000. Second, the receptor and TBPA may have evolved from a common gene, in which case certain elements contributing to the binding site may have been conserved. The confirmation of any possible relationship between these two proteins will require more detailed structural or sequence information on the nuclear receptor.

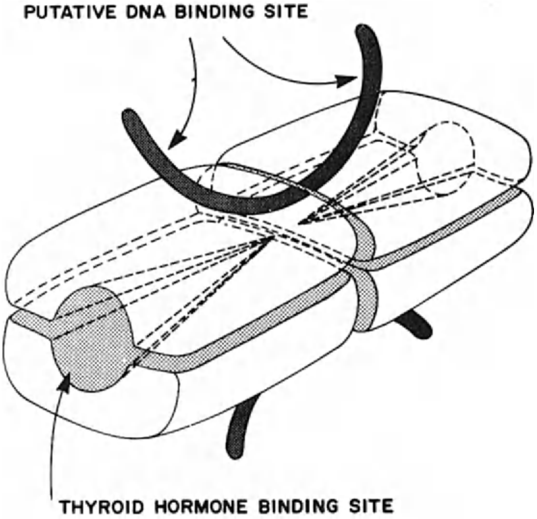

Fig. 5. Schematic model derived from X-ray crystallographic studies of prealbumin, illustrating the interaction of four identical subunits that form a channel through the interior of the molecule, creating two identical thyroid hormone-binding sites. The channel narrows at the center of the molecule. Although the binding sites are identical, T_4 binds cooperatively; occupation by T_4 at one site presumably alters the conformation of the second site which results in a lower affinity for the second T_4 binding interaction. Depicted on the upper and lower side of the molecules is a symmetrical β-pleated sheet structure which is relatively rich in amino acids with ionic side chains and which also contains tryphophan molecules. Based on computergraphic modeling studies, this site has been proposed to be a DNA-binding site (adapted from Blake and Oatley, 1977).

IV. PHYSICAL CHARACTERISTICS OF THE SOLUBILIZED NUCLEAR RECEPTORS

The receptor has been solubilized from chromatin by several methods. In the earliest studies (Surks *et al.*, 1973; Samuels *et al.*, 1974; DeGroot *et al.*, 1974), 0.4 *M* KCl was used. Latham *et al.* (1976) have extracted receptor from chromatin employing salt conditions (0.2 *M* ammonium sulfate) similar to those commonly used to extract RNA polymerase from chromatin. Incubation of nuclei under hypotonic conditions has been shown to release a small fraction of the receptors from chromatin (Bernal and DeGroot, 1977); however, this is not an efficient extraction process for obtaining the receptor. Nevertheless, this observation is an important consideration when evaluating quantitative studies utilizing nuclei to measure receptor levels.

The receptor extracted from nuclei with salt has a reported sedimentation coefficient of 3.5 S (Latham *et al.*, 1976; Jump and Oppenheimer, 1980) or 3.8 S

(Samuels *et al.*, 1980). As mentioned previously, the receptor can also be released from nuclei or chromatin by nucleases. Samuels *et al.* (1980) found that micrococcal nuclease excises the receptor from GH_1 cell nuclei as an abundant 6.5 S form and a less abundant 12.5 S form. It was suggested that the 12.5 S form represents receptor excised in association with mononucleosome particles, whereas the 6.5 S form represents receptor excised in association with a DNA fragment of approximately 36 base pairs (Perlman *et al.*, 1982), and present either as an oligomer or in association with other proteins complexed with DNA. The 6.5 S form could be converted to a 3.8 S species by digestion with DNase I or by treatment with 0.4 *M* KCl. Jump and Oppenheimer (1980) and Gruol (1980) obtained similar results in nuclease studies on thyroid hormone receptors in liver nuclei.

The protein nature of the receptor was demonstrated in studies employing treatment with proteolytic enzymes and nucleases (Surks *et al.*, 1973; Samuels *et al.*, 1974). The T_3-binding activity of the receptor was inactivated by trypsin, chymotrypsin, or pronase, but not by DNase or RNase. The receptor was more resistant to trypsin than to chymotrypsin or pronase. This finding suggested that the receptor was a nonhistone protein, since nonhistone proteins have a lower content of arginyl and lysyl residues than histones. This conclusion was supported by the finding that the receptor was extracted more efficiently at slightly alkaline pH (8.5) than at mildly acidic pH (6.0) (Surks *et al.*, 1973). In addition, the receptor binds to anion exchange resins (Latham *et al.*, 1976; Silva *et al.*, 1977) and behaves like a nonhistone protein on a weak cation exchange resin such as Bio-Rex 70 (Samuels *et al.*, 1974).

Finally, isoelectric focusing studies support the conclusion that the receptor is an acidic, nonhistone protein, and also suggest that the receptor interacts with basic proteins. As shown in Fig. 6A, when the receptor was focused under nonequilibrium conditions, two forms with isoelectric points of 5.8 and 7.8 were observed. By contrast, in equilibrium isoelectric focusing experiments (Fig. 6B) most of the bound hormone focused at pH 5.8 with greatly reduced levels of the form with an isoelectric point of 7.8. These results suggest that the acidic form of the receptor may be initially associated with a more basic protein(s) that dissociates from it under equilibrium focusing conditions.

When the receptor is initially solubilized from chromatin by salt extraction, it retains the same hormone binding properties that it possessed in chromatin. The binding affinity of the solubilized receptor for T_3 ($K_d \simeq 0.2$–1.0 n*M*) and T_4 ($K_d \simeq 2$–10 n*M*) may be somewhat lower than that measured in intact cells (K_d [T_3] $\simeq 29$ p*M*) or isolated nuclei (K_d [T_3] $\simeq 0.16$–0.21 n*M*). This result may be explained in part by differences in experimental conditions. Nevertheless, the relative affinities of a number of thyroid hormone analogs for binding to these solubilized proteins are similar to those measured *in vivo* or in isolated nuclei (Thomopoulos *et al.*, 1974; Jorgensen *et al.*, 1976).

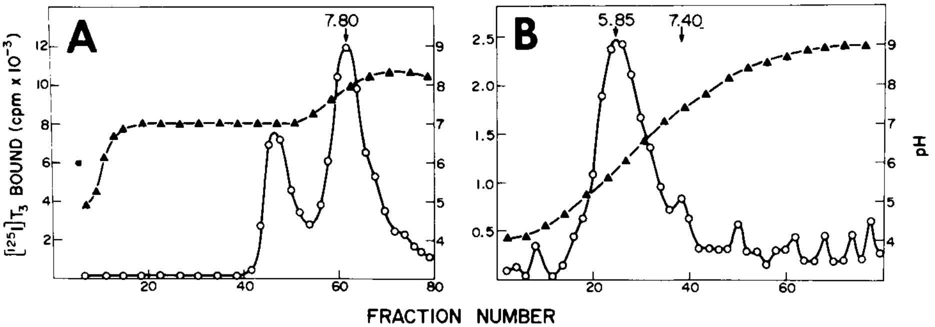

Fig. 6. Nonequilibrium (A) and equilibrium (B) isoelectric focusing of a nuclear thyroid hormone receptor-containing extract. Nuclear extracts were desalted on Sephadex G-25 and then electrofocused according to the method of O'Brien *et al.* (1976) except for nonequilibrium conditions in which case samples were electrofocused for 1 hour. $[^{125}I]T_3$-binding activity (○) and pH (▲) were measured in individual fractions. Data from N. L. Eberhardt and L. K. Johnson (unpublished).

Sephadex G-100 chromatography of receptor-containing crude nuclear extracts incubated with $[^{125}I]T_3$ yields two peaks of binding activity: an excluded peak corresponding to a molecular weight greater than 100,000 and an included peak corresponding to a molecular weight of 50,000–70,000 (Surks *et al.*, 1973; Latham *et al.*, 1976). The 50,000 form of the receptor is considered to be the major form of the receptor, since it is not observed in the presence of a large excess of unlabeled T_3 and contains most of the specifically bound radioactivity (Latham *et al.*, 1976). Also, the 50,000 form is the major form observed after partial purification of receptors by QAE-Sephadex chromatography (Latham *et al.*, 1976). The $[^{125}I]T_3$ bound to the higher molecular weight fractions is partially due to nonspecifically bound hormone, and partially due to species that bind T_4 with an affinity that is higher than T_3, and may contain some aggregated receptors (Latham *et al.*, 1976). Based on Sephadex G-100 chromatography and equilibrium density gradient centrifugation, it was calculated that the receptor has a frictional coefficient of 1.4 and is slightly asymmetric in shape (Latham *et al.*, 1976). These studies were conducted at high ionic strengths; at lower ionic strengths, the proteins appear to aggregate (N.L. Eberhardt, unpublished results).

Using ultracentrifugation in D_2O-containing gradients and Sepharose CL-6B chromatography, Perlman *et al.* (1982) determined that the salt extracted receptor from GH_1 cells has a molecular weight of 54,000, a Stokes radius of 3.3 nm, a density of 1.36 g/cm^3, and a frictional ratio of 1.212. Gruol and Kempner (1982) used radiation inactivation to show that the receptor has a single target size of 59,000 daltons in the solubilized 3.8 S form, in the 5–6 S micrococcal nuclease-released form, and in sonicated chromatin fragments that sedimented faster than 30 S. These studies indicate that the 59,000 dalton form of the receptor is the smallest functional unit required to bind thyroid hormones. Nikodem *et al.* (1980) found that SDS polyacrylamide gel electrophoresis of the nuclear receptor labeled with *N*-bromoacetyl derivatives of $[^{125}I]T_3$ or $[^{125}I]T_4$ showed one major radioactive component with a molecular weight of 56,000.

V. INTERACTION OF THE SOLUBILIZED NUCLEAR RECEPTOR WITH THYROID HORMONE

There are generally equivalent concentrations of T_3 and T_4 binding sites in solubilized extracts from either rat liver (Latham *et al.*, 1976; Eberhardt *et al.*, 1979a) or GH_1 cell nuclei (Samuels and Tsai, 1973) as measured by Scatchard analysis of the binding data (Fig. 7). This suggests that T_3 and T_4 may be bound by the same proteins in the nuclear extract. A number of laboratories have demonstrated that $[^{125}I]T_3$ binding by nuclear extracts is inhibited by unlabeled T_4 (Samuels and Tsai, 1973; DeGroot and Strausser, 1974; Oppenheimer *et al.*, 1974; Koerner *et al.*, 1975; Latham *et al.*, 1976). Moreover, the binding of $[^{125}I]T_4$ by nuclear extracts is completely inhibited by unlabeled T_3 and T_4 (Latham *et al.*, 1976; Eberhardt *et al.*, 1979a) and T_3 is the more avid competitor (Eberhardt *et al.*, 1979a) (see Fig. 13A). Thus, both hormones are bound by the same protein(s), which has a higher affinity for T_3 than T_4.

The importance of sulfhydryl groups for the binding of T_3 to the receptor has been demonstrated in two different laboratories. DeGroot *et al.*, (1974) have shown that T_3 binding is inhibited by *p*-chloromercuribenzoate. Eberhardt *et al.*

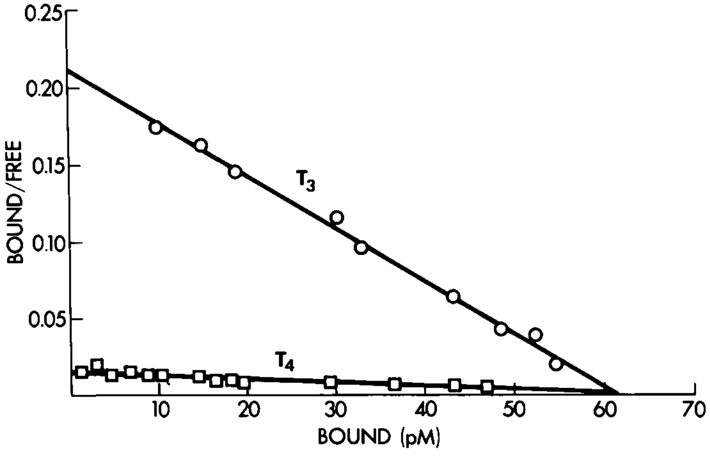

Fig. 7. Scatchard analysis of the binding of $[^{125}I]T_3$ (○) and $[^{125}I]T_4$ (□) by a crude nuclear extract derived from purified rat liver nuclei. Isolation and assay of this solubilized receptor preparation were performed according to Latham *et al.* (1976). Data reprinted from Baxter *et al.* (1979).

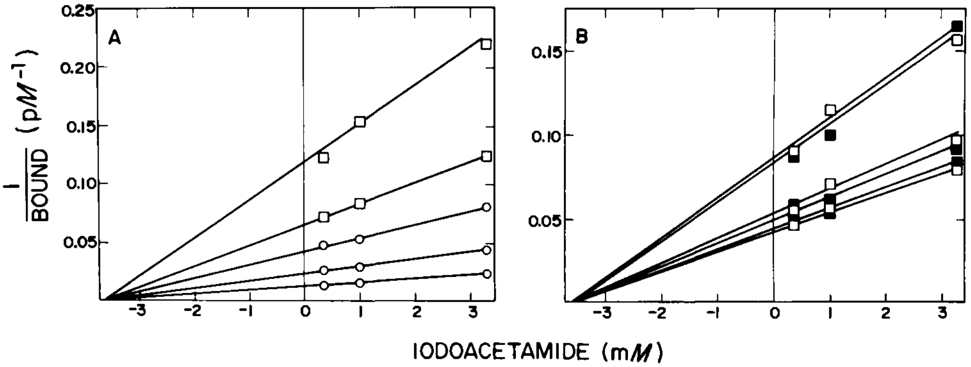

Fig. 8. Dixon analysis of iodoacetamide inhibition of receptor hormone-binding activity. (A) Dixon analysis of the inhibition of the binding of $[^{125}I]T_3$ (○) and $[^{125}I]T_4$ (□) to the initial chromatin extract from rat liver nuclei by iodoacetamide. (B) Dixon analysis of the inhibition of the $[^{125}I]T_4$-binding of control (□) and heated (■; 50°C for 10 minutes) chromatin extracts from rat liver nuclei by iodoacetamide. Iodoacetamide was included in the binding assays at the indicated concentrations, the concentrations of $[^{125}I]T_3$ and $[^{125}I]T_4$ were kept constant at 1 n*M*, and the receptor concentration was varied. Linear regression analysis of the data for the initial extract indicates that the inhibition constant was 3.87 ± 0.25 m*M* (SD, $N = 3$) when measured with T_3 and 3.53 ± 0.15 m*M* (SD, $N = 5$) when measured with T_4; the inhibition constant was 3.45 ± 0.25 (SD, $N = 3$) when measured with T_4 in the heated extract. Data reprinted from Eberhardt *et al.* (1979a).

(1979a) found that T_3 and T_4 binding is also inhibited to iodoacetamide (Fig. 8A). Thus, cysteine residues appear to be involved in maintenance of receptor integrity. Additionally, as shown in Fig. 8A, the inhibition constant for iodoacetamide was identical for T_3 or T_4 binding (Eberhardt *et al.*, 1979a), a finding that is consistent with the idea that T_3 and T_4 are bound by the same protein.

The pH optima for T_3 and T_4 binding are 7.6–8.5 and 6.0, respectively (Latham *et al.*, 1976). Based on the difference in the pK_a of the phenolic hydroxyl generated by the 3′- and 3′,5′-halogen analogs of thyroid hormones, it has been suggested that the ionization of the phenolic hydroxyl may account in part for the decreased binding of T_4 relative to T_3 at physiological pH (Latham *et al.*, 1976). However, 3′- and 3′,5′-methyl derivatives are biologically active thyroid hormone analogs but exhibit little effect on the pK_a of the phenolic hydroxyl group. Accordingly, the presence of a 5′ substituent on the hormone has been postulated to interfere more directly with binding by the receptor and the role of the ionization of the phenolic hydroxyl is uncertain (Jorgensen, 1978). Furthermore, recent evidence (Eberhardt *et al.*, 1979a) suggests that pH may also influence receptor properties. As shown in Fig. 9B, Scatchard analysis of $[^{125}I]T_3$ and $[^{125}I]T_4$ binding by the receptor at pH 6.0 indicates a greatly reduced high affinity T_3-binding capacity relative to the T_4-binding capacity whereas at pH 7.6 the binding capacities for T_3 and T_4 are identical (Fig. 9A) (Eberhardt *et al.*, 1979a). Nevertheless, at pH 6.0 unlabeled T_3 was still capable

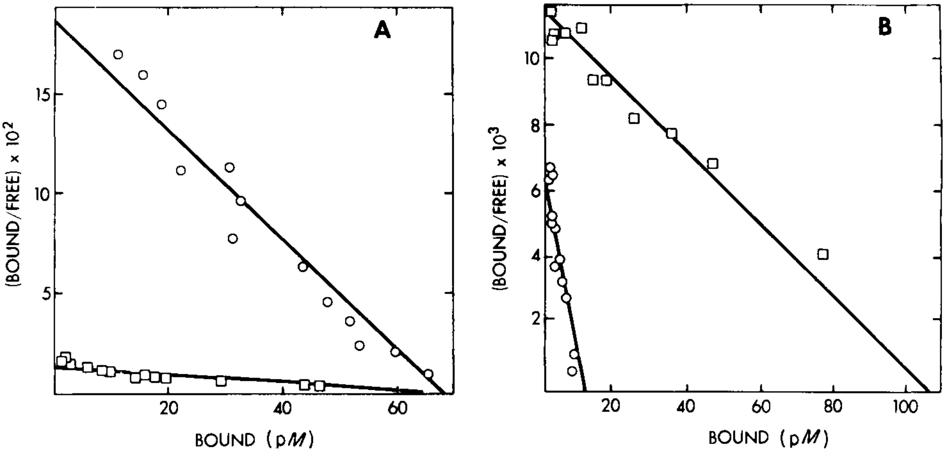

Fig. 9. Scatchard analysis of binding of [^{125}I]T_3 (○) and [^{125}I]T_4 (☐) by the initial chromatin extract from rat liver nuclei at pH 7.6 (A) and pH 6.0 (B). Samples of the chromatin extracts were dialyzed against buffer, pH 7.6 or pH 6.0, for 12 hours at 4°C. Following dialysis, the samples were centrifuged and aliquots were assayed at pH 7.6 or 6.0. The equilibrium dissociation constants for [^{125}I]T_3 and [^{125}I]T_4 binding by the nuclear extracts at pH 7.6 are 0.37 and 4.6 n*M*, respectively. The corresponding values at pH 6.0 are 2.1 and 9.3 n*M*, respectively. Data reprinted from Eberhardt *et al.* (1979a).

of displacing all the [^{125}I]T_4 bound by the receptor. Under these acidic conditions, however, the majority of the T_3 binding occurred with an affinity that was 10-fold lower than that of T_4. Thus, the possibility that pH affects the receptor directly should be considered.

Figure 10 shows that binding of both T_3 and T_4 to the solubilized receptor is diminished in a parallel fashion as the ionic strength is increased above 0.2 *M* (Eberhardt *et al.*, 1979a). Chloride was more effective than sulfate in promoting the loss of binding activity. Scatchard analysis of the effect of ionic strength on hormone binding (Fig. 11) indicates that the affinities of both T_3 and T_4 for binding to the receptor are decreased with higher ionic strength, while there is no major effect on the binding capacity.

A thyroid hormone binding protein with hormone binding characteristics that differ from those of the receptor appears to be generated during QAE-Sephadex chromatography of crude nuclear extracts containing the receptor (Latham *et al.*, 1976). This binding protein appears to exhibit a high affinity for T_4 (about the same as the receptor for T_4) and a low affinity for T_3. This is not seen in the starting material, in which all the T_4 binding is by sites that exhibit a higher affinity for T_3 (Fig. 13A).

In order to study this curious effect systematically, a number of experimental manipulations of the initial nuclear extracts were performed and their influences on the binding of T_3 and T_4 were examined. Figure 12 shows that when crude

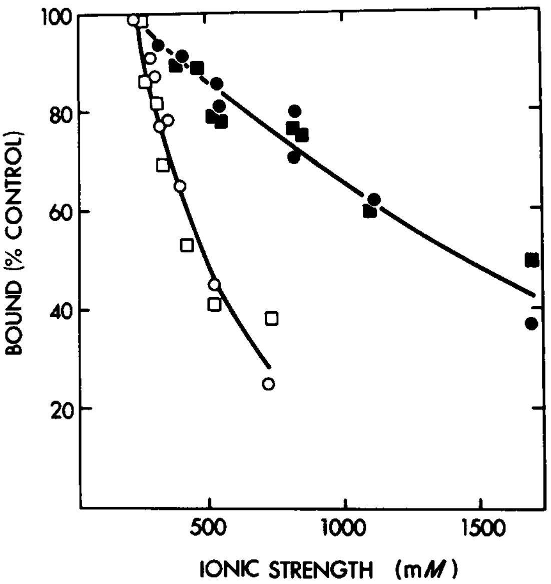

Fig. 10. Effect of increasing ionic strength on the binding of $[^{125}I]T_3$ (○, ●) and $[^{125}I]T_4$ (□, ■) by the initial chromatin extract from rat liver nuclei. The initial buffer composition was 50 m*M* sodium phosphate (pH 7.6), 20 m*M* ammonium sulfate, 1 m*M* EDTA, 0.2 m*M* dithiothreitol, and the ionic strength was increased by adding aliquots of 1 *M* KCl (○, □) or 1 *M* ammonium sulfate (●, ■). Data reprinted from Eberhardt *et al.* (1979a).

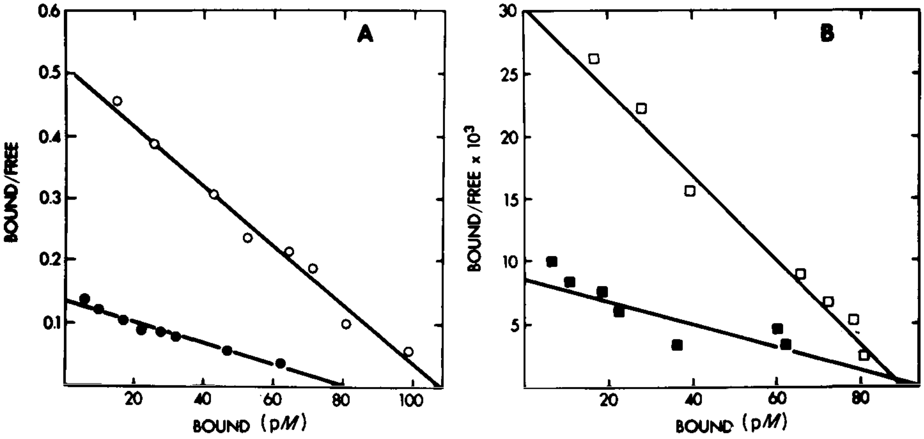

Fig. 11. Scatchard analysis of the binding of $[^{125}I]T_3$ (A) and $[^{125}I]T_4$ (B) by the initial chromatin extract from rat liver nuclei at an ionic strength of 206 m*M* (○, □) and 706 m*M* (●, ■). Buffer conditions were as described in the legend to Fig. 9 and KCl was used to increase ionic strength. Data reprinted from Eberhardt *et al.* (1979a).

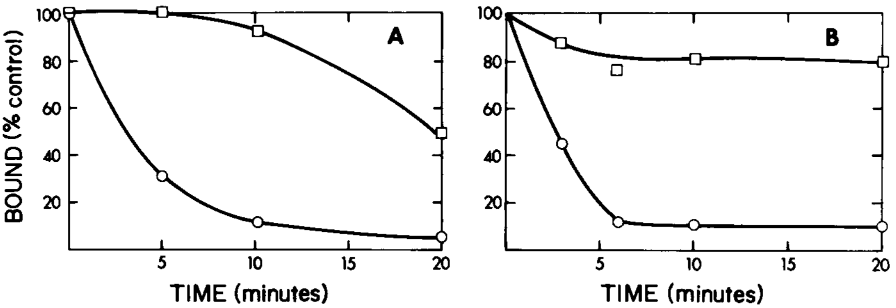

Fig. 12. Effect of heating on binding of $[^{125}I]T_3$ (○) and $[^{125}I]T_4$ (□) by solubilized rat liver (A) and GH_3 cell (B) nuclear thyroid hormone receptors. Aliquots of the heated extracts were removed at the specific times, chilled on ice, and centrifuged prior to assay to remove precipitated protein. The binding is plotted as the percent of binding in the unheated control. Data reprinted from Eberhardt *et al.* (1979a).

extracts were heated at 50°C for various times, there was a marked loss of T_3-binding activity (Eberhardt *et al.*, 1979a). Scatchard analysis of such extracts after 10 minutes of heating indicated that the number of high affinity T_4-binding sites and their affinity for T_4 were essentially unaltered; however, the concentration of high affinity T_3-binding sites was reduced by 90%. Competition studies of the binding of $[^{125}I]T_4$ in the presence of increasing concentrations of unlabeled T_3 and T_4 after heating indicated that the T_4-binding sites still bound T_3, but with an affinity that was 1000-fold lower than that of the receptor in the initial extract (Fig. 13A and B). As discussed previously (Section V), qualitatively similar results were observed when extracts were acidified at pH 6.0 (Eberhardt *et al.*, 1979a) or diluted (Eberhardt *et al.*, 1979b). The observed

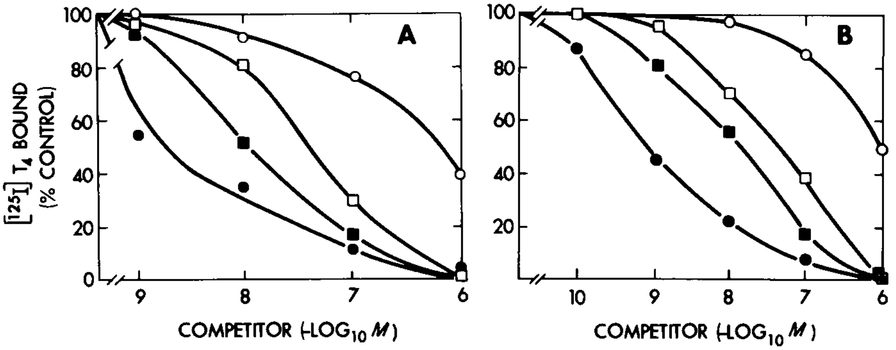

Fig. 13. Competition for $[^{125}I]T_4$ binding by unlabeled T_3 (○, ●) and T_4 (□, ■) in the initial nuclear extracts (filled symbols) and in an extract that was heated at 50°C for 10 minutes (open symbols). The receptor-containing extracts in A were from rat liver nuclei. Those in B were obtained from cultured anterior pituitary cell (subline GH_3) nuclei. Reprinted from Eberhardt *et al.* (1979a).

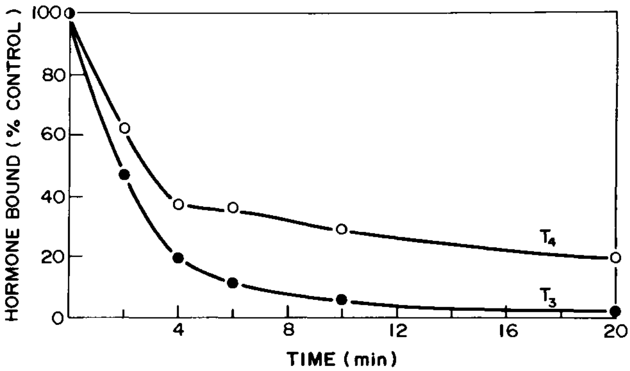

Fig. 14. Effect of heating on binding of $[^{125}I]T_3$ (●) and $[^{125}I]T_4$ (○) by the included peak from Sephadex G-100 chromatography of rat liver crude nuclear extracts. Samples were incubated in a water bath at 50°C, aliquots were removed at the specified, times and treated as described in Fig. 12. Data from J. Apriletti (unpublished).

changes in binding properties by heat or acidification cannot be explained by the loss of a binding species with preferentially binds T_3 and T_4, as the original T_3-binding sites all bound T_4 with as high an affinity as the residual T_4-binding sites and there was no decrease in the concentration of these high affinity T_4-binding sites. The data could be explained, however, if there were a simultaneous generation of new T_4-binding sites and loss of preexisting T_4-binding sites.

Although the simultaneous generation of a new binding species after heating was considered unlikely (Eberhardt *et al.*, 1979a), this possibility was examined further in a recent study (Apriletti *et al.*, 1982). When crude nuclear receptor prepared by the procedure of Eberhardt *et al.*, 1979a, was purified 6-fold by Sephadex G-100 chromatography prior to the heat treatment, both T_3- and T_4-binding activities were inactivated by heating at 50°C (Fig. 14).

In these studies the nuclear extracts were also prepared with slight modifications of the original procedure (Eberhardt *et al.*, 1979a). The modifications included increasing the sonication time for releasing the receptor from chromatin and freezing the rat livers with dry ice instead of liquid nitrogen.

As shown in Fig. 15, heat treatment of this extract (50°C for various times) resulted in a 3-fold increase in T_4 binding, whereas the T_3-binding activity was rapidly destroyed. When this crude extract was separated into two fractions by Sephadex G-100 chromatography, both T_3 and T_4 binding in the included fraction (containing the 50K dalton receptor) decreased in parallel during heating (Fig. 15). By contrast, heat treatment of the excluded fraction (containing proteins >100,000 daltons), which contained most of the protein but was depleted with respect to the high affinity receptor, increased both T_3 and T_4 binding after heating (Fig. 15). Scatchard analysis of T_3 and T_4 binding by the crude nuclear extract before and after heat treatment indicated that the high affinity, limited

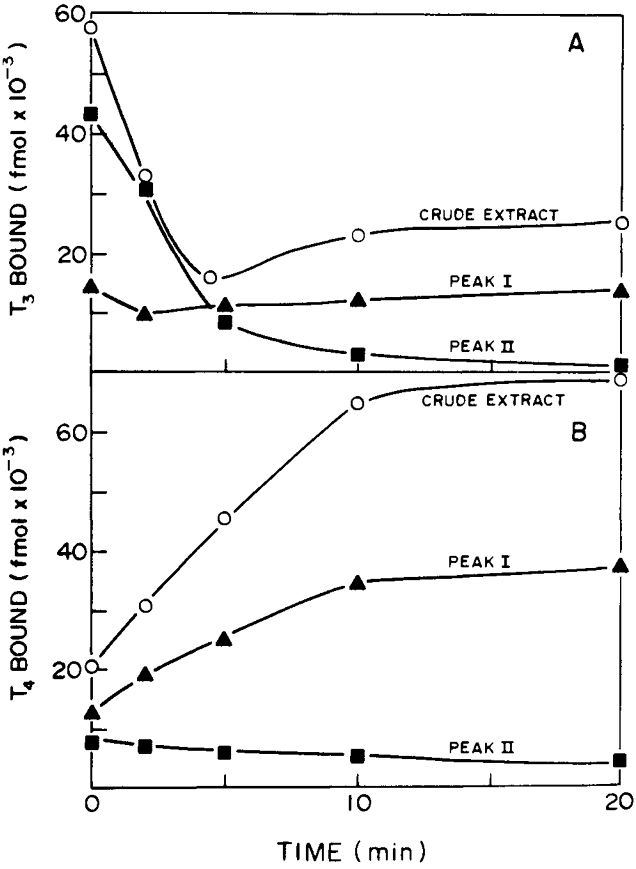

Fig. 15. Effect of heating on binding of $[^{125}I]T_3$ (A) and $[^{125}I]T_4$ (B) by the crude nuclear extract (○), and by the excluded peak (peak I) (▲) and the included peak (peak II) (■) from Sephadex G-100 chromatography of nuclear extract prepared by sonicating nuclei 3 × 15 seconds in buffer containing 0.2 *M* ammonium sulfate. Samples were treated as described in Fig. 14. Data from J. Apriletti (unpublished).

capacity binding sites for both T_3 and T_4 decreased markedly after heating. The T_4 binding that remained was primarily of low affinity and high capacity. These results suggested that during heat treatment the high affinity receptors were inactivated, and at the same time nonspecific low affinity, high capacity T_4-binding sites were generated or exposed.

In further studies by Eberhardt *et al.* (1979b), it was found that the high affinity T_3-binding activity that was lost as a result of dilution could be reconstituted by the addition of a chromatin extract that had been heated at 60°C to destroy endogenous T_3- and T_4-binding activity. The factor responsible for stimulation of T_3-binding activity was shown to be a protein, since its activity was destroyed by proteases but not RNase or DNase. The factor also appeared to be a specific protein since a number of acidic or basic proteins, lysozyme, ovalbumin, cytochrome *c*, and poly-L-lysine, or DNA, RNA, or various macromolecular ligands had no influence on T_3-binding activity.

It was subsequently discovered that the addition of purified histone fractions to partially purified receptor also resulted in a stimulation of T_3-binding activity. As shown in Fig. 16, core histones (H2A + H2B + H3 + H4) stimulated T_3-binding activity at much lower concentrations than a preparation of histone H_1 containing no identifiable core histones (Eberhardt *et al.*, 1979b). In addition, the loss of T_3-binding activity that occurred upon dilution was prevented by the addition of core histones. Thus, it was proposed that a histone or histone-like species acted as a factor that conferred high affinity T_3-binding activity on the receptor in chromatin.

In the more recent studies (Apriletti *et al.*, 1982) this phenomenon was reexamined using a receptor preparation that had been purified more than 500-fold by a combination of Sephadex G-100, affinity, and DEAE-Sephadex chromatogra-

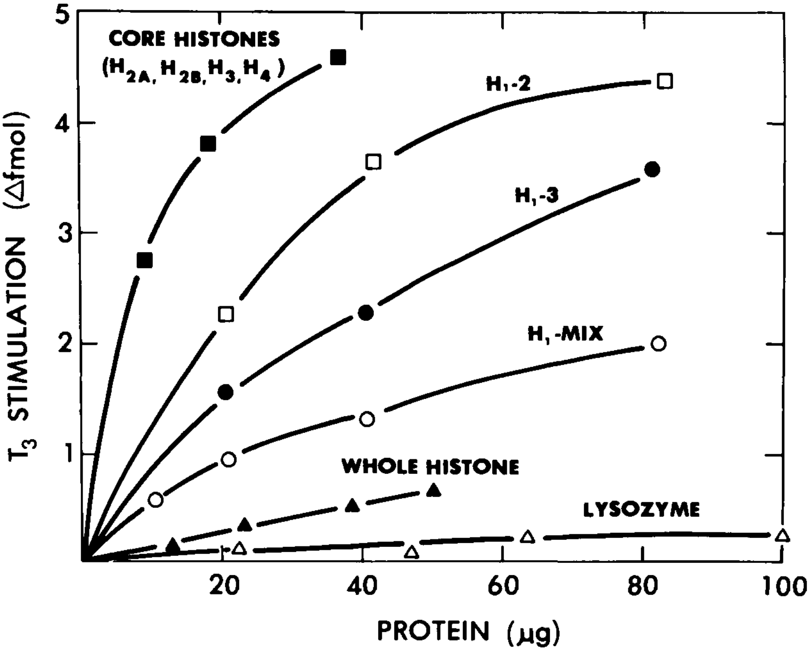

Fig. 16. Reconstitution of [^{125}I]T_3-binding activity in the Sephadex G-100 included receptor fraction by core histones (■) ($H_{2A} + H_{2B} + H_3 + H_4$) purified H_1 subfractions (□, ●) (gift from R. D. Cole, Berkeley, California), a mixture of H_1 subfractions (○) (5% perchloric acid extract of whose histone), whole histone (▲), and lysozyme (△). Reprinted from Eberhardt *et al.* (1979b).

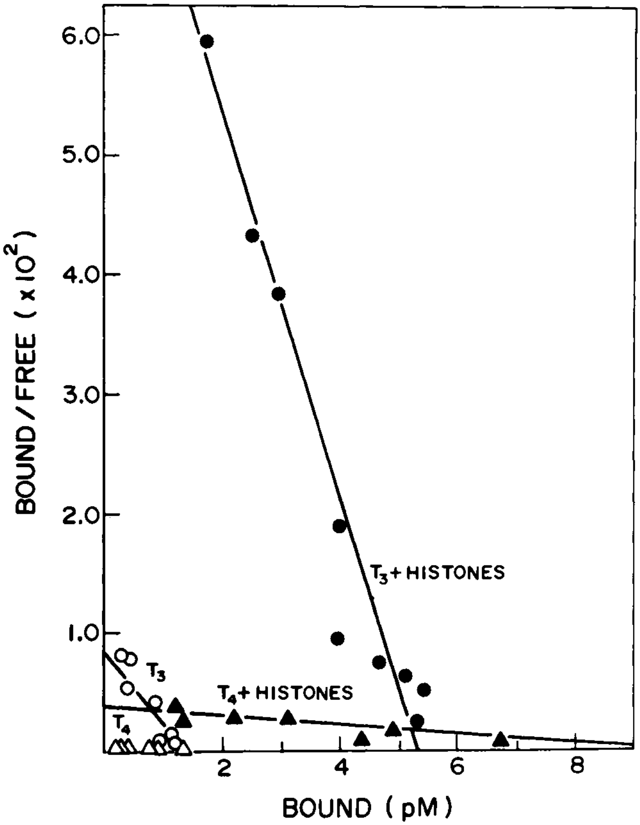

Fig. 17. Scatchard analysis of the binding of [^{125}I]T$_3$ (○, ●) and [^{125}I]T$_4$ (△, ▲) by the Sephadex G-100 purified receptor in the presence (filled symbols) or absence (open symbols) of core histones (50 μg/ml), Data from Y. David-Inouye (unpublished).

phy (Apriletti *et al.*, 1981). It was observed that addition of core histones stimulated both T$_3$ and T$_4$ binding by the affinity purified receptor. In addition, Scatchard analysis of the binding of T$_3$ and T$_4$ by diluted Sephadex G-100 purified receptor in the presence or absence of core histones demonstrated that the number of T$_3$- and T$_4$-binding sites were equally increased by the addition of histones, with no significant change in the equilibrium dissociation constants (Fig. 17).

In experiments investigating the nature of this stimulation of hormone binding, it was found that treatment of the Sephadex G-25 assay columns with histones prior to use in the hormone-binding assay was sufficient to produce the apparent stimulation in T$_3$ binding. Pretreatment of the columns with histones also prevented the loss of binding activity when receptor-containing preparations at low protein concentrations were filtered over Sephadex columns (Fig. 18). Thus, a possible explanation for these results is that the receptors are binding to Sephadex (accounting for the loss in binding activity) and that this binding is prevented by incubation of the Sephadex with histone or by inclusion of histones in the reaction mixture. Interestingly, core histones were more efficient than any other protein for reducing receptor losses during Sephadex G-25 filtration.

When the effects of core histones on the affinity purified receptors were examined, it was found that the histones also act to stabilize the T_3-binding capacity of the affinity purified receptor as well as prevent its binding to Sephadex G-25. Incubation of the affinity purified receptor at 25°C in the absence of histones resulted in loss of more than 50% of the binding activity within 1 hour; greater than 80% of the binding activity was lost after 4 hours. This loss was prevented by the addition of core histones, which were more effective than any other proteins tested for stabilizing the receptor. When the individually purified core histones (H2A, H2B, H3, or H4) were tested for their ability to stabilize the affinity purified receptor, or to block binding of diluted Sephadex G-100 purified receptor to the Sephadex G-25 columns, no significant differences were seen among the various histones. When the core histone fractions were added directly to the Sephadex G-100 purified receptor during the T_3-binding incubation, H3 and H4 were less effective than H2A and H2B for stimulating the T_3 binding. However, interpretation of these results was complicated by high nonspecific T_3 binding in the individual core histone fractions (Apriletti *et al.*, 1982).

The apparent specificity for the stabilization of receptor by core histones

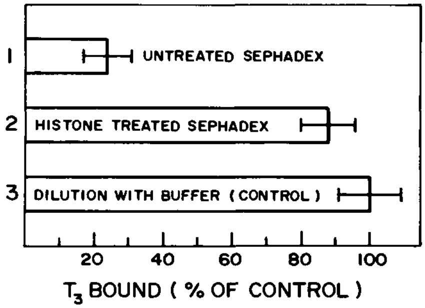

Fig. 18. Prevention of receptor loss by pretreatment of Sephadex G-25 assay columns with core histones. For samples 1 and 2, 0.4 ml of Sephadex G-100 purified receptor was filtered over a 2 ml bed volume of Sephadex G-25 in a Pasteur pipette, and 0.8 ml of eluate was collected as in a standard T_3-binding assay (Latham *et al.*, 1976). The column for sample 2 was pretreated by washing with buffer containing core histones, followed by washing with buffer until no detectable protein was being eluted. The column for sample 1 was only washed with buffer before applying the receptor. Instead of being filtered on Sephadex G-25, as a control, sample 3 was diluted to 0.8 ml by the addition of 0.4 ml buffer. Then, aliquots from all three conditions were assayed for T_3 binding using histone treated columns for all assays. Data from J. Apriletti (unpublished).

suggested that there may be a selective interaction between the receptor and core histones. In order to test this possibility, Sepharose gels were prepared containing immobilized core histones, ovalbumin, or insulin. The receptor was capable of binding to the core histone–Sepharose matrix, but not ovalbumin–Sepharose or insulin–Sepharose matrices. Binding to histone–Sepharose could be prevented by the addition of free core histones or 0.5 *M* NaCl. These results indicate that the receptors do bind to histones; however, they do not indicate whether this binding occurs by specific interactions or by nonspecific ionic interactions.

Another conceivable indication that the thyroid hormone receptor interacts with histones is suggested in studies using butyrate treatment of cells. Thus, treatment of GH_1 cells (Samuels *et al.*, 1980) or GH_3 cells (Eberhardt *et al.*, 1982) with *n*-butyrate, which increases the acetylation of histones H_3 and H_4, reduces the concentration of nuclear T_3-binding sites under conditions in which there is very little inhibition of total protein synthesis. Furthermore, this manipulation does not decrease the concentration of glucocorticoid receptors. However, in spite of the known effects of butyrate to increase the acetylation of histones by inhibiting a deacetylase activity, it remains to be established that the effect on receptor depletion is mediated by histone acetylation and not another effect of butyrate. For example, butyrate treatment of GH_3 cells (Eberhardt *et al.*, 1982) leads to alterations in the rate of synthesis of a number of specific proteins as measured by two-dimensional gel electrophoresis of $[^{35}S]$methionine pulse-labeled GH_3 cell proteins.

Measurements of the rate of growth hormone synthesis in butyrate-treated GH_3 cells by two-dimensional gel electrophoresis of $[^{35}S]$methionine pulse-labeled proteins indicated that the T_3-mediated induction of growth hormone synthesis was not altered by butyrate treatment. Under the conditions of these experiments the intranuclear thyroid hormone receptor concentration was reduced to 20% of the control values. These data could be interpreted to suggest that all the receptors present in GH_3 cells are not involved in mediating the hormone's biological responses. It could be that there are distinct classes of receptors with different butyrate sensitivities and that only a subclass of receptors is involved in the pathway of thyroid hormone action; this class could be physically distinct or else could be compartmentalized for instance through its association with specific sites in the chromatin.

VI. PURIFICATION OF THE NUCLEAR THYROID HORMONE RECEPTOR

Partial purification of the receptor has been obtained by means of molecular sieve chromatography (Latham *et al.*, 1976; Silva *et al.*, 1977) DNA-Sepharose chromatography (Torresani and Anselmet, 1978), high pressure liquid chromatography (Nikodem *et al.*, 1980) and, most recently, by affinity chromatography (Latham *et al.*, 1981; Apriletti *et al.*, 1981).

Latham *et al.* (1976) used chromatography on Sephadex G-100 and QAE-Sephadex to partially purify the receptor. The receptor eluted from Sephadex G-100 in an included fraction corresponding to a molecular weight of 50,000. Up to 6-fold purification has been obtained using this technique (Eberhardt *et al.*, 1979b). Latham *et al.* (1976) also used chromatography on the strongly basic anion exchange resin QAE-Sephadex to obtain a twofold purification of the receptor; the receptor was eluted from the resin stepwise with 0.2 *M* ammonium sulfate.

Silva *et al.* (1977), using a linear NaCl gradient to elute the receptor, obtained a 10- to 25-fold purification with DEAE-Sephadex chromatography. They found that the binding of T_3 to the receptor altered its chromatographic mobility; receptor labeled with $[^{125}I]T_3$ prior to chromatography eluted at 0.15 *M* NaCl, whereas unoccupied receptor eluted at 0.18 *M* NaCl. The reason for this change in mobility is unknown, although it was proposed that conformational changes of the receptor induced by T_3 binding could account for the differential elution (Silva *et al.*, 1977). In the procedure developed by Silva *et al.* (1977), dialysis of the crude nuclear extract resulted in the precipitation of nonspecific proteins, yielding a 2- to 5-fold further increase in specific binding activity prior to chromatography. Combined with the purification obtained during DEAE-Sephadex chromatography, this resulted in a net receptor purification of 60- to 125-fold. Torresani and Anselmet (1978) were able to obtain an additional 4-fold purification of the DEAE-Sephadex purified material by chromatography on DNA-Sepharose. Under these conditions the receptor eluted at approximately 0.2 *M* KCl. Nikodem *et al.* (1980) have used high pressure liquid chromatography to achieve a 100-fold purification of the receptor. The purification obtained by these techniques is still far short of the 25,000- to 50,000-fold purification required to achieve homogeneity.

In order to design affinity support matrices suitable for receptor purification, certain structural requirements for the binding of thyroid hormone to the receptors were determined (Latham *et al.*, 1981). These studies indicated that the 4′-hydroxyl and carboxyl groups of T_3 were involved in hormone binding, but that loss of the amino group did not decrease binding to the receptor. This suggested that linkage of T_3 to an affinity matrix through the amino group would produce the most efficient affinity adsorbent. To test this, amine substituted hormone analogues were prepared by reacting D-T_3 or L-T_3 with the diactivated succinimide esters of either glutaric or pimelic acid (Fig. 19A).

The ability of T_3 receptors to bind these derivatives is shown in Fig. 20. Confirming earlier reports (Oppenheimer *et al.*, 1973), L-T_3 binds to the receptor more avidly than does D-T_3. Also L-T_4 binds with a somewhat lower affinity than either of the T_3 isomers. The amino-substituted derivatives tested can com-

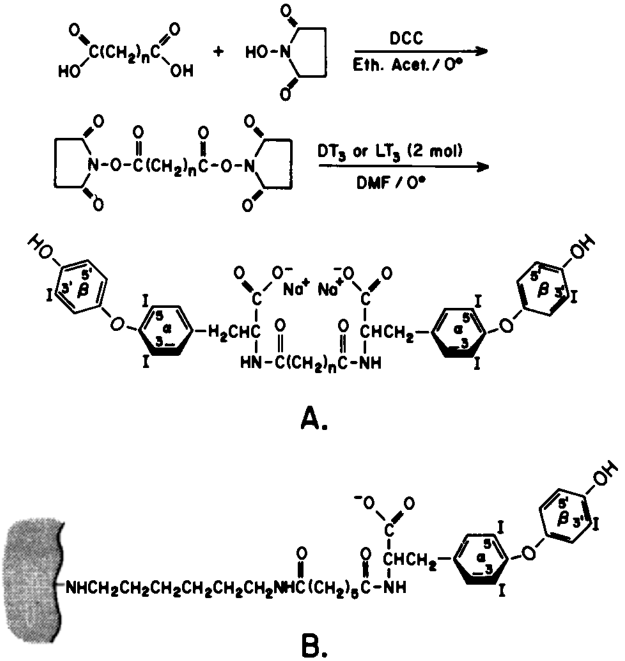

Fig. 19. Scheme for the synthesis of amino-substituted thyroid hormone analogs (A) and affinity gel matrices (B). Thyroid hormone "monomers" and "dimers" were prepared from the D- and L-stereoisomers of T_3 (denoted in the figure as DT_3 and LT_3) using the diactivated succinimide esters of either glutaric or pimelic acids (Anderson *et al.*, 1964). D-T_3 or L-T_3 were coupled through their amino groups to the terminal primary amino groups of AH-Sepharose (Pharmacia) using the diactivated succinimide ester derived from glutaric acid. Data reprinted from Baxter *et al.* (1979).

petitively inhibit the binding of T_3 by the receptors, but substitution of the bulky group at this position diminishes their effectiveness. Surprisingly, in contrast to the parent hormones, it was found that the amino-substituted derivatives of D-T_3 bind to the receptor more tightly than do the analogous derivatives of L-T_3. This reversal in the enantiomeric specificity has also been observed by Somack *et al.* (1982) with the binding of a variety of *N*-acyl derivatives of T_3 and T_4 to TBPA. These seemingly paradoxical results might be explained by the model shown in Fig. 21. For L-T_3 the amino group decreases the binding interaction either by unfavorable charge interactions (as indicated in Fig. 21) or by unfavorable steric interactions. This could explain the relatively higher affinities of deaminated hormone analogs, such as triac, for the receptor (Oppenheimer *et al.*, 1973). In the case of D-T_3, either the charge interactions (Fig. 21) or steric interactions are less favorable than for L-T_3, thereby leading to a relative decrease in the binding affinity of D-T_3 for the receptor (Oppenheimer *et al.*, 1973). However, when a bulky substituent is coupled to the amino groups, there may be greater steric

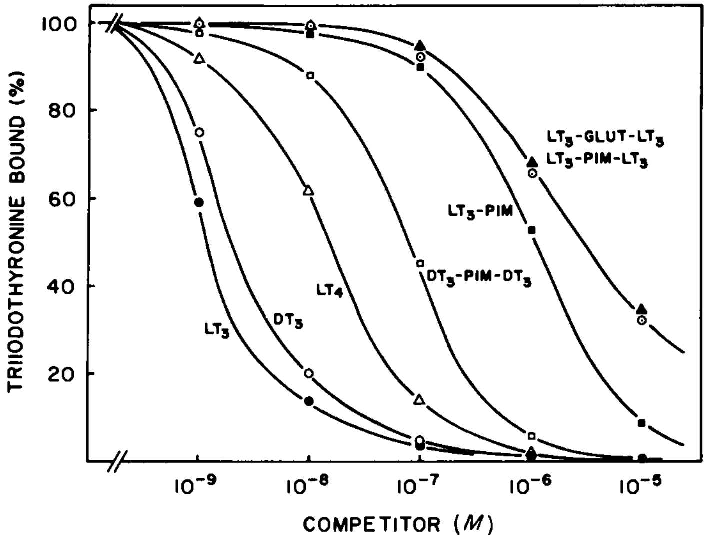

Fig. 20. Competition for [^{125}I]T_3 binding in the nuclear extract (Latham *et al.*, 1976) by unlabeled T_3 (●), D-T_3 (DT_3, ○), T_4 (△), D-T_3 pimelate dimer (□), L-T_3 (LT_3) pimelate mononer (■), L-T_3 pimelate dimer (○), and L-T_3 glutarate dimer (▲). Data reprinted from Baxter *et al.* (1979).

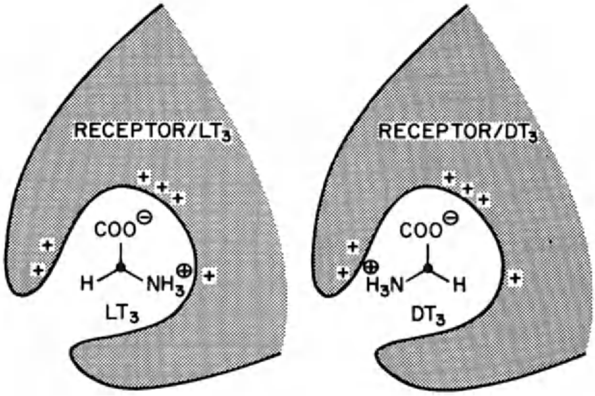

Fig. 21. Model of the receptor binding site indicating receptor-thyroid hormone interactions. Normally, L-T_3 (LT_3) binds to the binding site with a higher affinity than D-T_3 (DT_3). Substitution of a bulky substituent on the amino group, however, reverses the sterospecificity of this interaction such that D-T_3 derivatives bind with a higher affinity than L-T_3 derivatives. Because deaminated thyroid hormone analogs bind with equal or greater avidity to the receptor than the parent hormones (Oppenheimer *et al.*, 1973), the interaction of the amino group with the receptor is viewed as a relatively negative interaction (shown here as a charge interaction) for L-T_3. D-T_3 binds with lower affinity than L-T_3 owing to steric restriction and/or increased negative charge interactions (shown on the right). Substitution of the amino group increases steric restriction. However, in this case there is a marked preference for the D-T_3 derivative owing either to more favorable charge and/or to steric interactions. Reprinted from Baxter *et al.* (1979).

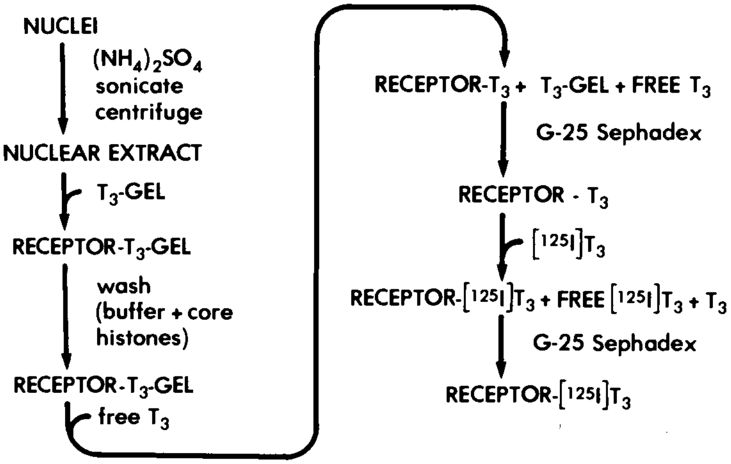
NUCLEI
$(NH_4)_2SO_4$
sonicate
centrifuge
NUCLEAR EXTRACT
T_3-GEL
RECEPTOR-T_3-GEL
wash
(buffer + core
histones)
RECEPTOR-T_3-GEL
free T_3
RECEPTOR-T_3 + T_3-GEL + FREE T_3
G-25 Sephadex
RECEPTOR - T_3
$[^{125}I]T_3$
RECEPTOR-$[^{125}I]T_3$ + FREE $[^{125}I]T_3$ + T_3
G-25 Sephadex
RECEPTOR-$[^{125}I]T_3$

Fig. 22. Scheme for the purification of the nuclear receptor by affinity chromatography. Details are presented in the text. Reprinted from Baxter *et al.* (1979).

hindrance when the molecule is in the L than in the D configuration. Thus, the affinity for the receptor of the D-T_3 derivative is higher than that of the L-T_3 derivative.

Using the same synthetic techniques, affinity gels were prepared by linking thyroid hormone via its amino group and the diactivated ester of glutaric acid to the primary amino groups of diaminohexane-Sepharose (Fig. 19B). These gels bound solubilized nuclear receptor, and the receptor adsorption efficiency of the various matrices paralleled the binding affinity of the thyroid hormone analogue used in the synthesis of the matrix (Baxter *et al.*, 1979; Latham *et al.*, 1981). Binding of receptor to these gels is biospecific, that is, not due to nonspecific uptake, since excess free hormone could block receptor adsorption by the gel.

The scheme which has been developed for using this affinity gel to purify the receptor (Apriletti *et al.*, 1981) is presented in Fig. 22. After the receptors were solubilized from nuclei and bound to the gel, the gel was washed with a high and then a low ionic strength buffer. Receptors could then be eluted by incubating the gel with free T_3. The T_3-binding capacity of the affinity purified receptor was measured by an exchange assay using $[^{125}I]T_3$; after most of the unlabeled T_3 in the affinity gel eluate was removed by chromatography on Sephadex G-25, the small amount of nonradioactive T_3 remaining was displaced from the receptor by exchange with $[^{125}I]T_3$. The recovery of the receptors from the affinity gel was stimulated approximately 5-fold by the addition of purified core histones (H2A + H2B + H3 + H4, themselves lacking T_3-binding activity) to the wash and elution buffers. Other proteins, including ovalbumin and lysozyme, did not enhance receptor recovery. In the presence of 25–100 μg/ml core histones, recovery of the receptor bound to the gel was 10–40% with a purification of more than 500-fold (Table I).

In the studies of the ability of unlabelled hormones to compete with $[^{125}I]T_3$ for binding to the affinity purified receptor, the rank order of competitive inhibition of $[^{125}I]T_3$ binding was $T_3 \geq$ isopropyl $T_2 > T_4 >$ reverse T_3 (Fig. 23). This is identical to that of the crude nuclear thyroid hormone receptor. Importantly, this competition study and the finding that unlabeled T_3 bound to the eluted receptors can be exchanged by $[^{125}I]T_3$ demonstrates that the affinity purified material reversibly binds hormones, thereby permitting a more detailed study of the binding characteristics using a purer form of the receptor. Scatchard analysis of hormone binding by the affinity-purified material indicated apparent K_d's of 50 pM to T_3 and 1 nM for thyroxine, which are approximately the same as those of the initial nuclear extract (Fig. 24). The chromatographic properties of the

TABLE I

Purification of Rat Liver and Sheep Liver Nuclear Receptor[a]

Experiment	Specific activity (pmol/mg protein)	Total purification (fold)	Total T_3 binding (pmol)	Yield for step (%)	% initial extract
1. Rat liver					
Nuclear extract	0.43		46.5		(100)
Sephadex G-100	1.4	3.3	22.4	48	48
Affinity gel			4.02	18	9
DEAE-Sephadex	220	510	2.5	62	5
2. Rat liver					
Nuclear extract	0.43		49.9		(100)
Sephadex G-100	1.22	2.8	21.2	42	42
Affinity gel			3.24	15	6.5
DEAE-Sephadex	247	570	1.99	61	4
3. Sheep liver					
Nuclear extract	0.094		3.4		(100)
Sephadex G-100	0.18	1.9	0.78	23	23
Affinity gel			0.115	15	3.4
DEAE-Sephadex	44	460	0.044	37	1.3

[a] In experiments 1 and 2, 45 ml of rat liver nuclear extract was chromatographed on Sephadex G-100, and the peak fractions (135 ml) were applied to a T_3 affinity gel (10 ml bed volume). The affinity columns were washed, receptor was eluted, and 5 ml samples of the eluate were chromatographed on DEAE-Sephadex, using step elution. In experiment 3, 45 ml of sheep liver nuclear extract was chromatographed on Sephadex G-100, and the peak fractions (155 ml) were applied to a 3 ml T_3 affinity gel. After washing and elution from the column, 4 ml samples were chromatographed on DEAE-Sephadex. All T_3-binding assays were performed at 1 nM $[^{125}I]T_3$. Reprinted from Apriletti *et al.* (1981).

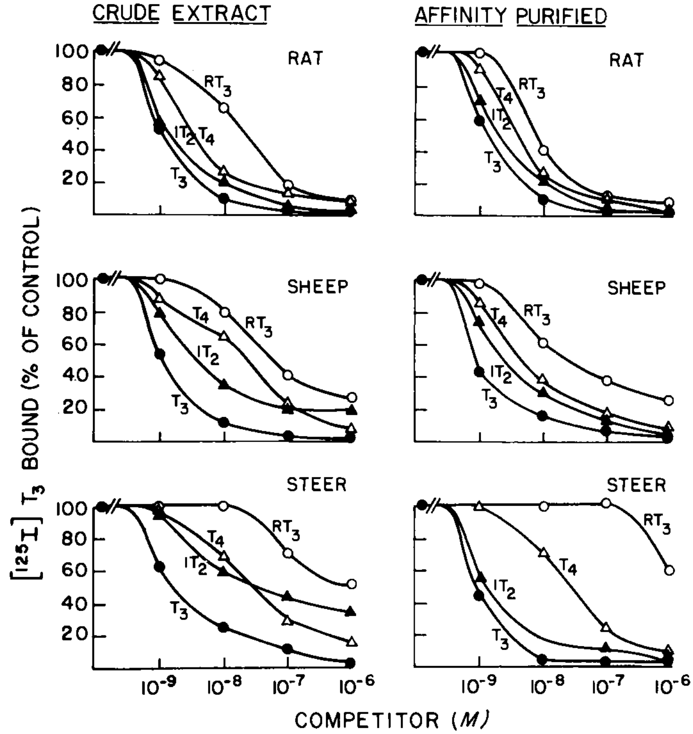

Fig. 23. Competition for [^{125}I]triiodothyronine binding in crude nuclear extract and affinity purified thyroid hormone receptor. Standard binding reactions were prepared containing 1 n*M* [^{125}I]T_3 and either 50 μl of crude nuclear extract from rat, sheep, or steer liver, or 100 μl of receptor which had been purified from nuclear extract by affinity chromatography. Various concentrations of competing unlabeled T_3 (●), T_4 (△), isopropyl T_2 (▲), and reverse T_3 (○) were added, and, after incubation for 2 hours at 22°C and then overnight at 4°C, the reactions were assayed for bound radioactive hormone by the standard procedures. Reprinted from Apriletti *et al.* (1981).

affinity purified receptor on Sephadex G-100 and DEAE-Sephadex were also identical to those of the crude receptor (Latham *et al.*, 1976; Silva *et al.*, 1977) providing additional confirmation that the purified material contains the nuclear receptor. Further work will be required before homogeneous receptors will be available.

VII. SUMMARY

The present chapter summarizes current knowledge of the physicochemical properties of the intranuclear thyroid hormone receptor. The receptor appears to be an intrinsic chromosomal protein; its localization within chromatin is not dependent on the hormone. The localization of the receptor in chromatin is correlated with known thyroid hormone influences on RNA (particularly mRNA) metabolism, a finding that suggests a role for the receptor in mediating certain thyroid hormone responses. Although a small fraction of receptors may be concentrated in template-active regions of chromatin, the majority of receptors appear to be localized in inactive fractions. The localization of the receptor within chromatin may be due to direct binding interactions with DNA and/or DNA-binding proteins.

The solubilized receptor has been shown to be an acidic, non histone protein composed of a single polypeptide chain with a molecular weight of approximately 50,000. It binds thyroid hormones and analogs in direct proportion to the hormones' biological potency. This latter finding and the general existence of this protein in thyroid hormone-responsive tissues further supports the concept that the receptor is involved in mediating certain of the hormones' physiological responses in target cells.

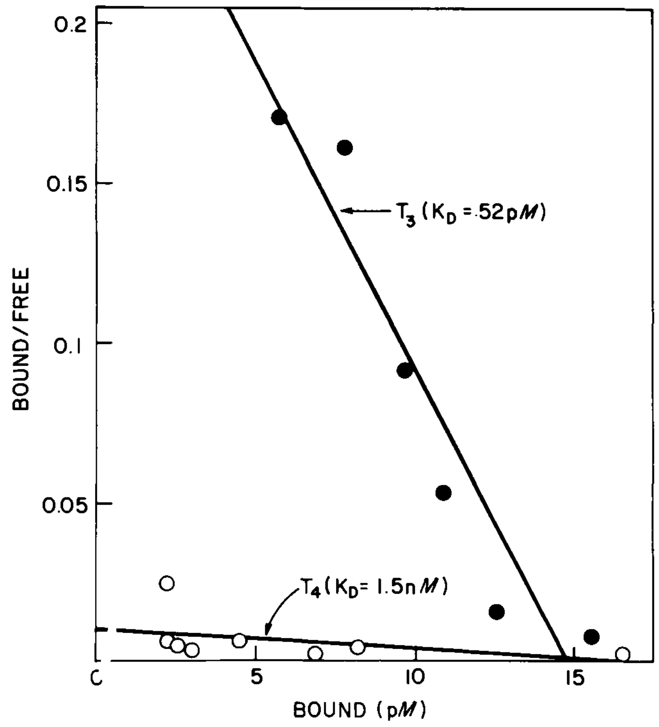

Fig. 24. Scatchard analysis of $[^{125}I]T_3$ (●) and $[^{125}I]T_4$ (○) binding by rat liver nuclear thyroid hormone receptor after affinity chromatography purification. The eluate from the affinity column described in Table I, experiment 1, was assayed for hormone binding and the concentrations of $[^{125}I]T_3$ and $[^{125}I]T_4$ were varied from 20 to 2000 and 100 to 10,000 p*M*, respectively. Reprinted from Apriletti *et al.* (1981).

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4

Thyroid Hormone Receptor Synthesis and Degradation and Interaction with Chromatin Components

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I. INTRODUCTION

A. Thyroid Hormone Receptor Levels Can Be Influenced by a Variety of Factors

As described in Chapter 2 as well as in other chapters in this volume, the thyroid hormone receptor is a chromatin-associated protein which appears to mediate the actions of the thyroid hormones in mammalian cells. Evidence in several systems indicates that thyroid hormone stimulates the synthesis of proteins by increasing the accumulation of their respective mRNA molecules (Kurtz *et al.*, 1976; Roy *et al.*, 1976; Seo *et al.*, 1977; Martial *et al.*, 1977; Shapiro *et al.*, 1978; Towle *et al.*, 1980). The modulation of specific mRNA molecules by thyroid hormone likely reflects a transcriptional or post-transcriptional nuclear event which appears to be controlled by the thyroid hormone–receptor complex. Unlike steroid hormones (O'Malley and Means, 1974), a cytoplasmic form of the receptor has not been identified or characterized (Samuels *et al.*, 1974; Oppenheimer and Surks, 1975). Furthermore, thyroid hormone does not appear to elicit an increase in the concentration of nuclear associated receptors as a result of a cytosolic to nuclear translocation as has been reported for the steroid hormones.

Evidence supporting this comes from the observation that the concentration of nuclear associated receptors is not decreased in the hypothyroid state compared to the euthyroid state in rat liver (Oppenheimer *et al.*, 1975). GH_1 cells cultured in the absence of thyroid hormone contain approximately 15,000 copies of receptors per cell nucleus, and the level of the receptor is reduced by thyroid hormone in a time- and dose-dependent fashion. This reduction of receptor involves a mechanism that is dependent on the association of hormone with the receptor binding site (Samuels *et al.*, 1976, 1979b). In addition to thyroid hormone, sodium butyrate and other short chain aliphatic carboxylic acids elicit a reduction of receptor in GH_1 cells. This reduction appears to be inversely related to the extent of acetylation of chromosomal proteins which is increased by these compounds (Samuels *et al.*, 1980). Therefore, nuclear receptor levels likely reflect a dynamic steady state, which is influenced by (1) the rate of receptor degradation, (2) the rate of receptor synthesis, and (3) the functional state of chromatin, which may be influenced by postsynthetic modifications of specific chromatin domains with which the receptor associates.

Evidence that the receptor does not represent a static population also comes from the observation that a variety of factors may influence the concentration of hepatic receptors *in vivo*. Starvation or a decrease in carbohydrate intake decreases the level of nuclear associated receptors (DeGroot *et al.*, 1977; Burman *et al.*, 1977). Furthermore, administration of pharmacological concentrations of glucagon elicits a rapid reduction in hepatic receptor (Dillmann *et al.*, 1978a) and a decrease in concentration of receptor is observed in rat liver after partial hepatectomy which results in liver regeneration (Dillman *et al.*, 1978b). Furthermore, the administration of cycloheximide, an inhibitor of protein synthesis, to rats results in rapid decrease in hepatic levels of receptor to approximately 50% of the control levels within 5 hours of administration (Jaffe and Means, 1977). Therefore, both *in vivo* studies in animals as well as studies in cultured cells indicate that the thyroid hormone nuclear receptor likely represents a dynamic rather than a static population in which the concentration of receptor may be influenced by a variety of physiological and pharmacological factors.

These observations raise a number of questions regarding the dynamics of the receptor in the cell. Do receptor concentrations reflect steady-state values that are solely dependent on the rate of receptor synthesis and degradation? If receptor concentrations represent steady-state values, is there evidence for equilibrium of nuclear bound receptor with a significant pool of cytoplasmic receptor that cannot be detected, but develops an affinity for ligand after association with chromatin? Is there evidence for several populations of chromatin bound receptors having different affinities for chromatin or do all chromatin associated receptors turn over with an identical half-life? If the level of nuclear associated receptor reflects a steady state, is the modulation of receptor due to changes in receptor synthesis, degradation, or a combination of both parameters?

B. Questions Relating to Chromatin Interactions of the Thyroid Hormone Receptor

The thyroid hormone nuclear receptor can be extracted from chromatin by high ionic strength buffer (Samuels *et al.*, 1974; Latham *et al.*, 1976). The salt extracted form of the receptor from rat liver has been estimated to have a sedimentation coefficient of 3.5 S and a Stokes radius of 3.5 nm (Latham *et al.*, 1976). Assuming a partial specific volume of 0.725 cm^3/g, a molecular weight

(M_r) for the rat liver receptor was estimated to be 50,500. Similar parameters have been observed for salt solubilized receptors from GH_1 cell chromatin which has an estimated sedimentation coefficient of 3.8 S, a Stokes radius of 3.3 nm, and an M_r of 54,000 (Perlman *et al.*, 1982). Although these studies provide some information regarding the basic structure of the receptor binding site, they provide no information as to whether receptor is organized in a subunit structure in chromatin with other protein components since this would likely be disaggregated by high salt conditions.

Although the receptor can be identified only in the nuclei of cells, very little is understood regarding the interaction of the receptor with chromatin components. We do not know if the receptor is homogeneously organized in chromatin or is localized to restricted chromatin domains. Furthermore, although the salt extracted receptor can associate with DNA *in vitro* (MacLeod and Baxter, 1976), the question remains whether the receptor associates with DNA in the intact cell? In addition to possible interactions with DNA does the receptor interact with other unique regulatory proteins which may play an important role in the action of the thyroid hormone–receptor complex?

This chapter reviews recent developments in which the dynamics of receptor synthesis and degradation have been quantitated and the mechanism of receptor regulation explored. In addition, studies are also reviewed in which the interaction of the receptor with chromatin components has been probed using nuclease digestion. Evidence is presented to indicate that the level of nuclear bound receptor is solely determined by the rate of receptor synthesis and receptor degradation and that the receptor may be organized in chromatin in a multimeric form.

II. QUANTITATION OF RECEPTOR HALF-LIFE AND ESTIMATION OF SYNTHETIC RATES USING DENSE AMINO ACID LABELING IN GH_1 CELLS

A. Principles of Dense Amino Acid Labeling of Receptors

As described above, the nuclear concentration of thyroid hormone receptor appears to be influenced by a variety of parameters *in vivo* and in particular by thyroid hormone and certain aliphatic carboxylic acids in GH_1 cells. The modulation of receptor levels could occur by several mechanisms. These include (1) an increase in receptor degradation, (2) a decrease in the rate of receptor synthesis, (3) an alteration in the conformational state of the receptor such that it no longer recognizes the ligand, and (4) modification in the structure of chromatin such that newly synthesized receptor does not associate. Studies on the regulation of thyroid hormone nuclear receptor levels may improve our understanding of the biology of the receptor as well as provide information regarding the association of receptor with chromatin components and its action.

The standard techniques used to quantitate the rate of synthesis or degradation of a protein involve radioactivie amino acid incorporation followed by selective isolation of the peptide with specific antiserum, identification by gel electrophoresis, or a combination of both procedures. If the peptide is present in low abundance (e.g., receptor proteins) it may be technically difficult to quantitate synthetic and/or degradation rates using this approach even if highly purified antisera is used.

It is possible to directly quantitate the half-life of the receptor and estimate the rate of receptor synthesis by density labeling cell proteins with amino acids uniformly labeled with the nonradioactive dense isotopes ^{15}N, ^{13}C, and ^{2}H. Newly synthesized proteins will be of higher density than the pre-existing proteins and can be separated using gradient centrifugation techniques (Hunttermann and Wendelberger, 1976). The densities of most proteins are approximately 1.3–1,4 g/cm^3. The total substitution of amino acids containing all three heavy isotopes results in a maximal density shift of 0.123 g/cm^3 which increases the density of proteins approximately 8%. The velocity of sedimentation of a particle is directly related to the difference between the particle density (ρ) and the density of the gradient (ρ_0). Since the density of most proteins is approximately 1.35 g/cm^3, an 8% increase will yield protein with a density of 1.47 g/cm^3. If a gradient can be constructed such that the average density is 1.2 g/ml, the value of $\rho - \rho_0$ for the dense protein is 0.27 while the value for the protein of normal density is 0.16.

Thus, under the correct gradient conditions an 8% increase in the density of the protein can result in a 1.5-fold increase in the velocity of sedimentation. To achieve a gradient density of approximately 1.2 g/ml sucrose gradients can be constructed using D_2O instead of H_2O. The newly synthesized protein of higher density can be separated from the preexisting protein of normal density by velocity sedimentation and the respective populations of dense and normal receptor are identified using radiolabeled ligand. This approach was initially used to quantitate the half-life and the synthetic rate of the acetylcholine receptor (Devreotes *et al.* (1977) and the position of receptor of different densities was identified using [^{125}I]α-bungarotoxin.

This approach has been adapted to quantitate thyroid hormone receptor synthesis and half-life in cultured GH_1 cells (Raaka and Samuels, 1981). As described in the Chapter 2, physiological concentrations of the thyroid hormones stimulate growth hormone synthesis and mRNA in the GH_1 cells (Tsai and Samuels, 1974; Samuels and Shapiro, 1976; Shapiro *et al.*, 1978; Samuels *et al.*, 1979a). In addition to modulating the growth hormone response, thyroid hormone also reduces the concentration of its receptor in GH_1 cells and the kinetics of disappearance appear to be a direct linear function of the occupancy of the receptor by L-triiodothyronine (L-T_3) as well as other thyroid hormone analogs (Samuels *et al.*, 1977, 1979b). Dense amino acid labeling was utilized in GH_1 cells to quantitate receptor half-life and synthetic rates and to examine the mechanism by which thyroid hormone modulates receptor levels in these cells. Using this approach receptor half-life is measured without the use of protein synthetic inhibitors such as cycloheximide, which may itself alter the rate of protein turnover (Hershko and Tomkins, 1971). Because purification of the protein of interest is not required, density labeling is particularly useful in studies of receptor proteins of low abundance.

B. Separation and Quantitation of Normal and Dense Thyroid Hormone Receptors in Sucrose–D_2O Gradients

GH_1 cells were cultured in medium containing dense amino acids for 1.5, 5, or 20 hours (Raaka and Samuels, 1981). One hour prior to harvesting the cells, 5 n*M* L-[^{125}I]T_3 was added to the culture medium to identify and quantitate the receptor. This concentration of L-T_3 occupies greater than 95% of the receptor population and gives an estimate of total receptor levels (Samuels *et al.*, 1976). Normal and dense receptors were then extracted from isolated GH_1 cell nuclei with 0.4 *M* KCl and separated by velocity sedimentation in 17–32% sucrose gradients constructed in D_2O (Fig. 1). After 1.5 hours in dense amino acid medium approximately 90% of the receptor is of normal density and only a small, faster sedimenting peak corresponding to newly synthesized dense receptor is detected. After 5 hours in dense medium, the receptor of normal density is reduced and an almost equal amount of dense receptor is present. Finally, after 20 hours about 95% of the receptor is of high density. By quantitating the amount of receptor of normal density after different times of incubation with dense amino acids the receptor half-life can be determined. Furthermore, by following the kinetics of appearance of receptor of high density, the rate of receptor synthesis can be calculated.

Figure 2 illustrates the disappearance of receptor of normal density over a 20 hour incubation of cells with dense amino acid medium. The receptor of normal density disappears with a half-life of about 5 hours when the cells are cultured in the absence of L-T_3, but received L-[^{125}I]T_3 for only 1 hour to estimate receptor levels. After 20 hours in dense amino acid medium less than 5% of the total receptor is of normal density. The receptor decay curve indicates that the receptor of normal density begins to decrease after lag time of less than 30 minutes following addition of dense amino acids to the cells. Since the concentration of total receptor during the experiment remains constant at 205 fmole/100 μg DNA, the concentration of receptor reflects a steady state determined by the 5 hour half-life and the synthetic rate.

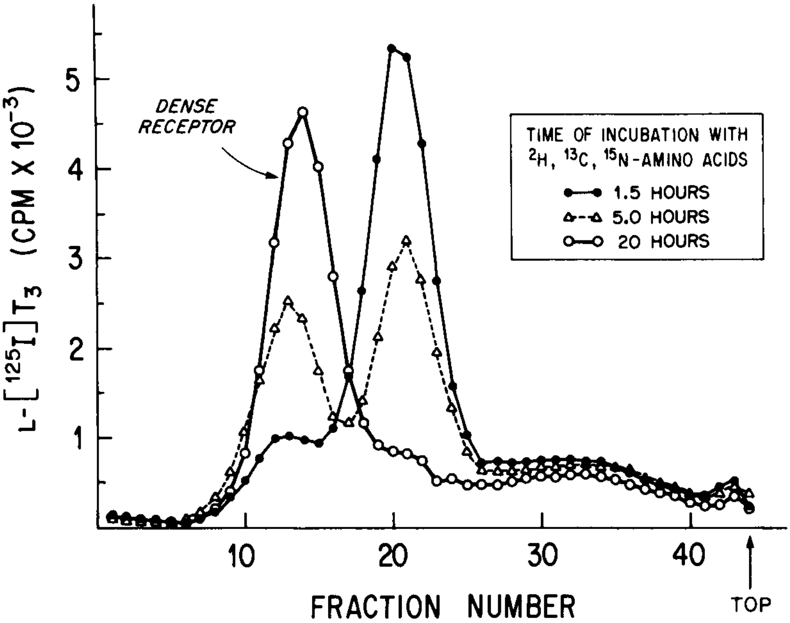

Fig. 1. Separation of normal and dense thyroid hormone receptors in sucrose gradients. GH_1 cells were cultured in medium without thyroid hormones for a total of 43 hours. Normal medium was replaced with dense medium 1.5, 5, or 20 hours prior to harvesting the cells. The dense medium was supplemented with 5 n*M* L-[^{125}I]T_3 1 hour before harvesting the cells to identify the receptor. Extraction of hormone–receptor complexes from isolated nuclei and conditions for sucrose gradient centrifugation are described in the text. The direction of sedimentation is from right to left. From Raaka and Samuels (1981).

The synthetic rate (k_s) can be calculated from the degradation rate constant (k_d) ($k_d = 0.693/t_{1/2}$) and the steady-state amount of receptor (R) by the formula $k_s = k_d R$ (Schimke, 1975). The k_s was calculated to be 29 fmole of receptor/100 μg DNA/hour which is equivalent to the synthesis of 1700 molecules of receptor/hour/cell. This value was directly confirmed by examining the kinetics of appearance of newly synthesized receptor and is described below. Furthermore, the virtual absence of a lag period and the straight line semilogarithmic plot indicate that an appreciable storage pool of extranuclear receptor that cannot be detected but can associate with nuclei and become activated to bind thyroid hormone does not exist. Moreover greater than 95% of the receptor falls off with an identical half-life indicating that no appreciable nuclear associated receptor pools exist with varying half-lives. This does not exclude the possibility that a very small chromatin associated pool (less than 5%) may be tightly bound to chromatin and be associated with a subset of chromatin components (Raaka and Samuels, 1981).

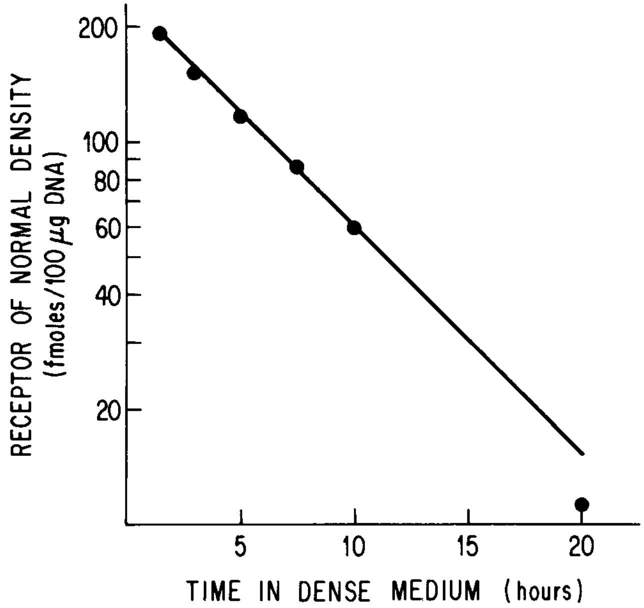

Fig. 2. Half-life of receptor of normal density in cells cultured in dense medium. GH_1 cells were cultured for a total of 44 hours in medium without thyroid hormone. Normal medium was replaced with dense medium at the times indicated prior to harvesting the cells. The dense medium was supplemented with 5 n*M* L-[^{125}I]T_3 1 hour before harvesting the cells to identify the receptor. Dense and normal receptors were separated and quantitated as described in Fig. 1. From Raaka and Samuels (1981).

C. Effects of Thyroid Hormone on Receptor Half-Life and Appearance Rate during Steady-State Conditions

The dense amino acid labeling technique (Raaka and Samuels, 1981) was utilized to study the mechanism by which L-T_3 and other iodothyronine compounds cause a dose-dependent reduction in the amount of thyroid hormone receptor in GH_1 cells (Samuels *et al.*, 1976, 1977, 1979b). To observe receptor depletion, cells are first cultured for 24 hours in medium lacking thyroid hormone. Following addition of 5 n*M* L-[^{125}I]T_3, the amount of receptor gradually decreases reaching a new steady-state level after 24 hours which is 55–60% of the value in cells cultured without thyroid hormone (Fig. 3). This reduction in receptor is not a result of a redistribution of receptor in the cell, a change in the affinity of the receptor for hormone, or a decrease in cell uptake of iodothyronine (Samuels *et al.*, 1976). The steady-state amount of receptor appears to be determined by the rate of receptor synthesis and the rate of receptor degradation. Therefore, in order to reduce the level of nuclear receptor, thyroid hormone must effect one or both of these processes.

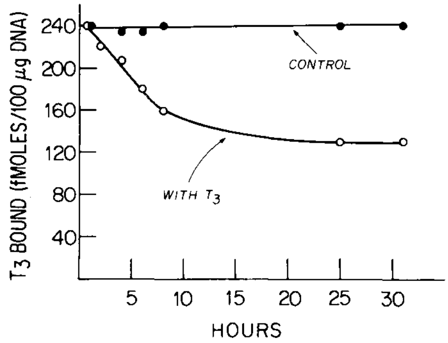

Fig. 3. Time course of receptor depletion in cells incubated with 5 nM L-[^{125}I]T$_3$. GH$_1$ cells were cultured for 24 hours in Ham's F-10 medium without thyroid hormone. At zero time on the figure, the medium was supplemented with 5 nM L-[^{125}I]T$_3$ in the cell cultures shown with open circles. Cells were harvested at the times indicated and radioactivity bound to the cell nuclei was determined. Control cells, shown with closed circles, were treated identically except that 5 nM L-[^{125}I]T$_3$ was added to the culture medium only 1 hour before harvesting the cells to identify and quantitate the receptor. From Raaka and Samuels (1981).

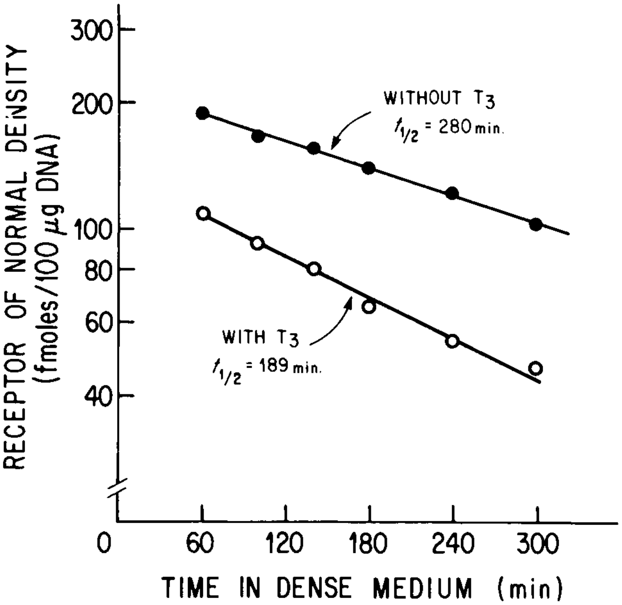

Fig. 4. Measurement of thyroid hormone receptor half-life under steady-state conditions in cells cultured with or without 5 nM L-[^{125}I]T$_3$. GH$_1$ cells that had been previously depleted of thyroid hormone were cultured in medium containing 5 nM L-[^{125}I]T$_3$ for 29 hours before harvesting. At times ranging from 1 to 5 hours before harvesting the cells, normal amino acid medium was replaced with dense medium. Control cells were treated identically except that 5 nM L-[^{125}I]T$_3$ was added to the culture medium only 1 hour before harvesting the cells. Normal and dense receptors were separated and quantitated as described in Fig. 1 (Raaka and Samuels, 1981).

To distinguish between these possibilities, dense amino acid labeling was used to compare receptor half-life in control cells cultured without thyroid hormone to cells cultured with 5 n*M* L-[^{125}I]T$_3$ for a total of 29 hours to reduce the steady-state amount of receptor (Fig. 4). Receptor half-life was 280 minutes in control cells cultured without hormone, and 189 minutes in cells cultured with 5 n*M* L-[^{125}I]T$_3$. The steady-state amounts of receptor in control and hormone-treated cells were 206 and 123 fmol/100 μg DNA, respectively. If the rate of appearance of newly synthesized receptor was not affected by thyroid hormone, the observed decrease of receptor half-life would reduce the steady-state amount of receptor to (189/280) minutes × 100 or 68% of the control value. Since the observed amount of receptor in the presence of hormone was (123/206) × 100 or 60% of the control value these results suggest that the rate of receptor appearance as well as half-life was influenced by hormone exposure.

D. Effects of Thyroid Hormone on Receptor during Approach to Steady-State Conditions

The mechanism by which L-T$_3$ shortens the half-life of its receptor is not known. The change in half-life could be an immediate result of the binding of hormone to receptor to induce a conformational change which could decrease the affinity of the receptor for chromatin and increase the susceptibility of receptor to proteolytic cleavage. Alternatively the change in half-life could be a delayed effect of the binding interaction such as a gradual modification of chromatin structures to which the receptor binds, or to the induction of a receptor-specific protease.

To distinguish between immediate and delayed effects, the density labeling technique was used to estimate receptor half-life under non-steady-state conditions in cells cultured for a total of 6 or 15 hours with 5 n*M* L-[^{125}I]T$_3$ (Raaka and Samuels, 1981). As can be seen from Fig. 3, a substantial reduction of receptor occurs within 6 to 15 hours of L-T$_3$ incubation. During these incubation times receptor reduction occurred without any substantial change in receptor half-life. Receptor half-life in cells incubated for a total of 6 hours with L-T$_3$ was identical to control cells, and cells incubated for 15 hours with hormone demonstrated a half-life which was only 9% decreased (Raaka and Samuels, 1981). This demonstrates that a change in receptor half-life does not occur immediately upon binding of hormone to the receptor, and a significant decrease in the amount of the receptor occurs before any change in receptor half-life occurs. Figure 5 demonstrates that the decrease in the amount of receptor occurring at early times during incubation of cells with L-T$_3$ is primarily due to a decrease in the accumulation of newly synthesized receptor. The rates of appearance of newly synthesized receptor during the final 5 hours of 6, 15, and 29 hour incubations with L-[^{125}I]T$_3$ were calculated from the accumulation data and these rates were

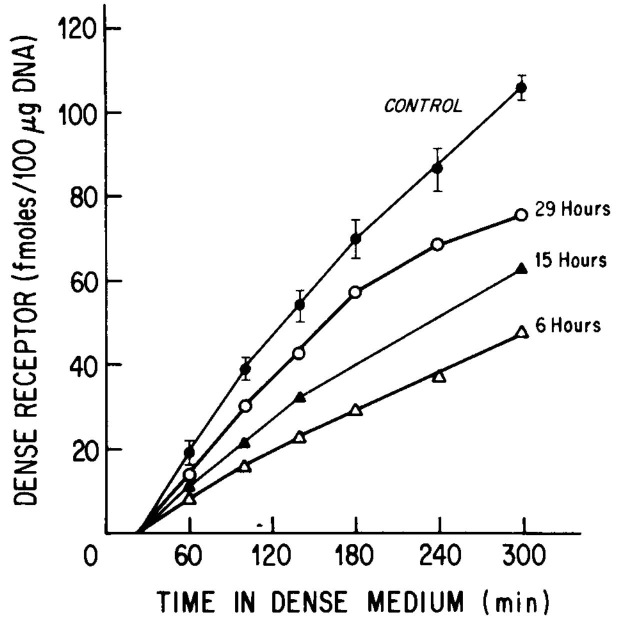

Fig. 5. Accumulation of newly synthesized dense receptor in cells incubated with 5 n*M* L-[^{125}I]T$_3$ for 6, 15, or 29 hours. The rates of appearance of newly synthesized dense receptor (k_s) can be calculated from the accumulation results using the formula $k_s = k_d R_t/(l - e^{-k_d t})$, where R_t is the amount of newly synthesized dense receptor present in the nucleus at any time (*t*) after addition of dense amino acids to cells. The degradation rate constants ($k_d = 0.693/t_{1/2}$) for the 6, 15, and 29 hour, and control experiments were calculated from the half-life values given in the text (Raaka and Samuels, 1981).

13.6, 18.7, and 26.1 fmole/100 μg of DNA/hour, respectively. The rate in control cells cultured without thyroid hormone was 31.2 fmole/100 μg of DNA/hour. Therefore L-T$_3$ initially inhibits the accumulation of newly synthesized receptor by as much as 56%, but the magnitude of this inhibition gradually diminishes to about 16% during the final 5 hours of a 29 hour L-T$_3$ incubation. This rapid inhibition of receptor appearance is not due to a general inhibition of protein synthesis since 5 n*M* L-T$_3$ does not alter the rate of incorporation of L-[^{3}H]leucine into total cell protein (Samuels and Shapiro, 1976).

A dose–response study further demonstrated that during a 5-hour incubation, thyroid hormone reduced the accumulation of newly synthesized receptor without affecting the half-life of preexisting receptor (Fig. 6). Cells were incubated for 6 hours in dense amino acid medium. During the first 5 hours of this incubation the medium contained the indicated concentrations of L-[^{125}I]T$_3$. The hormone concentration was adjusted to 3.8 n*M* in each cell culture for the final hour of the incubation, which gives a good estimate of total receptor levels. Half-

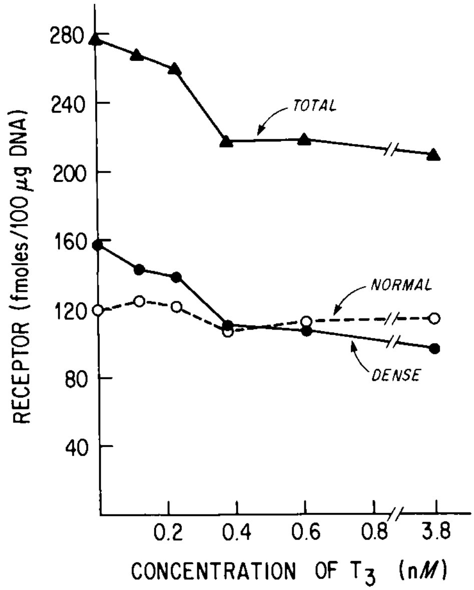

Fig. 6. Effect of L-[^{125}I]T$_3$ concentration on accumulation of newly synthesized dense receptor. GH$_1$ cells were grown in normal amino acid medium without thyroid hormone for 24 hours. Exactly 6 hours before harvesting the cells, the normal medium was replaced with dense medium containing the indicated concentration of L-[^{125}I]T$_3$. The concentration of L-[^{125}I]T$_3$ was adjusted to 3.8 n*M* in each culture 1 hour before harvesting the cells to identify and quantitate receptor. Normal and dense receptors were separated and quantitated as described in Fig. 1. The contribution of normal and dense receptors to the total amount of nuclear receptor at each concentration of L-T$_3$ is shown on the figure (Raaka and Samuels, 1981).

maximal depletion of total receptor occurred at about 0.3 n*M* L-T$_3$. This value was similar to the L-T$_3$ concentration giving half-maximal occupancy of receptor binding sites (Samuels *et al.*, 1976), confirming that receptor occupancy is necessary for receptor depletion. From the amounts of total receptor and receptor of normal density in the cells cultured without L-T$_3$, the receptor half-life was 280 minutes which is in the expected range in cells cultured without hormones (Fig. 4). Since the amount of receptor of normal density remained constant as the hormone concentration was increased, receptor half-life was not influenced by L-T$_3$ during the 6-hour incubation. A hormone-dependent decrease in the accumulation of newly synthesized dense receptor completely accounted for the decrease in total receptor levels.

The reduction of receptor caused by L-T$_3$ can be reversed by removing the hormone (Fig. 7). GH$_1$ cells were cultured in medium containing 1 n*M* L-[^{125}I]T$_3$ for 24 hours. This resulted in a 55% decrease in the amount of receptor compared to control cells that were not incubated with hormone. After removing hormone from cells by exchanging the culture medium several times over a 30 minute period with hormone-free medium, the cells were cultured in dense medium without hormone. The amount of receptor progressively increased after hormone removal and at 18 hours attained 92% of the value in control cells. Although the total amount of receptor increased, the amount of receptor of normal density

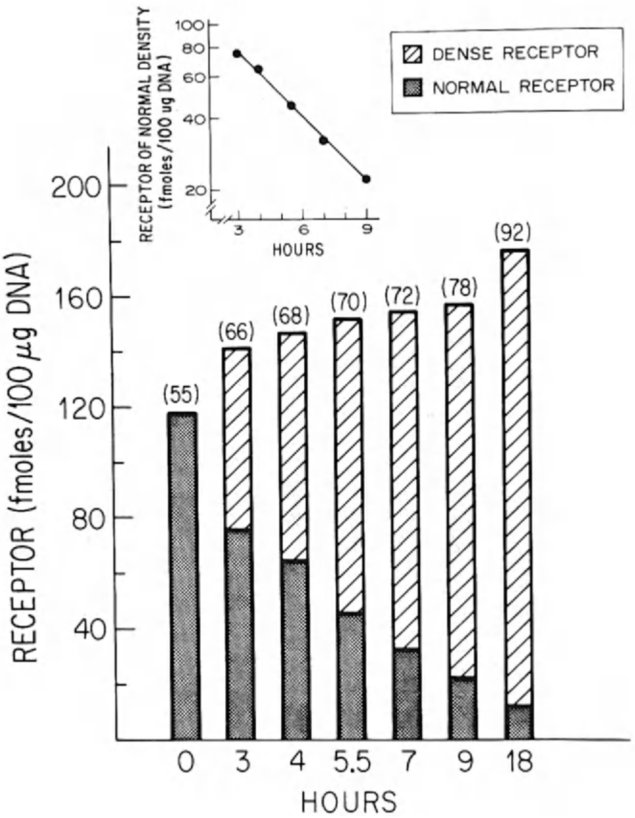

Fig. 7. Reappearance of receptor after removal of L-[^{125}I]T$_3$ from cells. GH$_1$ cells that had been previously depleted of thyroid hormone were incubated for 24 hours with normal amino acid medium containing 1 n*M* L-[^{125}I]T$_3$. Hormone was then removed from the cell cultures by replacing the culture medium 3 times over a 30 minute period with hormone-free medium. After this washing procedure, which removed greater than 97% of the radioactivity from the cultures, cells were incubated with dense medium for the times indicated. All cells were incubated with 5 n*M* L-[^{125}I]T$_3$ for 1 hour prior to harvesting to identify receptor. The numbers in parentheses indicate the amount of receptor in hormone-treated cells as a percentage of receptor in control cells that did not receive hormone but were otherwise treated identically (Raaka and Samuels, 1981).

decreased with a half-life of about 198 minutes during the first 9 hours after the removal of hormone which is characteristic of receptor in cells cultured for 24 hours or longer with L-T_3 (Fig. 4).

Therefore, when hormone is removed from cells, receptor half-life does not immediately increase to the value of 280 minutes found in cells cultured without thyroid hormone. The increase in amount of receptor following removal of hormone is solely due to accumulation of newly synthesized dense receptor (Fig. 7). The rate of appearance of receptor during the first 9 hours of removal of hormone from cells was calculated to be 31.5 fmole/100 μg of DNA/hour using a receptor half-life value of 198 minutes. This rate is essentially identical to the rate found in cells cultured without thyroid hormone, indicating that the rate of receptor appearance rapidly increases following removal of hormone from cells. Thus, during both the onset of and the recovery of thyroid hormone-mediated receptor depletion, the change in the appearance rate of newly synthesized receptor occurs rapidly whereas the change in receptor half-life occurs more slowly. With longer incubation times receptor half-life decreases approximately 30% while the appearance rate in newly synthesized receptor partially recovers but does not completely return to that observed in control cells.

E. Possible Mechanisms and Implications of Receptor Level Regulation

A model to explain the observed results must account for the observation that L-T_3, as a result of binding to its nuclear receptor, causes a rapid but partially transient decrease in the rate of appearance of newly synthesized receptor and a slow increase in the rate of receptor degradation. One possible model assumes that the expression of the gene for the receptor protein is negatively controlled by the thyroid hormone–receptor complex, and that the cytosolic mRNA for the receptor protein has a short half-life. When L-T_3 is added to cells previously depleted of hormone, the production of receptor mRNA rapidly decreases and cytosolic mRNA levels consequently decrease, causing a reduction in receptor synthesis. This would account for the decreased rate of appearance of newly synthesized receptor in the nucleus after short incubation times with hormone. As the receptor concentration in the nucleus falls, the amount of hormone–receptor complex present may no longer be sufficient to exert maximal negative control on receptor gene expression. This would cause a partial increase in receptor mRNA levels and a consequent increase in receptor synthesis to a level approaching that of control cells cultured without hormone.

This partial recovery of receptor synthesis does not lead to an increase in the amount of receptor since it is compensated for by the slower increase in the rate of receptor degradation. When thyroid hormone is removed from the cells, hormone rapidly dissociates from the receptor and the negative control of receptor gene would be completely relieved. This would cause a rapid increase in receptor synthesis to a level identical to that with control cells cultured without hormone. Again, the change in receptor half-life would occur more slowly following removal of hormone by reversal of the process responsible for shortening receptor half-life in the presence of hormone.

Although the binding of 5 n*M* L-T_3 to the nuclear receptor reaches equilibrium within 1 hour, the increase in receptor degradation occurs more slowly and is not seen until after 15 hours of incubation. For this reason the shortened half-life is not a direct consequence of the binding of hormone to receptor which would elicit a conformational change and make the receptor more susceptible to proteolytic degradation. Although the time course of change in the receptor half-life is consistent with the L-T_3 induction of a protease specific for receptor, the change in half-life might be an indirect result of late effects induced by thyroid hormone. For example, increases in the rate of cell growth and glucose consumption become measurable about 20 hours after addition of L-T_3 to GH_1 cells (Samuels *et al.*, 1973). Such general changes in cell metabolism might alter the amounts or activity of many cellular proteins including those responsible for receptor degradation.

In summary, in cultured GH_1 cells, chromatin associated thyroid hormone receptors are a dynamic population in which the steady-state value is solely dependent on rates of receptor synthesis and degradation. Thyroid hormone appears to decrease steady-state levels of receptor by initially inhibiting the appearance rate of newly synthesized receptor, and subsequently elicits a change in receptor half-life. Evidence that receptor concentrations *in vivo* reflect a dynamic rather than a static population comes from the observation of Jaffe and Means (1977) in which the hepatic receptor decreases rapidly after administration of cycloheximide. Other studies have demonstrated that the receptor can be reduced quite rapidly after pharamcological doses of glucagon (Dillmann *et al.*, 1978a) or after partial hepatectomy (Dillmann *et al.*, 1978b).

With a half-life of approximately 5 hours over 95% of the receptor is exchanged in a 24-hour period. Therefore, receptor does not remain fixed to specific regions of chromatin but is constantly exchanged both by loss of receptor from chromatin and replenishment by newly synthesized receptor. In addition it is likely that mobility of receptor occurs within chromatin from one region to another. Although the parameters of receptor turnover have recently been defined (Raaka and Samuels, 1981), an important aspect involving thyroid hormone action relates to the interaction of the receptor with chromatin components. Although the receptor can be extracted in a soluble form by high salt conditions, very little is understood regarding the specific chromatin domains or structures with which the receptor interacts. Recent developments from several laboratories have provided some insights in this area.

III. ORGANIZATION OF THE RECEPTOR IN CHROMATIN

A. Organization of Chromatin

1. Nucleosome Structure

It is now generally accepted that DNA in chromatin is organized into repeating subunits referred to as nucleosomes (Kornberg, 1977; Felsensfeld, 1978). This repeating subunit structure can be identified in extended chromatin by electron microscopy in which the nucleosome particles appear to be connected by strands of linker DNA (Olins and Olins, 1974). Further evidence for this structure comes from digestion of nuclei or chromatin with nucleases. Micrococcal nuclease digestion of chromatin or nuclei followed by chelation of divalent ions with EDTA releases a soluble chromatin fraction, which when separated on sucrose gradients yields a series of particles varying between 11 and 30 S (Noll, 1974; Kornberg, 1974).

Analysis of the DNA size of the individual particles indicates that the 11–11.5 S species represents mononucleosome particles containing between 140 and 240 base pairs (b.p.) of DNA, and the DNA sizes of the larger sedimenting fractions appear to be multiples of these values (Kornberg, 1977). Analysis of the protein composition of the mononucleosome fraction formed during micrococcal nuclease digestion demonstrates that it is associated with histones H2A, H2B, H3, and H4 and as well as histone H1. In rat liver nuclei Noll and Kornberg (1977) showed that the average DNA repeat of this species is approximately 160 b.p. of DNA and upon further digestion this is trimmed down to a core particle which contains only histones H2A, H2B, H3, and H4 and 140 b.p. of DNA. The loss of approximately 20 base pairs from the species is associated with the release of histone H1.

Although the DNA size of nucleosomes released during early micrococcal nuclease digestion varies between 140 and 240 b.p. in a variety of species as well as from the same cell, further digestion with micrococcal nuclease in each case yields a core nucleosome particle containing 140 b.p. that is devoid of H1 histone (Morris, 1976a,b). The variability of the length of DNA in the nucleosome, therefore, appears to derive entirely from variation in the length of the DNA linker regions associated with the core particle rather than the core particle itself. These studies indicate, therefore, that micrococcal nuclease shows preference for cleaving linker DNA regions releasing core nucleosome particles, nucleosome particles containing linker DNA, and nucleosome particles connected by linker DNA such as dinucleosomes, trinucleosomes, and larger polynucleosomes (Fig. 8). Cleavage of linker DNA regions produces fragments of linker DNA associated with specific chromosomal proteins (Varshavsky *et al.*, 1978). These proteins may represent receptor proteins of low abundance or highly

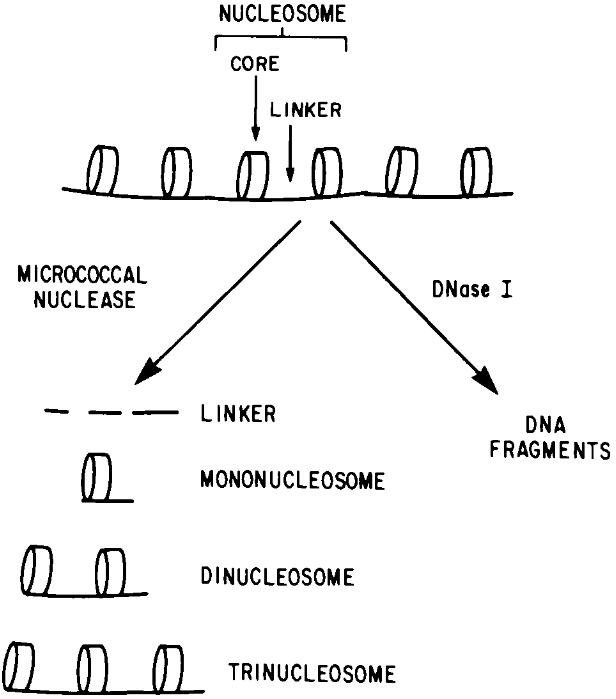

Fig. 8. Chromatin digestion by micrococcal nuclease and DNase I.

abundant nonhistone proteins such as the high mobility group proteins (HMG) initially described by Goodwin and Johns (1973). These proteins are approximately one-tenth as abundant as the histones and appear to be enriched in domains of chromatin that are involved in or have a history of being transcribed. In contrast to micrococcal nuclease, pancreatic deoxyribonuclease I (DNase I) does not show significant preference for DNA linker regions and cleaves the DNA of both the linker and the core particle generating smaller DNA fragments. The characteristics of chromatin digestion by micrococcal nuclease and DNase I are illustrated schematically in Fig. 8.

2. *Transcriptionally Competent Chromatin*

Although micrococcal nuclease and DNase I are nonspecific nucleases and cleave DNA without specific base sequence recognition they show preference for digesting domains of chromatin which are transcriptionally competent. For example, DNase I digests the globin genes of immature erthyrocytes at a greater rate than total chromatin DNA (Weintraub and Groudine, 1976). The increased sensitivity to DNase I digestion is not directly related to the transcriptional process since the enzyme also preferentially digests gene sequences that have been but are no longer transcribed (Weintraub and Groudine, 1976; Miller *et al.*, 1978), and also digests genes that are transcribed at different rates with equal sensitivity (Garel *et al.*, 1977). Therefore, the altered conformation of chromatin recognized by DNase I is imposed by specific genes as they are transcriptionally active and remain associated with these sequences following their inactivation. In contrast, Bloom and Anderson (1978, 1979) have shown that micrococcal nuclease recognizes transcriptionally active genes which appears to correlate with alterations in the expression of their sequences. Furthermore, unlike DNase I, which destroys active genes, micrococcal nuclease excises but does not degrade nucleosomes from transcriptionally active regions of chromatin (Bloom and Anderson, 1978, 1979; Levy *et al.*, 1979).

Since micrococcal nuclease and DNase I are nonspecific nucleases the enhanced sensitivity of transcriptionally competent chromatin to these enzymes implies that these chromatin domains exist in an altered conformation which are cleaved more rapidly than that of the bulk of chromatin. Therefore, both enzymes digest and/or excise transcriptionally competent chromatin preferentially during early digestion times, whereas with more extensive digestion other domains of chromatin which are accessible to these nucleases are either destroyed by DNase I or excised as mononucleosome particles by micrococcal nuclease. Even with extensive digestion, particularly at 4°C, a residual fraction of chromatin remains which is relatively insensitive to nuclease digestion. This fraction presumably represents the heterochromatin fraction observed by electron microscopy which has a highly compact structure. Chromatin, which is more accessible to nuclease digestion, appears to have a more extended structure and likely represent the dispersed euchromatin fraction observed by ultrastructural analysis. Therefore, sensitivity to nuclease digestion represents a continuum in which certain chromatin domains (e.g., transcriptionally competent chromatin) are attacked most rapidly by micrococcal nuclease or DNase I followed by cleavage of chromatin domains, which presumably exist in an extended conformation but are not necessarily enriched for transcriptionally active genes. Lastly, the fraction of chromatin which presumably reflects a highly compact domain is more resistent to cleavage by nucleases.

The precise factors that organize chromatin into domains with increased sensitivity to nonspecific nucleases are not completely defined, but increasing evidence suggests that the HMG proteins may play an important role in this organization. Several of these proteins have been purified and their amino acid sequence fully defined. Four major classes appear to be present in virtually all chromatin and are designated HMG-1 (M_r = 26,500); HMG-2 (M_r = 26,000); HMG-14 (M_r = 8000–10,000); and HMG-17 (M_r = 9247) (Goodwin *et al.*, 1975). HMG proteins are highly abundant at approximately 10^6 copies per nucleus (Goodwin and Johns, 1973) and their amino acid sequences have been highly conserved during evolution (Romani *et al.*, 1979). Jackson *et al.* (1979) demonstrated that mononucleosome fractions excised by micrococcal nuclease which were enriched for transcriptionally active genes lacked histone H1 and instead were associated with stoichiometric amounts of HMG-1 and HMG-2. These HMG proteins are likely associated with DNA linker regions since mild micrococcal nuclease digestion, which does not cleave the DNA of core particle, releases large amounts of HMG-1 and HMG-2 (Jackson *et al.*, 1979). Each protein sediments at approximately 5 to 6 S in sucrose gradients, which based on their M_r of approximately 26,000 suggests they remain bound to excised linker DNA (Varshavsky *et al.*, 1978). This is further supported by the observation that after micrococcal nuclease digestion, chromatin fractions that sediment to 4 to 7 S in sucrose gradients contain DNA fragments of 30–70 base pairs (Rill *et al.*, 1975).

In addition to the micrococcal nuclease studies, evidence that the HMG proteins are involved with gene activation comes with the observation that under mild digestion with DNase I (Vidali *et al.*, 1977) a high proportion of total chromatin associated HMG proteins is excised. In contrast to HMG-1 and HMG-2, the lower molecular weight HMG-14 and HMG-17 proteins appear to be associated with the DNA of a core particle and not with linker DNA (Mathew *et al.*, 1979). Furthermore, extraction of HMG proteins with 0.35 *M* NaCl prevents the selective digestion of transcriptional competent genes by nonspecific nuclease. Addition of purified HMG-14 and HMG-17 to nuclei that have been extracted by salt demonstrated that these HMG proteins were fully effective in restoring nuclease sensitivity to transcriptionally competent genes (Weisbrod and Weintraub, 1979). Therefore, the HMG proteins represent a class of chromatin proteins that may play an important role in modifying domains of chromatin to generate altered conformations associated with gene transcription and also show increased sensitivity to micrococcal nuclease and DNase I.

B. Interaction of Thyroid Hormone Receptors with Chromatin Components

From the above analysis it is apparant that major advances in understanding chromatin structure have been achieved in the past 5 years. Most of this work, however, has related to the fundamental structure of the nucleosome core particle and an examination of the organization of the HMG proteins with this basic structure. Other questions to be resolved relate to how nuclear associated receptor proteins (e.g., thyroid and steroid hormone receptors) are organized within this basic structure to modulate changes in chromatin function and possibly its structure, for example: (1) Is the thyroid hormone homogeneously distributed throughout all chromatin domains or is it primarily restricted to chromatin conformations which are transcriptionally competent? (2) Is the receptor associated solely with linker DNA regions or is it associated primarily with the core nucleosome particle? (3) Does the receptor associate with chromatin primarily by directly binding to DNA or is the receptor organized with other specific chromosomal proteins to form a multimer that is both structurally and functionally important in the action of the thyroid hormone–receptor complex? Such a putative functional multimeric species would not be detected by 0.4 *M* KCl extraction of nuclei since its structure would likely be disaggregated by high salt conditions. By examining what chromatin forms of the receptor are excised by micrococcal nuclease and/or DNase I as a function of total chromatin digestion, the above questions may be resolved. Furthermore, since nuclease digestion can be carried out at low ionic strength, an assessment can be made as to whether the receptor interacts primarily with linker DNA, DNA of the core particle, or with other unique chromosomal proteins within these chromatin domains. Recently, several laboratories have examined the organization of the receptor in chromatin using micrococcal nuclease or DNase I as a probe (Samuels *et al.*, 1980; Jump and Oppenheimer, 1980; Groul, 1980; Perlman *et al.*, 1982).

C. Micrococcal Nuclease Excises the Receptor as a Predominent 6.5 S Form and as a Less Abundant 12.5 S Species from GH_1 Cell Nuclei

Using low ionic strength conditions, Samuels *et al.* (1980) first reported that micrococcal nuclease excises the receptor from GH_1 cells as an abundant 6.5 S form and as a less abundant 12.5 S species, which sediments slightly more rapidly than the bulk of the mononucleosomes generated (11.5 S). Similar studies on thyroid hormone receptors in rat liver were subsequently reported by Jump and Oppenheimer (1980) and Groul (1980). These investigators reported that micrococcal nuclease released the receptor as a particle of approximately 6.0 S. These results contrast with the observation that 0.4 *M* KCl extraction of GH_1 cell and rat liver nuclei yield receptor species that sediment as 3.8 and 3.5 S, respectively (Samuels *et al.*, 1980; Latham *et al.*, 1976). Based on micrococcal nuclease digestion of GH_1 cell nuclei, we suggested that the 12.5 S receptor form represents receptor excised in association with a subset of mononucleosome particles while the 6.5 S form represents receptor excised in association with linker DNA regions and possibly other protein components (Samuels *et al.*, 1980).

Figure 9 illustrates an isokinetic sucrose gradient of chromatin solubilized from GH_1 cell nuclei after a 10 minute digestion at 0°C followed by centrifugation at 32,000 rpm in an SW 41 rotor for 16 hours. In all studies intact GH_1 cells are first incubated with L-$[^{125}I]T_3$ for 1–2 hours followed by nuclear isolation and chromatin digestion (Samuels *et al.*, 1980). In this experiment approximately 10% of the total chromatin was excised as a soluble fraction based on the A_{260} nm units released. Ultracentrifugation resolves the released chromatin fraction into bulk nucleosomal species which can be quantitated by the DNA content

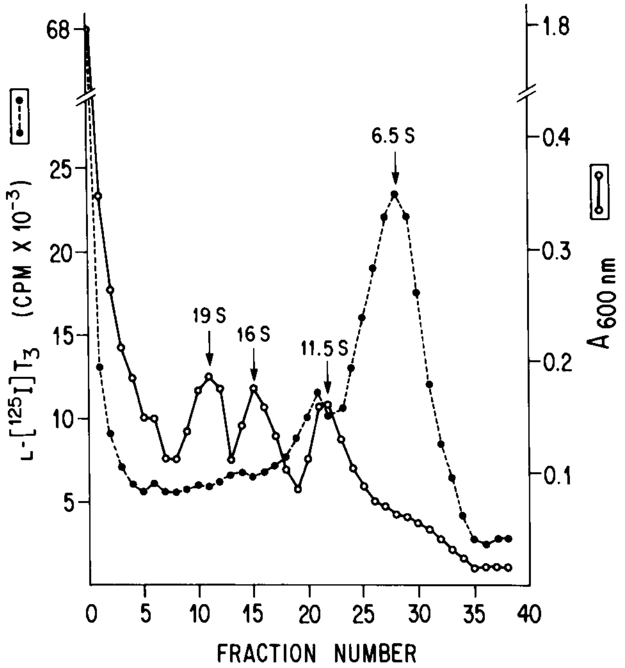

Fig. 9. Isokinetic gradient sedimentation of L-[^{125}I]T$_3$ bound to chromatin solubilized by micrococcal nuclease digestion. GH$_1$ cells cultured in roller bottles were incubated with L-[^{125}I]T$_3$ for 1 hour. Isolated nuclei equivalent to 35 A$_{260\text{ nm}}$ units were digested in 0.7 ml for 10 minutes with 15 units of enzyme/1 A$_{260\text{nm}}$ unit at 0°C. Solubilized chromatin (250 μl) was centrifuged in isokinetic gradients at 0°C at 32,000 rpm for 16 hours. The gradient was fractionated into 38 equal fractions and trichloroacetic acid was added to each fraction to achieve a final concentration of 15% (w/v). The L-[^{125}I]T$_3$ bound (●) was determined in a refrigerated Auto-Gamma spectrometer and the samples were then centrifuged at 3000 *g* for 20 minutes. The dry pellets were then analyzed for DNA content (○) by the method of Burton (1956) by measuring the absorbance at 600 nm. One unit of absorbance at 600 nm is equal to 75 ug of DNA. From Samuels *et al.* (1980).

within the gradient. The study in Fig. 9 resolves the excised chromatin into 11.5 mononucleosomes, 16 S dinucleosomes, and 19 S trinucleosomes, while higher molecular weight forms sediment toward the bottom of the gradient. Fifty percent of the excised L-[^{125}I]T$_3$ sediments as a predominant 6.5 S peak. A less abundant peak of 12–12.5 S is also observed and sediments slightly more rapidly than the bulk of the mononucleosomes generated (11.5 S). There is also a suggestion of a small L-[^{125}I]T$_3$ peak of 17 S migrating slightly faster than the 16 S dinucleosome. Figure 10A indicates that the L-[^{125}I]T$_3$ peak of 12.5 S is a discrete form and can be resolved from the abundant 6.5 S receptor form. Figure 10B illustrates the sedimentation profile of receptor which was extracted from nuclei with 0.4 *M* KCl and then sedimented in the low salt isokinetic sucrose gradient. In this case only a 3.8 S form is resolved (Samuels *et al.*, 1980).

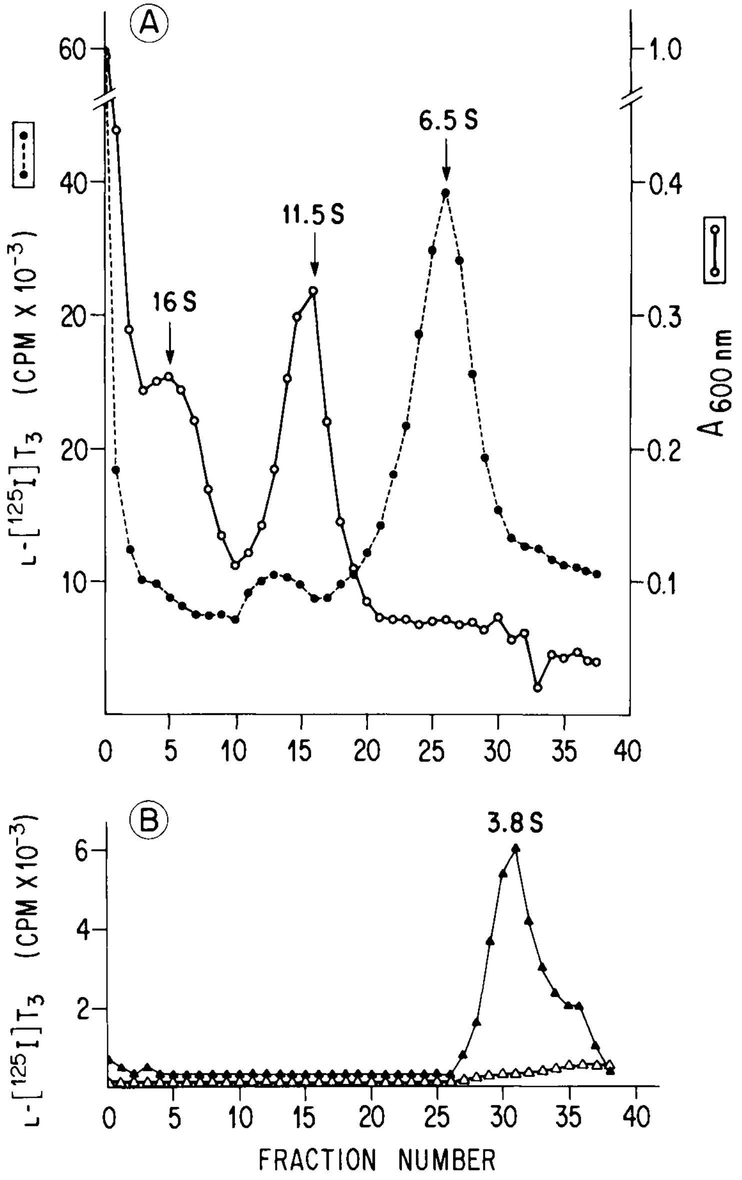

Fig. 10. Isokinetic gradient sedimentation of receptor released from chromatin solubilized by micrococcal nuclease digestion (A) and receptor extracted from nuclei with 0.4 *M* KCl (B). The soluble chromatin fraction was prepared by enzyme digestion at 0°C using nuclei derived from cells incubated with L-[^{125}I]T3 for 1 hour. The incubation conditions were the same as described in the legend to Fig. 9 except that nuclei equivalent to 54 A_{260nm} units were digested for 20 minutes. In a parallel study, thyroid hormone receptors were solubilized from nuclei by extraction with 0.4 *M* KCl and then dialyzed at 0°C to lower the salt concentration. Both the enzyme solubilized chromatin fraction and the nuclear extract were centrifuged at 37,000 rpm for 16 hours at 0°C. (A) absorbance at 600 nm (○); L-[^{125}I]T_3 (●). (B) L-[^{125}I]T_3 (▲); L-[^{125}I]T_3 plus a 1000-fold excess of nonradioactive L-T_3 (△). From Samuels *et al.* (1980).

These observations on the sedimentation profile of the micrococcal nuclease excised thyroid hormone receptor are similar to those reported for the estradiol receptor in rat uterine chromatin by Senior and Frankel (1978) and hen oviduct chromatin by Massol *et al.* (1978). In both studies the level of estradiol receptor was observed to be greater in the mononucleosome region compared to the dinucleosome and trinucleosome fractions. An estradiol peak sedimented somewhat more rapidly than the bulk of the mononucleosomes generated. In addition, the estradiol receptor was released as a 6.9 S form which sedimented more rapidly than the estradiol receptor dissociated from nuclei with high salt (Senior and Frankel, 1978). Excluding the thyroid hormone receptor associated with the high molecular weight chromatin at the bottom of the gradient, the 6.5 S form represents approximately 80% and the 12.5 S form 10% of the receptor resolved in the gradient. In addition, assuming one receptor moiety per nucleosome species, the subset of 12.5 S mononucleosomes associated with receptor represents approximately 0.006% of the bulk mononucleosomes generated.

Martin *et al.* (1977) demonstrated that micrococcal nuclease digestion yields mononucleosomes of heterogenous size and that polynucleosomes, which have longer DNA linker regions, are processed to mononucleosomes more rapidly than those with short linker regions. Furthermore, the DNA repeat lengths of these mononucleosomes are greater than 140 b.p. and appear to have intact linker DNA associated with histone H1 and nonhistone proteins (Todd and Garrard, 1979). Therefore, the 12.5 S L-T_3 receptor peak may represent a species in which the thyroid hormone receptor is associated with mononucleosomes with intact linker DNA regions. Since micrococcal nuclease shows preference for cleavage and excision of linker DNA regions, the abundant 6.5 S receptor species likely represents the 3.8 S receptor in association with linker DNA fragments and/or other chromatin components.

D. Kinetics of Excision of Chromatin Receptor Forms by Micrococcal Nuclease

Since the studies in Fig. 9 and 10 examined the forms of the receptor excised with minimal micrococcal nuclease digestion at a single time, no inferences can be made whether the thyroid hormone receptor is uniformly distributed throughout the total chromatin or whether it is heterogenously organized such that a high percent of total receptor is excised when only a small fraction of total chromatin is digested. Furthermore, it does not establish whether both the 6.5 and 12.5 S receptor forms are generated from the same chromatin domain or represent the release of receptor from discrete and different regions of chromatin. Therefore, kinetic studies were carried out to examine the rate of appearance of 12.5 and 6.5 S receptor in relation to the digestion of total chromatin [as measured by the percent of total DNA rendered perchloric acid soluble (PCA soluble)] and to the

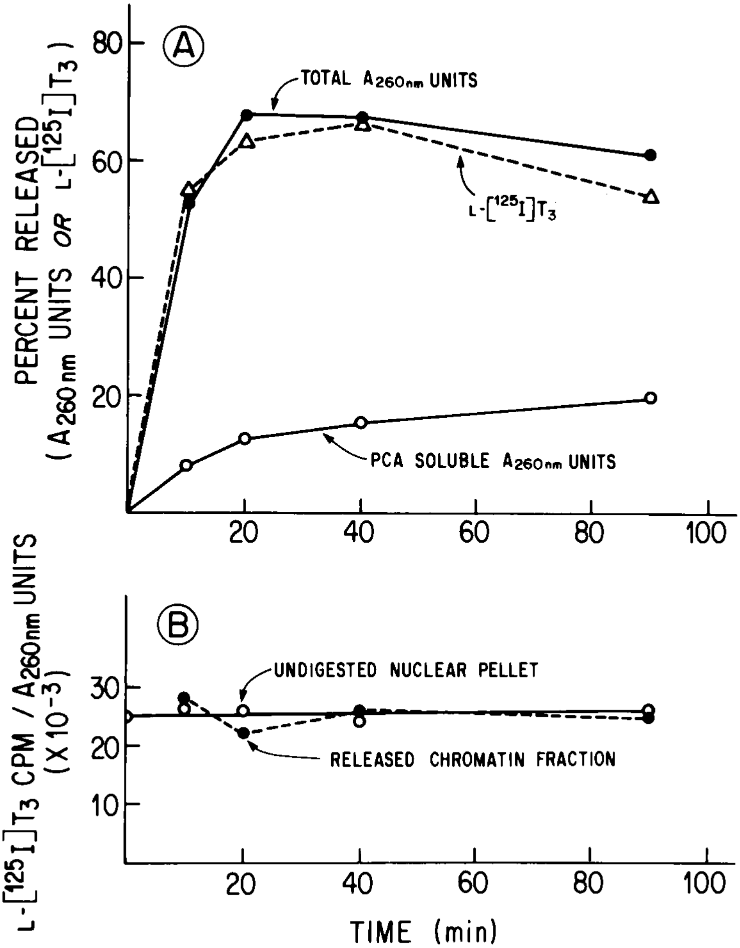

Fig. 11. Kinetics of release of receptor and chromatin by micrococcal nuclease. GH_1 cells were incubated 1 n*M* L-$[^{125}I]T_3$ for 1 hour. The derived nuclei were incubated at 0°C with micrococcal nuclease at 15 units of enzyme/$A_{260\ nm}$ units of nuclei. At 10, 20, 40, and 90 minutes, 10 m*M* EDTA was added to stop the reaction. The samples were centrifuged at 3000 *g* for 15 minutes. The absorbance of the supernatant and residual pellet were determined at 260 nm and an aliquot of the supernatant was precipitated with 5% perchloric acid (PCA). (A) Percent of total receptor and chromatin ($A_{260\ nm}$ units) released. (B) Concentration of L-$[^{125}I]T_3$ in the released and insoluble chromatin fraction.

generation of the bulk mononucleosomes. Figure 11 illustrates the effect of micrococcal nuclease digestion on the kinetics of release of the total L-$[^{125}I]T_3$ receptor, total solubilized chromatin released, and the extent of digestion as assessed by the percent of total A_{260} nm absorbance units which are rendered PCA soluble. As shown in Figure 11A the total L-$[^{125}I]T_3$ receptor excised by micrococcal nuclease parallels the percent of total chromatin solubilized by the enzyme. Although not illustrated, digestion of only 2–5% of total chromatin yields a similar parallelism in the release of chromatin and receptor. Figure 11B compares the concentration of receptor per A_{260} nm absorbance units in the released chromatin fraction, and in the residual fraction which was not solubilized by micrococcal nuclease digestion. At all digestion times the concentration of receptor is the same and identical to that of the starting nuclear (zero time) preparation prior to digestion. This indicates that the bulk of the receptor is homogenously dispersed throught the chromatin.

However, it remains possible that a small fraction of the total receptor exists in a chromatin domain which is preferentially excised by micrococcal nuclease. To explore this possibility the kinetics of appearance of the nuclease derived receptor forms was examined using isokinetic sucrose gradients. Figure 12 illustrates the gradient profile of receptor and chromatin released by micrococcal nuclease digestion from the experiment in Fig. 11. Between 10 and 90 minutes of digestion, the 6.5 S receptor form progressively increases in magnitude and appears to parallel the abundance of the bulk mononucleosome peak generated from chromatin. In contrast the 12.5 S receptor form plateaus within 10 minutes of digestion and remains relatively constant thereafter.

Figure 13 compares the kinetics of appearance of the 6.5 S receptor, the rate of formation of the bulk mononucleosomes, and the kinetics of appearance of the 12.5 S receptor form.

At virtually all digestion times the kinetics of formation of the 6.5 S receptor form parallels the excision of the mononucleosomes from the bulk of the chromatin. These rates also parallel the rates of total digestion as assessed by the percent of chromatin rendered PCA soluble by micrococcal nuclease. In contrast, the 12.5 S receptor species is generated rapidly and attains a plateau value within 10 minutes of incubation. In other studies (not illustrated) the 12.5 S receptor form was shown to attain a maximal value within 3 minutes of micrococcal nuclease digestion. Therefore, if the 12.5 S receptor form represents receptor in association with mononucleosome particles, this species is excised far more rapidly than the mononucleosomes of bulk chromatin. This suggests that it is derived from a chromatin domain that is exceedingly sensitive to micrococcal nuclease digestion. Based on the studies of Bloom and Anderson (1978) the 12.5 S receptor form likely represents receptor in association with mononucleosomes derived from a putative transcriptionally active region of chromatin. Although this is suggested by the digestion kinetics, this will have to be confirmed by

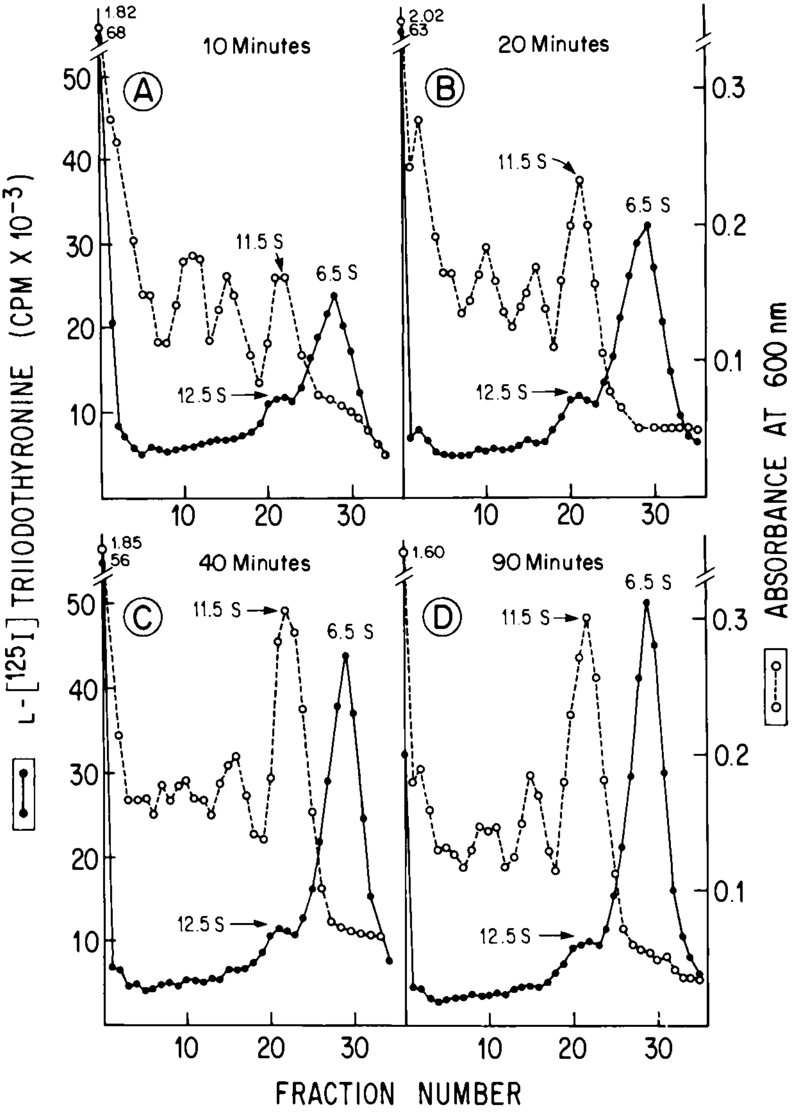

Fig. 12. Kinetics of release of chromatin receptor forms by micrococcal nuclease. The supernatent fractions from the study in Fig. 11 were centrifuged in isokinetic sucrose gradients at 32,000 rpm for 16 hours in an SW-41 Rotor. Micrococcal nuclease digestion times; (A) 10 minutes, (B) 20 minutes, (C) 40 minutes, (D) 90 minutes.

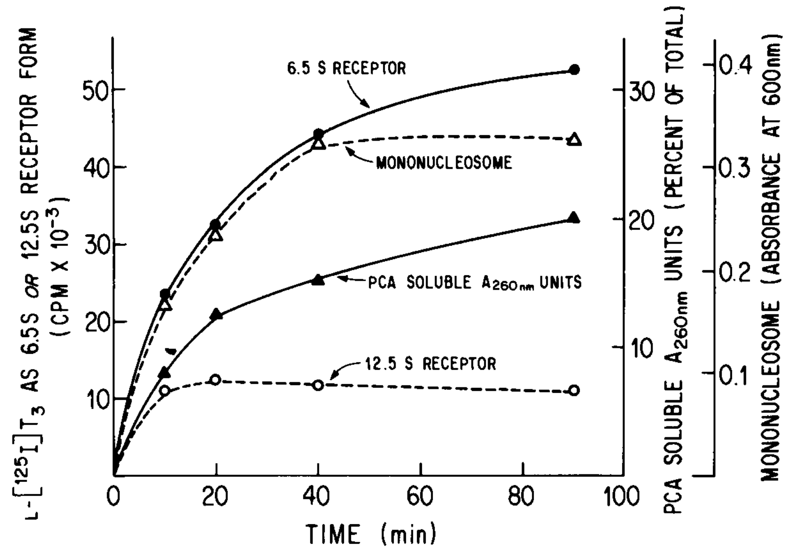

Fig. 13. Comparison of the rate of appearance of the 6.5 S receptor, 11.5 S bulk mononucleosomes, and 12.5 S receptor. The results were derived from Figs. 11 and 12.

determining the rate of appearance of transcriptionally active sequences in the mononucleosome region as a function of the kinetics of appearance of the 12.5 S form.

E. The 6.5 S Receptor Form Contains a DNA Fragment and May Exist as a Multimeric Species

The observation that the kinetics of appearance of the 6.5 S form parallels that of the bulk mononucleosomes generated suggest that most of this receptor form is derived from a separate domain in chromatin than the 12.5 S receptor. If the 6.5 S receptor represented receptor bound to linker DNA regions and was homogenously dispersed throughout the chromatin, the kinetics of appearance of this form and the bulk mononucleosome species would be expected to be parallel since cleavage of DNA linker regions would excise the 6.5 S receptor and also release chromatin particles as mononucleosomes. The 12.5 S receptor form is of too low abundance to isolate and further characterize. However, the 6.5 S form can be totally isolated from other forms of the receptor excised by micrococcal nuclease digestion. This can be achieved either by isolating this receptor form by density gradient centrifugation or by micrococcal nuclease digestion in the presence of 5 m*M* $MgCl_2$. At this concentration of Mg^{2+}, mononucleosome particles and higher chromatin forms remain insoluble and the receptor is released only as a 6.5 S species. Studies were therefore performed to (1) characterize the 6.5 S

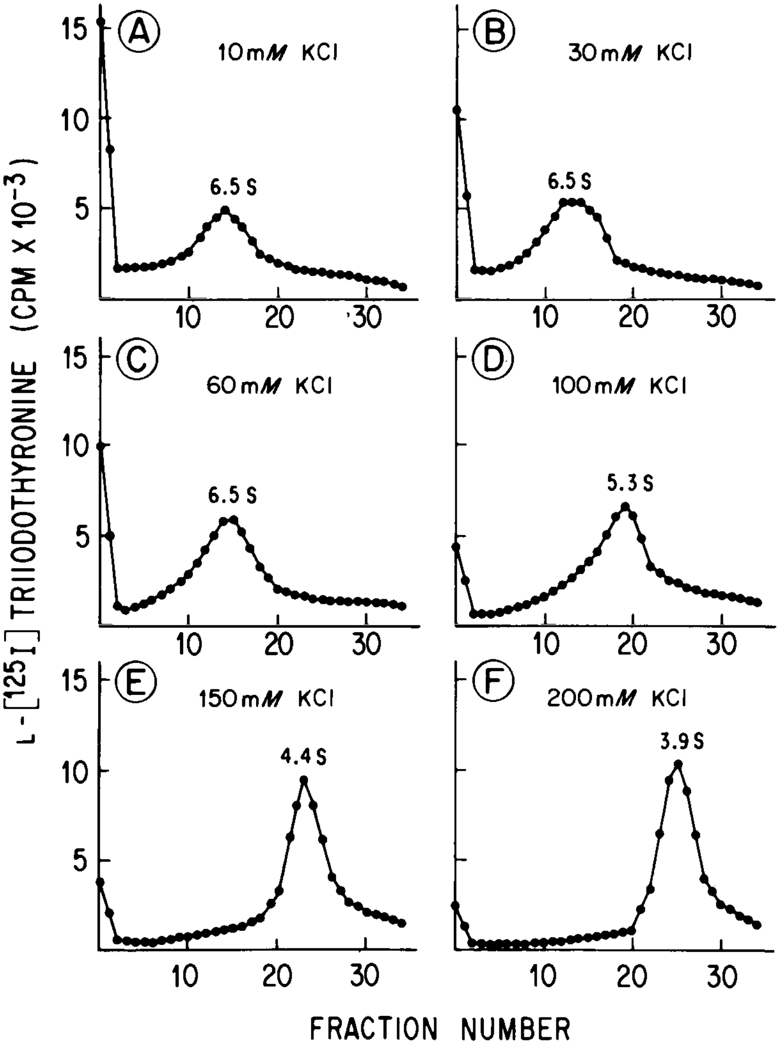

Fig. 14. Conversion of the 6.5 S receptor to slower sedimentating forms by increasing KCl concentrations. From Perlman *et al.* (1982).

receptor, (2) to determine if it is excised in association with a DNA fragment, and (3) to assess whether this structure may represent a multimeric species of 3.8 S salt extracted receptor in association with other protein components.

This was initially assessed by determining the influence of increasing salt concentrations on the sedimentation profile of the 6.5 S receptor (Perlman *et al.*, 1982). Figure 14 illustrates the sedimentation profile of the 6.5 S receptor which was adjusted to KCl concentrations between 10 and 200 m*M* prior to sedimentation in gradients containing the same KCl concentration. At KCl concentrations of 60 m*M* or less (Fig. 14A–C) the receptor sediments as a 6.5 S form. Above 60 m*M* KCl (Figure 14 D–F) there is a progressive stepwise decrease in the sedimentation coefficient. The 6.5 S receptor sediments as a 5.3 S species at 100 m*M* KCl and as a 4.4 S form at 150 m*M* KCl. At 200 m*M* KCl the 6.5 S receptor sediments as a 3.9 S form which is essentially identical to that extracted from nuclei by high salt conditions (3.8 S). The decrease in the sedimentation coefficient with increasing salt concentrations does not reflect a conformational change of a single component species since at high salt concentrations the receptor component would be more compact and would sediment at an increased, and not decreased, rate (Sherman *et al.*, 1980). If the 6.5 S receptor was only 3.8 S receptor bound to only one other component (i.e., à DNA fragment), increasing KCl concentrations would result only in a decrease in the magnitude of the 6.5 S receptor with a reciprocal increase in the 3.8 S species. Therefore, the stepwise change in the sedimentation coefficient with increasing KCl concentrations is compatible with the notion that the 6.5 S receptor is in association with at least two other components which are progressively and sequentially disaggregated by high salt conditions.

Evidence that one of these components is a DNA fragment comes from studies that examined the receptor forms released by DNase I (Perlman *et al.*, 1982). Figure 15 illustrates the effect of DNase I concentrations on the magnitude and the sedimentation profile of the receptor forms released. In contrast with micrococcal nuclease, receptor forms sedimenting greater than 6.5 S are not observed. With DNase I concentrations from 0.5 to 5-enzyme units, the 6.5 S species is symmetrical and progressively increases in magnitude (Fig. 15A–D). At 10-enzyme units (Fig. 15E) the 6.5 S peak broadens and decreases in height. At 20-enzyme units (Fig. 15F) the 6.5 S peak is reduced in magnitude and an apparent 4.9 S shoulder is also present. Therefore, DNase I appears to initially excise the receptor only as a 6.5 S species, and at high enzyme concentrations the magitude of the 6.5 S form diminishes within an apparent conversion to a slower sedimenting species. With further DNase I digestion the 6.5 S receptor is converted to a 3.8 S form (see Fig. 16). These results suggest that DNase I initially excises the receptor as a 6.5 S form containing a DNA fragment. Further digestion of the DNA fragment converts the 6.5 S receptor to a series of slower sedimenting forms and upon complete cleavage of the DNA fragment yields 3.8 S receptor which has the same sedimentation properties as the receptor extracted from nuclei by high salt conditions.

Fruther documentation that the 6.5 S receptor form contains a DNA fragment comes from studies which examined the ability of receptor species of various *s* values to associate with highly polymerized DNA (Perlman *et al.*, 1982). Figure

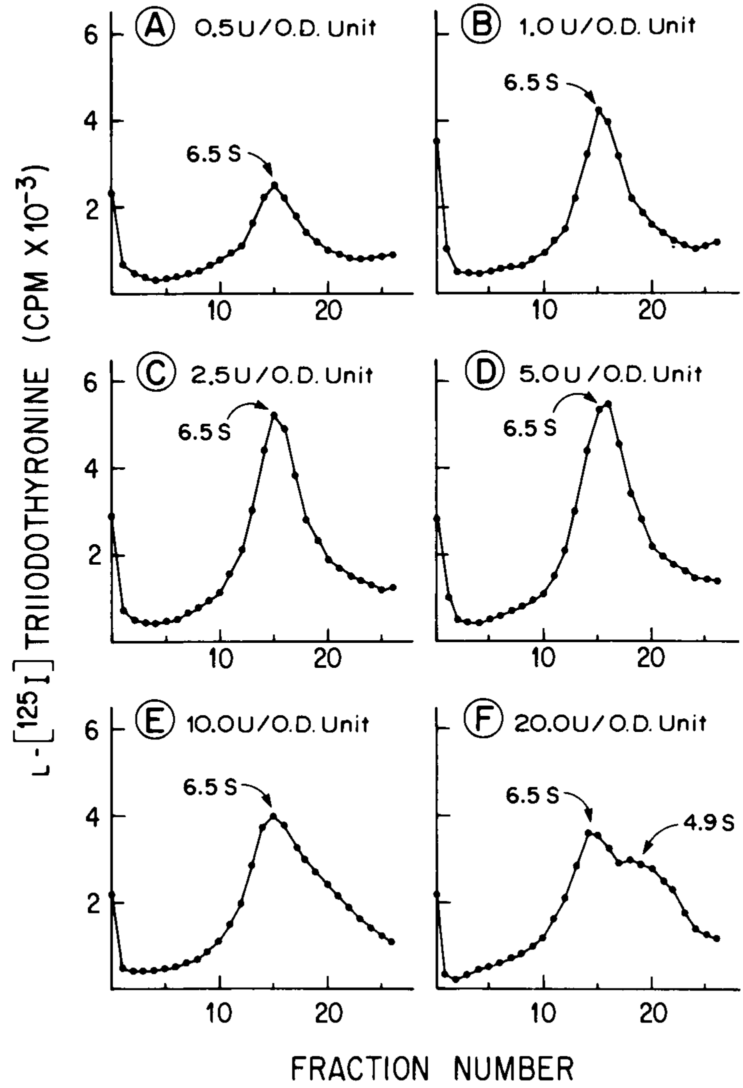

Fig. 15. Effect of DNase I concentration on the release of the receptor from GH_1 cells. From Perlman *et al.* (1982).

16 illustrates a study that examined the DNase I digestion of the 6.5 S species and the DNA binding characteristics of the derived forms. Nuclei were first incubated with 50 units of DNase I for 30 minutes at 0°C. The supernatant

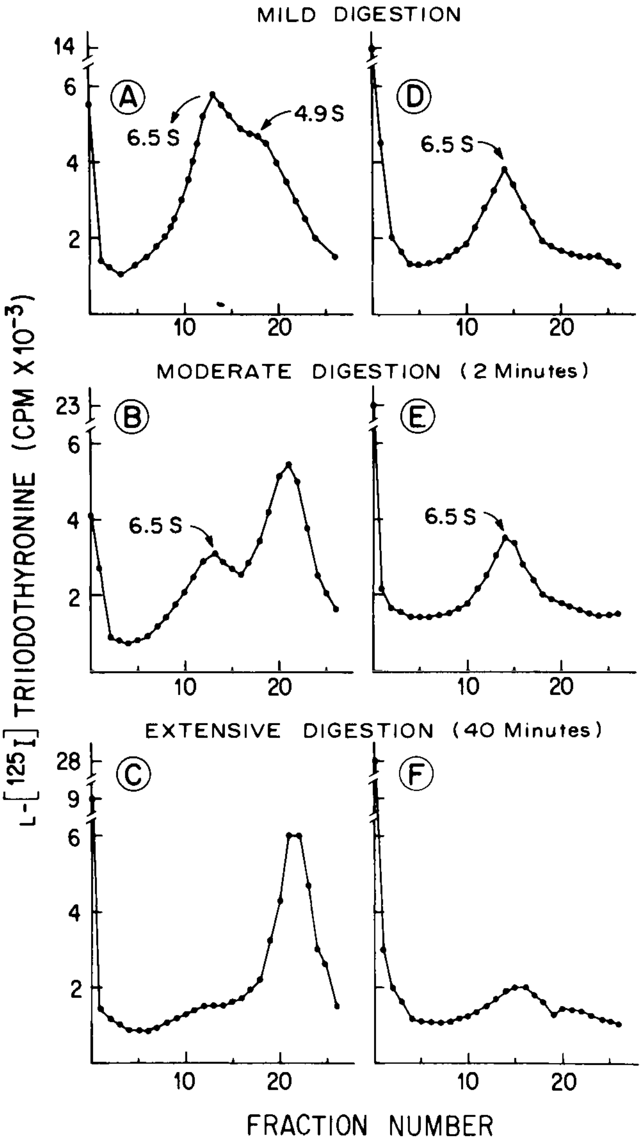

Fig. 16. Interaction of DNase I generated receptor forms with duplex DNA. From Perlman *et al.* (1982).

fraction was divided into three groups and the digestion was inhibited in one group with 10 m*M* EDTA to yield "mild digestion." "Moderate" and "extensive'' digestion were obtained by additional incubation with 2500 units of DNase I for 2 and 40 minutes, after which the nuclease activity was inhibited. Each digestion was divided into two parts. To one part of each (A–C) 50 μl of nuclease buffer was added. To parallel samples, (D–F) 50 ul of buffer containing 300 μg of purified highly polymerized duplex calf thymus DNA was added. The samples were incubated for 30 minutes at 0°C and were centrifuged in isokinetic gradients at 37,000 rpm for 40 hours. DNase I progressively reduced the size of the 6.5 S receptor to a limit value of 3.8 S (Fig. 16B, C) and at early times both a 6.5 S and a shoulder estimated at 4.9 S are present (Fig. 16A). The 6.5 S form does not appear to bind to exogenous DNA while the slower sedimenting forms sediment with the DNA to the bottom of the gradient (Fig. 16D–F). In similar studies (data not illustrated) the 6.5 S receptor form excised with micrococcal nuclease did not associate with exogenous duplex DNA while the 3.8 S receptor isolated by 0.4 *M* KCl was a DNA binding species. These studies suggest that the 6.5 S chromatin derived receptor form contains a DNA fragment which is cleaved by progressive DNase I digestion and exposes regions of the receptor component(s) which have high affinity for duplex DNA.

F. Estimation of the Particle Density, Stokes Radii, and Molecular Weights of the 3.8 S and 6.5 S Receptor Forms: Implications for the Structure of the Receptor in Chromatin

Other evidence that the 6.5 S receptor contains a DNA fragment comes from the high density of the 6.5 S species compared to the 3.8 S form (Perlman *et al.*, 1982). The density of each of the receptor species was estimated by ultracentrifugation in isokinetic sucrose gradients where the density was varied by replacing H_2O by D_2O (Edelstein and Schachman, 1967). Devreotes *et al.* (1977) and Meunier *et al.* (1972) have used this approach to estimate the density of the acetylcholine receptor. The velocity of sedimentation of a particle is directly related to the difference between the particle density (ρ) and the density of the sedimentation medium (ρ_0). Since the density of D_2O is 10.56% greater than H_2O, a particle will sediment slower in sucrose gradients constructed in D_2O compared to H_2O. Figure 17 illustrates the sedimentation of the 6.5 S receptor is isokinetic sucrose gradients constructed in either 100% D_2O or 100% H_2O. The receptor sediments approximately 1.5-fold further when the gradient is constructed with H_2O compared to D_2O.

The distance that a particle sediments from the meniscus in millimeters (X_m) = (X_0tK/ρ) (ρ-ρ_0), where X_0 is the distance from the center of rotation of the meniscus, t is the time of centrifugation in hours, K is a constant determined by rotor speed, temperature, and M_r;ρ_0 is the average density of the gradient; and ρ is the density of the macromolecule. Therefore, if the particle is sedimented into

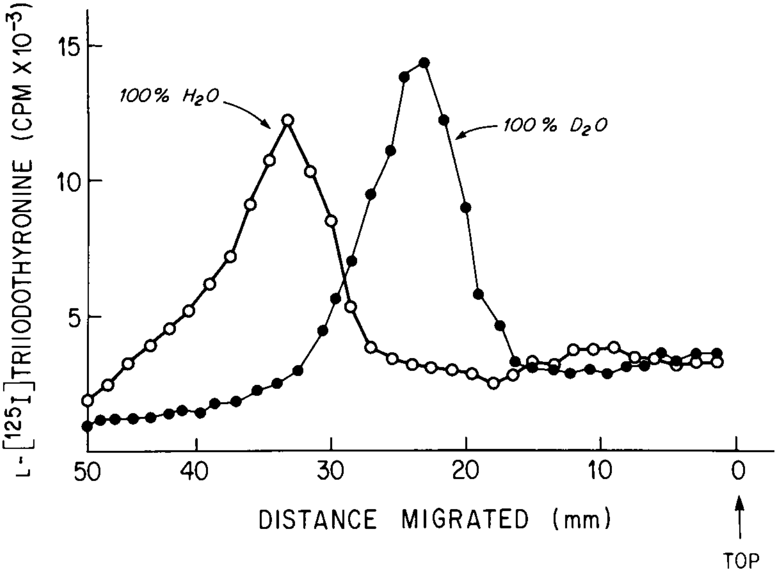

Fig. 17. Sedimentation of the 6.5 S receptor in gradients constructed with 100% H_2O or 100% D_2O. The samples were centrifuged at 44,000 rpm at 5°C for 17.5 hours in an SW-60 rotor.

a series of gradients having different densities, linear plots of X_m versus ρ_0 intersect the horozontal axis at the solvent density at which the macromolecule does not migrate. This gives an estimate of the density of the macromolecule since when $X_m = 0$, $\rho = \rho_0$. Figure 18 illustrates a study in which the 6.5 S and 3.8 S receptor forms were sedimented into gradients composed of H_20, D_20, or D_20:H_20 mixtures of 25, 50, or 75% D_20 (Perlman *et al.*, 1982). Based on the intersection with the horizontal axis, the estimated anhydrous density of the 6.5 S form was 1.42 g/cm^3 ± 0.02 ($p < 0.01$) and the 3.8 S form was 1.36 g/cm^3 ± 0.03 ($p < 0.01$). The density of most proteins is approximately 1.36 g/cm^3 (Sherman *et al.*, 1980), while that for DNA is 1.8 g/cm^3 (Luzzati *et al.*, 1967). Therefore, the 3.8 S receptor form has a density characteristic of protein, whereas the density of the 6.5 S species falls between that of protein and DNA. Assuming that the density increment of the 6.5 S form is due to an associated DNA fragment it can be calculated that the 6.5 S species consists of 15% DNA and 85% protein.

The Stokes radii were calculated by the elution profile of the 6.5 S and 3.8 S receptor on Sephrose CL-6B. Figure 19 illustrates these results. The distribution coefficient (K_d) was determined for each elution fraction and for protein standards of known Stokes radius (R_s). As illustrated in the inset to Fig. 19, a plot of $(K_d)^{1/3}$ versus R_s for the four protein standards is linear in agreement with the observations of Siegel and Monty (1966). Based on the K_d for the receptor

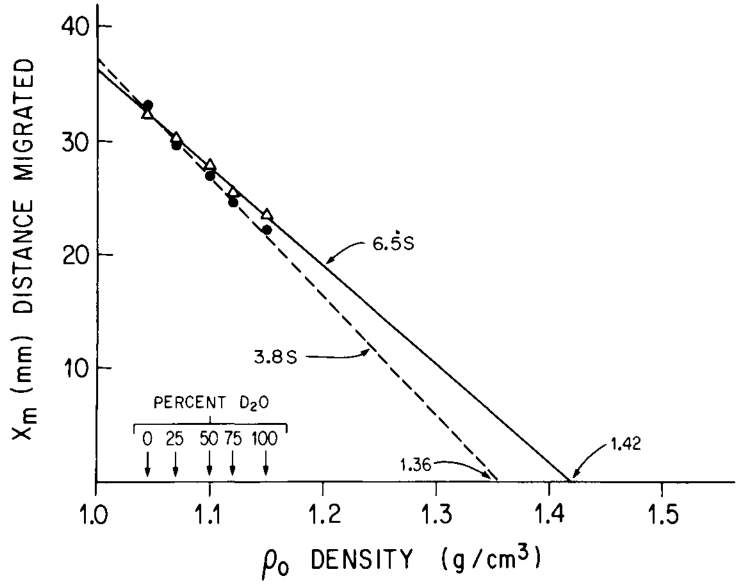

Fig. 18. Density determination of the 6.5 S and 3.8 S receptor forms. Sedimentation was in 5–20% sucrose gradients of various D_2O/H_2O proportions, and average densities (ρ_0) of 0% D_20 = 1.045 g/ml, 25% D_2O = 1.07 g/ml, 50% D_2O = 1.10 g/ml, 75% D_2O = 1.12 g/ml, and 100% D_2O = 1.15 g/ml Other details are given in the text. From Perlman *et al.* (1982).

forms, the R_s of the 3.8 and the 6.5 S receptor species were calculated to be 3.3 and 6.0 nm, respectively. Using the sedimentation coefficient, the R_s values and the partial specific volumes ($\bar{v}$) derived from the estimated anhydrous density ($\bar{v} = 1/\rho$), the M_r values were calculated to be 54,000 for the 3.8 S receptor and 149,000 for the 6.5 S form. The M_r and the R_s for the salt extracted receptor from GH_1 cell nuclei is in good agreement with that reported by Latham *et al.* (1976) for the thyroid hormone receptor extracted from rat liver nuclei (M_r = 50,500; R_s = 3.5 nm).

The DNase I digestion studies (Fig. 16) suggests that the 6.5 S receptor contains a DNA fragment and from the density measurement it can be calculated that the 6.5 S receptor is composed of 85% protein and 15% DNA. Based on the total M_r of the 6.5 S species of 149,000 this yields an M_r value for the protein component of 127,000. The M_r for the putative DNA component is therefore 22,000 which would be equivalent to 36 base pairs of DNA and is the size length expected if the 6.5 S receptor form were derived from linker DNA regions in chromatin. The estimated M_r of 127,000 for the protein component of the 6.5 S receptor is significantly greater than the M_r (54,000) for the hormone binding activity extracted from nuclei by 0.4 *M* KCl, suggesting that the receptor exists as a multimer in chromatin (Fig. 20). This is also supported by the stepwise

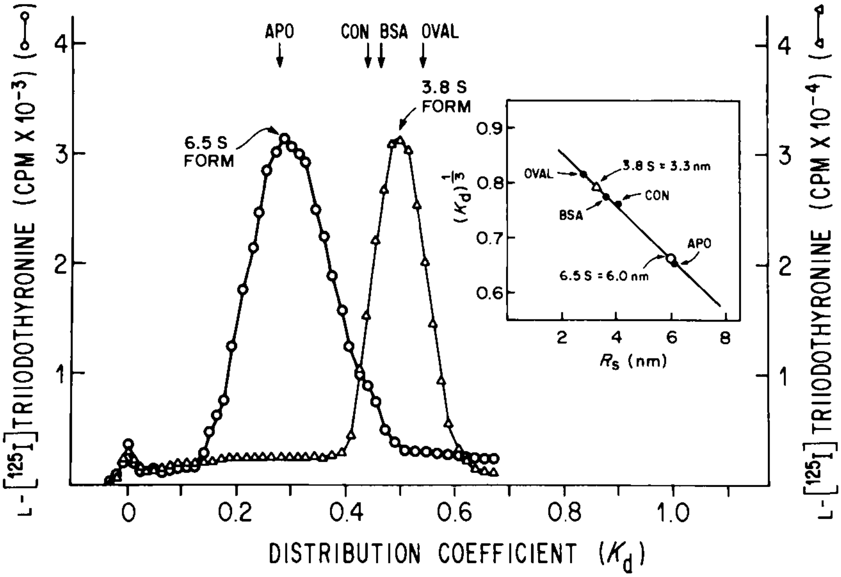

Fig. 19. Estimation of the Stokes radii of the 6.5 S and 3.8 S receptor forms by Sepharose CL-6B chromatography. The results are plotted as the distribution coefficient (K_d) of the receptor forms and protein standards of known Stokes radius (R_s). The insert shows the relationship between the R_s and the $(K_d)^{1/3}$ (Siegel and Monty, 1966) which was used to calculate the R_s of the receptor forms. From Perlman *et al.* (1982).

reduction in sedimentation coefficient seen with increasing salt concentrations (Fig. 14). The structure of the 6.5 S receptor form could represent the 54,000 M_r (3.8 S) receptor in dimeric form alone, or in association with other unique protein components bound to DNA. Alternatively, it could represent a single 54,000 M_r (3.8 S) receptor in association with other unique chromatin protein components which interact as a multimer with DNA (Fig. 20).

Whether these proteins are abundant chromosomal proteins (e.g., HMG proteins) or unknown proteins of low abundance will require purification of the

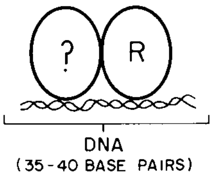

Fig. 20. Possible structure of the 6.5 S receptor form. R denotes the 3.8 S receptor. (?) represents additional chromosomal proteins with which the 3.8 S receptor interacts or another 3.8 S receptor molecule. The protein components are associated with DNA which is excised as a 35–40 b. p. fragment by micrococcal nuclease.

chromatin forms of the receptor. Clearly, identification of the protein composition of the 6.5 S receptor as well as the 12.5 S receptor form may provide important insights as to how the hormone–receptor complex initiates thyroid hormone action at the molecular level.

IV. SUMMARY AND CONCLUSIONS

Numerous studies indicate that the thyroid hormones stimulate the accumulation of specific mRNA molecules in cell culture as well as in tissues *in vivo*. This response appears to be mediated by a chromatin associated receptor, which presumably regulates a transcriptional and/or posttranscriptional event in the cell nucleus which is rate limiting for mRNA accumulation. The receptor can be extracted by high salt conditions from liver or GH_1 cell nuclei. This receptor form from liver has a Stokes radius of 3.5 nm, and a sedimentation coefficient of 3.5 S (Latham *et al.*, 1976). From GH_1 cells, the KCl extracted receptor has an estimated Stokes radius of 3.3 nm and a sedimentation coefficient of 3.8 S (Samuels *et al.*, 1980; Perlman *et al.*, 1982). The difference in the physical parameters of the salt extracted receptor from rat liver and GH_1 cells likely reflects differences in experimental measurements rather than intrinsic differences in the receptor binding component.

The half-life and synthetic rate of the 3.8 S receptor component has been estimated in GH_1 cells using a dense amino acid labeling technique (Raaka and Samuels, 1981). These studies show that the nuclear receptor level is solely a reflection of the rate of receptor degradation and the rate of accumulation of newly synthesized receptor. Furthermore, there is no evidence for a significant pool of masked cytoplasmic receptor which cannot bind the ligand prior to association with nuclei. Thyroid hormone lowers receptor levels in GH_1 cells (Samuels *et al.*, 1976,1977), although there is no evidence that hormone mediated receptor regulation occurs in the liver *in vivo* (Oppenheimer *et al.*, 1975). Using dense amino acid labeling we have shown that the reduction of receptor elicited by thyroid hormone in cultured GH_1 cells is primarily due to an inhibition in the accumulation of newly synthesized dense receptor at early incubation times with hormone. At longer incubation times (greater than 24 hours) thyroid hormone also results in a shortening of receptor half-life. This decrease in receptor half-life does not appear to result as a direct consequence of the hormone binding to the receptor but appears to reflect late and delayed effects of thyroid hormone on the cell which may result from changes in cell metabolism and/or chromatin structure. A model to explain the rapid thyroid hormone mediated decrease in accumulation of newly synthesized receptor is presented and it is suggested that the nuclear thyroid hormone receptor complex can inhibit the production rate of its own mRNA. Clearly, documentation of this model will require direct quantitation of the mRNA for the receptor.

Since the receptor has a half-life of about 5 hours the total nuclear receptor population appears to fully exchange within a 24-hour period. Therefore, the receptor does not remain fixed to specific regions of chromatin but is constantly exchanged both by loss of receptor from specific chromatin domains and replenishment by newly synthesized receptor. Studies using nuclease digestion demonstrated that the thyroid hormone receptor can be excised from nuclei as a predominent 6.5 S form and is a less abundant 12.5 S species (Samuels *et al.*, 1980; Perlman *et al.*, 1982). Studies in rat liver (Jump and Oppenheimer, 1980; Groul, 1980) also indicate that the predominent excised form of the receptor sediments at about 6.0 S. The 12.5 S form of the receptor likely represents association of receptor with a subset of mononucleosome particles that appear to be excised more rapidly than the bulk of the mononucleosomes generated. Based on kinetics of excision this fraction of nuclear bound receptor may represent receptor in association with mononucleosome particles that are derived from a transcriptionally competent region of chromatin. In GH_1 cells the remainder of the receptor population appears to be homogeneously distributed throughout the chromatin as assessed by micrococcal nuclease digestion. Whether this type of receptor distribution occurs *in vivo* will require further analysis.

Evidence is presented that the 6.5 S receptor form represents the 3.8 S receptor in association with other chromatin protein components which are excised as a complex bound to a DNA fragment of approximately 35 to 40 base pairs. Clearly studies of this type provide some basic information regarding the organization of the receptor in chromatin. Future approaches will require an analysis both on a functional and structural level. Such an analysis should include the following: (1) A determination as to whether the thyroid hormone–receptor complex directly regulates the transcriptional process or influences posttranscriptional modifications of specific genes transcripts. (2) Isolation of the mRNA for the receptor, synthesis of a cDNA, and cloning by recombinant DNA techniques. This will allow quantitation of the receptor mRNA as well as a function as a probe for isolation and analysis of receptor gene sequences in chromatin. (3) Studies to determine if the mononucleosome bound form of the receptor represents receptor in association with mononucleosome particles that are being actively transcribed. In addition, further analysis is required of the protein composition of the micrococcal nuclease excised form of the receptor since this may represent a functionally important multimer in which the receptor binding component is a subunit of a regulatory complex, which plays an important role in mediating the action of thyroid hormone at the nuclear level.

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5

Nuclear–Cytoplasmic Interrelationships

CHARLES P. BARSANO AND LESLIE J. DeGROOT

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The great multiplicity of effects on growth, development, and metabolism attributable to thyroid hormone suggests that this hormone acts through a fundamental mechanism common to many different tissues yet is capable of diversified function or expression. The cytoplasmic mechanism for protein synthesis distinctly possesses both the ubiquity and versatility suited to the biosynthetic implementation of the message carried to the cell by thyroid hormone. The protein synthetic mechanism is itself dependent on the nuclear RNA synthetic apparatus among other regulatory processes. Indeed both nuclear RNA synthesis and cytoplasmic protein synthesis have been shown to be quantitatively, and possibly qualitatively, affected by thyroid hormone administration. The interrelationships between these two fundamental processes and the mechanism through

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which thyroid hormone influences these processes are the subjects of this chapter.

Section I integrates the available data concerning thyroid hormone-induced changes in nuclear RNA synthesis and in cytoplasmic protein synthesis. Initially, changes in gross macromolecular synthesis not limited to one or a few species of mRNA's or proteins will be examined. Subsequently, more specific or limited T_3-induced effects on RNA and protein synthesis are discussed. Section II is concerned with the insights and controversies contributed to our understanding of nuclear and cytoplasmic interactions by the study of cellular thyroid hormone binding proteins.

I. COORDINATION OF THYROID HORMONE-INDUCED CHANGES IN NUCLEAR RNA SYNTHESIS AND CYTOPLASMIC PROTEIN SYNTHESIS

A. Gross Changes in RNA and Protein Synthesis

Historically, the roots of today's approaches to the investigation of the mechanism of action of thyroid hormone can be traced to early observations of the calorigenic effects of thyroid hormone excess or deficiency in man and in animals. Experimental thyrotoxicosis or hormonal treatment of hypothyroid animals could be shown to increase the basal metabolic rate (BMR) of animals within approximately 2 days. Attention was understandably focused on mitochondrial functions. A substantial number of investigations demonstrated the parallelism of both oxygen consumption (at the tissue and organelle levels) and mitochondrial enzyme activities with the BMR of the intact animal. An excellent review of these studies was published in 1964 (Tata, 1964a). Early on it was noted that cell-free, *in vitro* microsomal protein synthesis in hypothyroid animals treated with thyroid hormones was augmented in less time than was required to increase the BMR, preceding the BMR by approximately 24 hours (Tata *et al.*, 1963). The significance of microsomal protein synthesis as a necessary intermediate in the ultimate response to thyroid hormone was further strengthened by the demonstration that puromycin, an inhibitor of protein synthesis, could block the biological action of the hormone (Tata, 1963). In turn, the regulation of cellular protein synthesis was largely attributed to nuclear function by the observations of Widnell and Tata (1963) that *in vitro* liver nuclear RNA polymerase activity was accelerated in T_3-treated hypothyroid rats within 10 hours of T_3 administration and that actinomycin D, an inhibitor of nuclear RNA synthesis, could also block the calorigenic action of thyroid hormone (Tata, 1963). *In vivo* RNA polymerase activity, assessed by changes in the specific activity of extractable RNA after prior *in vivo* administration of [^{14}C]orotic acid, was shown to be increased in T_3-

treated hypothyroid rats as early as 3–6 hours after administration of hormone (Tata, 1964b; Tata and Widnell, 1966).

In the 15 years since these studies were performed, progress in defining the mechanism by which thyroid hormone accelerates and coordinates nuclear RNA synthesis and cytoplasmic protein synthesis has in some respects been disappointing but not at all fruitless. Eukaryotic RNA polymerases have been extensively studied and T_3-induced increases in polymerase activity can usually be ascribed to one or more of the three major nuclear polymerases. RNA polymerase I is active in low ionic strength media, insensitive to α-amanitin, and transcribes rRNA within the nucleolus. RNA polymerase II is active at high ionic strengths, is very sensitive to α-amanitin, and is thought to mediate the transcription of mRNA. RNA polymerase III is active at low ionic strength, only moderately sensitive to α-amanitin, and likely is responsible for the transcription of tRNA (Roeder, 1976). *In vitro* assay of nuclear RNA polymerase in isolated nuclei at specified ionic strength in the presence or absence of α-amanitin can be a measure of the quantity of one or more of the nuclear RNA polymerases, but also reflects the availability of DNA template initiation sites and the intrinsic transcribing efficiency of the enzyme–template complex. The complication imposed by the presence of potentially variable endogenous templates can be eliminated by assaying extractable polymerase activity on a defined, exogenous template. The capacity of the endogenous template to support RNA synthesis independent of variations in the quantity or efficiency of the endogenous nuclear polymerase is often determined by incubation of isolated chromatin with a saturating amount of a common exogenous RNA polymerase, e.g., *E. coli* polymerase.

Addition of rifampin to the polymerase–template preparation allows transcription only by polymerase molecules already complexed to the DNA at the initiation site or in the course of transcribing a DNA sequence, thus facilitating the calculation of the number of initiation sites per unit of template (M.-J. Tsai *et al.*, 1975). How the products of transcription regulate the rate and capacity of the cytoplasmic compartment to maintain protein synthesis is largely uncertain. Increased production of rRNA must be a preliminary to the proliferation of ribosomes and polysomes, and this proliferation has been observed during the period of augmented *in vitro* protein synthesis (Tata and Widnell, 1966). Surely, the regulation of protein synthesis is not a simple process. The factors that determine the general rate and capacity of the cell to synthesize the wide spectrum of cellular proteins must be different from the factors that regulate the synthesis of one or few specific proteins during times of unaltered total cell protein synthesis. The ribosomal population of the cell may be the dominant factor in determining the total or base line protein synthetic rate. An increased hepatocyte polysome population is observed in conjunction with the augmentation of total cytoplasmic protein synthesis after T_3 treatment of hypothyroid rats (Tata and Widnell, 1966), as noted above, and a decrease in the polysome population of mouse sarcoma cells is observed during the synthetic deceleration that accompanies starvation (Geoghegan *et al.*, 1979; McNurlan *et al.*, 1979). Transfer RNA production, in addition to mRNA production, is another proposed regulator of the capacity for net cellular protein synthesis (Osterman, 1979). The synthesis of specific proteins is probably regulated through one or more of a variety of pathways, dependent on the individual protein and the particular stimulus involved in its induction. Proposed regulatory mechanisms for synthesis of specific proteins are many and varied and include the actions of nuclear regulatory factors on the DNA template (MacLean and Hilder, 1977), or the nascent pre-mRNA transcript (Remington, 1979), and the activation of cytoplasmic messenger ribonucleoprotein particles (Geoghegan *et al.*, 1979), among other possibilities. Postulated mechanisms governing the induction of specific proteins by thyroid and other hormones are discussed at greater length elsewhere in this chapter and in this volume.

Studies to define the temporal coordination of nuclear RNA synthesis and cytoplasmic protein synthesis have yielded surprisingly similar results. Tata and Widnell (1966) have evaluated multiple liver nuclear and cytoplasmic functions at various times after administration of a single dose of T_3 to hypothyroid rats. RNA polymerase I activity in isolated nuclei was increased by 10–12 hours after T_3 administration and peaked at 45 hours. Polymerase II activity was elevated at 45 hours but was unchanged as late as 24 hours. Assay of rapidly labeled nuclear RNA suggested an increase in *in vivo* RNA polymerase activity (compared to untreated control rats) as early as 3–4 hours after T_3 treatment. Most of the rapidly labeled nuclear RNA was characterized as rRNA. Total cytoplasmic rRNA content was elevated 35–45 hours after T_3, which coincided with the peak of *in vitro* microsomal protein synthesis, and was associated with an increase in the number and size of polysomes. The time course of several of these functions is depicted in Fig. 1.

In a similar experiment Viarengo *et al.* (1975) also determined that the onset of augmented rat liver RNA polymerase I activity occurred 10 hours after T_3 treatment. The onset of increased polymerase II was noted 24 hours after treatment, which was also in accord with the study of Tata and Widnell (1966). Additionally, polymerase III, assayed as α-amanitin-resistant activity in the presence of a high ionic strength medium, was found to increase by 10 hours after treatment. In another study, the same group (Zoncheddu *et al.*, 1977) confirmed the increase in RNA polymerase I activity in isolated nucleoli 10 hours after T_3 treatment, and demonstrated that this increase was also detectable when the enzyme was solubilized and assayed on an exogenous DNA template. The conclusion that the increased polymerase I activity in isolated nucleoli was attributable to an increased content of the enzyme rather than to a T_3-induced change in the native template was supported by their finding that the number of template

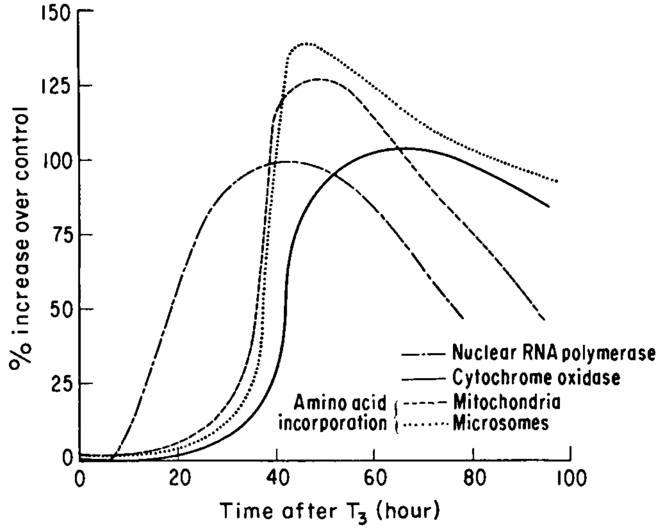

Fig. 1. Time course of T_3-stimulated cellular activities in hypothyroid rats. Thyroidectomized rats were injected with 20 μg T_3/100 g body weight and sacrificed at various times after T_3 administration. Nuclear RNA synthesis, microsomal protein synthesis, and mitochondrial protein synthesis and cytochrome oxidase activity were assayed *in vitro*. Activity is expressed as a percentage of the activity observed in uninjected thyroidectomized control rats. Reprinted from Tata (1964a) with permission of the publisher and author.

initiation sites was not changed 10 hours after treatment by a binding site assay employing [^{3}H]actinomycin D. Cycloheximide, administered 30–60 minutes prior to sacrifice to block *in vivo* protein synthesis, effectively inhibited the hormone-induced increase in polymerase I activity, but not the base line level of polymerase activity. Their suggestion that the thyroid hormone-induced increase in polymerase I activity was dependent on the presence of a concurrently synthesized protein with a short half-life was supported by a study of DeGroot *et al.* (1977c). In the latter study, the authors could show increases in total polymerase, polymerase I, and polymerase II activities in isolated liver nuclei 8–10 hours after treatment of hypothyroid rats with T_3. Determination of polyadenylated RNA content, a presumptive measure of mRNA content and, indirectly, polymerase II activity, was similarly elevated at the same interval after treatment. Cycloheximide administered at the same time as T_3 was effective in inhibiting the T_3-induced increase in polymerase activities, and also reduced the base line polymerase activities to slightly below those obtained in untreated hypothyroid rats. Mitochondrial α-glycerophosphate dehydrogenase activity was simultaneously measured as an example of a cytoplasmic response to T_3 and found to be enhanced after T_3 treatment and inhibited by cycloheximide treatment, implying its dependency on cytoplasmic protein synthesis. Dillmann *et al.*, (1978a) determined that rapidly labeled liver nuclear heterogeneous RNA and rapidly labeled polysomal mRNA were increased in hypothyroid rats as early as 6 hours after treatment with T_3. Jothy *et al.* (1975) observed an unusually rapid increase in *in vitro* polymerase II activity in liver nuclei 40–80 minutes after a single, low dose of T_3 was administered to hypothyroid rats. Unfortunately, this interesting finding has been lacking in confirmation.

Early T_3-induced generalized increases in protein synthesis have also been suggested by the finding of an increased specific activity of label recovered in nuclear proteins after the *in vivo* administration of labeled amino acids to T_3-treated hypothyroid animals. Implied in this type of experiment is that a more actively protein-synthesizing cell will add a greater amount of newly synthesized labeled protein to a pool of preexisting unlabeled protein. That varying amounts of preexisting protein, protein degradation rates, and substrate amino acid pools do not account for the observed differences in the specific activity of the recovered protein fraction is difficult to assure. Using this technique, Tata (1966) demonstrated an increase in *in vitro* synthesis of nonbasic liver nuclear proteins 10–11 hours after treatment of hypothyroid rats with a single dose of T_3. Bernal *et al.* (1978a) in a similar study demonstrated an increase in the synthesis of liver histone and nonhistone nuclear proteins as well as in pooled cytosol proteins within 5–8 hours of T_3 treatment in hypothyroid rats. Even assuming that these findings represent increased levels of *in vivo* protein synthesis, their significance as a unique, early response to thyroid hormone, or simply the more sensitive detection of the generalized increase in *in vitro* protein synthesis observed many hours later, is difficult to ascertain.

It seems reasonable to consider that the burst of liver nuclear RNA polymerase activity observed 8–12 hours after initiation of T_3 treatment in hypothyroid rats is at least partly responsible for the increase in cytoplasmic protein synthetic capacity seen 30–45 hours after treatment. The demonstration of increased quantities of extractable cytoplasmic rRNA, and of longer and more numerous polysomes, coincident with this peak of protein synthetic activity implies that more rRNA need be transcribed prior to the peak of activity. Further, it seems reasonable that more mRNA and tRNA is also required to this end. The approximately 24-hour lag period between the RNA and protein synthetic peaks is also consistent with this speculation. What is less tenable is the view that the peak of polymerase activity at 8–12 hours is independent of ongoing protein synthesis or antecedent to any T_3-induced effect on protein synthesis. The blockade of T_3-induced nuclear RNA polymerase activity by cycloheximide is good evidence that polymerase activity is highly dependent on ongoing protein synthesis, although it need not imply that the supportive protein synthesis is in some way related to the thyroid status of the animal. Mishima *et al.* (1979) have shown that cycloheximide or puromycin treatment markedly decreases nucleolar RNA synthesis in Ehrlich ascites tumor cells within 1 hour of drug administration. The studies of Yu and Feigelson (1972) and Lambert and Feigelson (1974) provided additional evidence for a short-lived protein supportive of RNA polymerase I activity. In these studies, it was found that the addition of poly d(A-T) to nuclei augments the activity of polymerase I. The poly d(A-T) did not inhibit or compete with the polymerase I transcribing the endogenous template, but appeared to provide an available template for unbound, nontranscribing polymerase I. *In vivo* administered cycloheximide inhibited the activity of the polymerase transcribing the native template, but did not affect the poly d(A-T)-transcribing enzyme. It was thus hypothesized that a rapidly turning-over protein may be continually regulating the activity of template-bound polymerase I, and that this activity can be quickly reduced by blocking the ongoing synthesis of this protein. The apparent dependence of hormone-induced synthesis of specific mRNA's on uninhibited, ongoing protein synthesis has more recently been demonstrated in several model systems. Chen and Feigelson (1979) have shown that cycloheximide treatment will block the dexamethasone-induced synthesis of α_{2u}-globulin mRNA in isolated rat hepatocytes. In other studies, inhibition of protein synthesis also blocks the induction of tryptophan oxygenase mRNA by hydrocortisone (DeLap and Feigelson, 1978) and the induction of ovalbumin mRNA by estrogen (McKnight, 1978).

If this hypothetical protein proved to be thyroid hormone responsive in its action, it might explain the increase in RNA polymerase activity in response to T_3 administration and the failure to see this increase in cycloheximide pretreated animals. It can be hypothesized that there may be a tonic stimulation of RNA polymerase activity by a rapidly turning-over protein. In a hypothyroid rat, 15 hours prior to T_3 treatment, for example, a small amount of this protein may be synthesized in the cytoplasm. Possibly acting on one or more nuclear RNA polymerases with a lag period of several hours, it could maintain synthesis of a small quantity of their respective RNAs. These transcription products are in turn used to support the synthesis of the various cellular proteins after another lag period of several hours. As depicted in Fig. 2, when T_3 is administered to the animal at time 0, there may be a brief lag period for absorption, binding to cell T_3 receptors, and for whatever processes are antecedent to an increase in the synthesis of, or the activation of, the RNA synthesis-supportive peptide. The resulting increase in quantity or efficiency of this peptide acting in the nucleus within a few hours of T_3 administration thus leads to an increase in RNA polymerase activity observed 8–10 hours after T_3 treatment. The increase in the various RNA's lead to enhanced protein synthetic capacity which becomes obvious by 30–45 hours after T_3 administration. When cycloheximide is administered with T_3, the synthesis of the RNA synthesis-supportive peptide is blocked as is the subsequent T_3-inducible burst of RNA synthesis approximately 8–10 hours later. The observation that cycloheximide blocks subsequent increases in T_3-responsive RNA polymerase activity (DeGroot *et al.*, 1977c; Zoncheddu *et al.*, 1977)

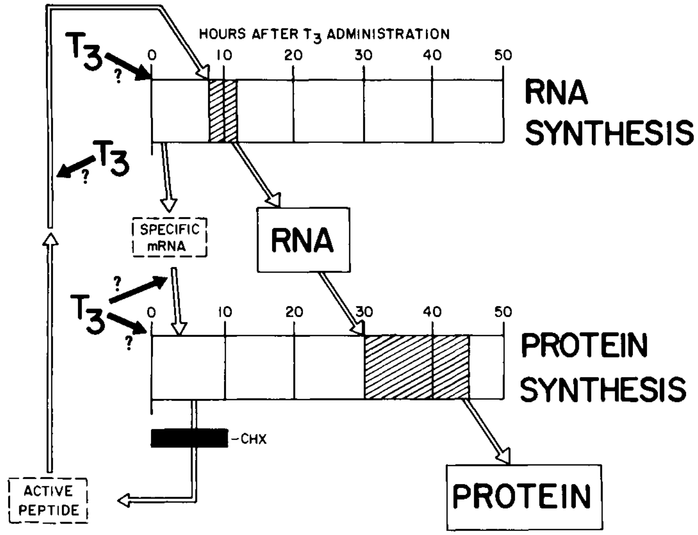

Fig. 2. Hypothetical schema for the role of T_3 in the induction of mass increases in total RNA and protein synthesis. A labile, active peptide that supports nuclear RNA synthesis is continuously synthesized in the cytoplasm directed by a specific mRNA. In hypothyroid animals, this peptide is synthesized in small quantities. Approximately 8 hours after T_3 administration to hypothyroid animals the quantity or activity of this peptide within the nucleus is enhanced, inducing subsequent increases in total RNA synthesis. By 30–45 hours after T_3 administration the accumulation of mRNA, tRNA, and rRNA permit increased activity in total cellular protein synthesis. Blockade of these T_3-induced effects of cycloheximide (CHX) administered with T_3 suggests that the initial action of T_3 precedes the cytoplasmic synthesis of the active peptide. This action may occur at specific mRNA transcription, at the level of mRNA processing, or at the ribosome itself. Alternatively, T_3 may function by activating a previously synthesized precursor of the active peptide, in which case the cycloheximide block of T_3 effects is a consequence of limiting production of the inactive precursor.

also argues that the action of T_3 precedes the synthesis of the hypothetical protein, perhaps at the level of its mRNA synthesis or processing. Alternatively, T_3 may serve to activate a previously synthesized but inactive precursor whose production would be limited by the cycloheximide blockade of protein synthesis. The increases in rapidly labeled RNA and rapidly labeled nuclear proteins observed within a few hours of T_3 administration (Bernal *et al.*, 1978a; Dillmann *et al.*, 1978a); Tata and Widnell, 1966) may relate to the synthesis of early intermediates of the action of T_3, but independent supportive evidence is not yet available.

In view of the finding that the augmented *in vitro* nuclear RNA synthetic capacity observed 8–10 hours after T_3 administration to hypothyroid rats is largely attributable to an increased presence of RNA polymerase, it is possible that the RNA synthesis-supportive peptide is itself RNA polymerase or a subunit thereof.

The nuclear T_3 receptor is unlikely to be the hypothetical peptide. Approximately one-half of these receptors are T_3 occupied in hypothyroid rat liver nuclei (Oppenheimer *et al.*, 1974b). Administration of a bolus of T_3 to these animals should lead to an increase in T_3–receptor complexes within minutes, long in advance of the post-T_3 increase in RNA synthetic capacity. The saturation of the nuclear receptors with T_3 is not, however, a cycloheximide-sensitive process as is the T_3-induced stimulation of nuclear RNA synthesis. Experimentally, T_3–receptor complexes added to rat liver nuclei *in vitro,* in contrast to estrogen–receptor complexes (Taylor and Smith, 1979), are ineffective in stimulating RNA synthetic capacity (DeGroot and Rue, 1980b).

More consistent with the chronicity and cycloheximide sensitivity of T_3-induced stimulation of nuclear RNA synthesis is the hypothesis that RNA synthesis is dependent on newly synthesized T_3 receptor which is somehow linked to the presence of T_3 itself. It is almost inescapable that the nuclear T_3 receptor, or its precursor, is synthesized in the cytoplasm and transported to the nucleus. Simple diffusion of the receptor from its intracytoplasmic site of synthesis into the nucleus does not seem economically sound unless restricted to small channels of endoplasmic reticulum leading into the nucleus. Studies by DeGroot and Rue (1980a) have shown that isolated rat liver nuclei take up T_3–receptor complexes with much greater avidity than a variety of other peptide hormones (Fig. 3). Although this uptake did not have the character of an energy-requiring process, the lower uptake rates of the presumably more diffusible smaller peptides suggest

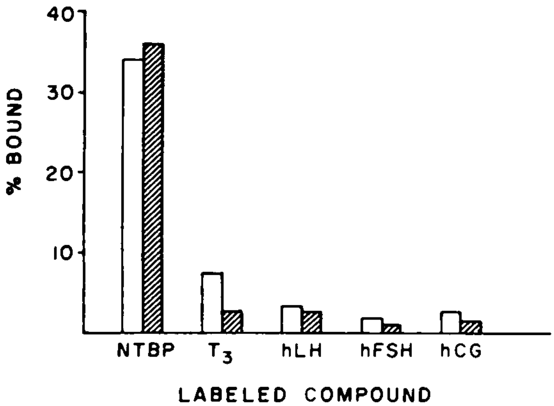

Fig. 3. *In vitro* nuclear uptake of rat liver T_3-nuclear T_3 binding protein complexes (NTBP). Rat liver nuclei were incubated in the presence of labeled NTBP, human luteinizing hormone (hLH), human follicle stimulating hormone (hFSH), human chorionic gonadotropin (hCG), or T_3. Duplicate tubes (shaded bars) additionally contained bovine serum albumin. After incubation for 2 hours, the nuclei were sedimented, washed, and counted. Nuclear uptake (binding) of label is expressed as a percent of the total label in the incubation mixture. Reprinted from DeGroot and Rue (1980a) with permission of the publisher and authors.

that there may be a specific nuclear membrane mechanism for sequestering extranuclear T_3 receptors. Whether this uptake in some fashion actively regulates the intranuclear activity of T_3 or T_3–receptor complexes is unknown. Alternatively, the nuclear uptake mechanism may serve only to maintain the nuclear supply of T_3 receptors.

There is in fact little if any additional evidence to argue that the synthesis of T_3 receptor is not itself a regulatory process rather than a loosely regulated means to replenish the nuclear stock of receptors. The inability to demonstrate in the cytoplasm a T_3 receptor physicochemically identical to the nuclear T_3 receptor implies that the newly synthesized binding protein is either inactive while in the cytoplasm or present in undetectably small quantities. Currently, it does not appear that acute T_3 treatment increases the nuclear content of T_3 receptors (Oppenheimer *et al.*, 1974a), although some (Hamada *et al.*, 1979; Nakamura *et al.*, 1979), but not all (Bernal *et al.*, 1978b; DeGroot and Rue, 1979), investigators find that experimental thyrotoxicosis does increase the nuclear content of T_3 receptors in rat liver. In cultured GH_1 pituitary cells, T_3 administration actually decreases the nuclear content of T_3 receptors (Samuels *et al.*, 1976, 1977).

Dillmann *et al.* (1977) have shown that α-amanitin can block the induction by T_3 of α-glycerophosphate dehydrogenase and malic enzyme. A presumptive "long-lived intermediate" of the induction was thought to be formed independent of RNA synthesis and to persist longer than the high intracellular content of free T_3. The intermediate thus served to promote the delayed synthesis of malic enzyme and α-glycerophosphate dehydrogenase mRNA's after the inhibition of RNA polymerase by α-amanitin was thought to have dissipated. If the messengers for malic enzyme and α-glycerophosphate dehydrogenase are among the mRNA's transcribed 8–12 hours after T_3 administration to hypothyroid rats, the "long-lived intermediate" may coincide with the peptide hypothesized to mediate the T_3-induced stimulation of nuclear RNA synthesis. Whether the formation of this intermediate is sensitive to cycloheximide is unknown, but it would be of significant interest.

Although perhaps promising, the status of our understanding of T_3 mediation of nuclear RNA synthesis is as yet inadequate. The evidence for the existence of the hypothetical peptide mediator of RNA synthesis described above, or that proposed by Dillmann *et al.* (1977) for the control of malic enzyme and α-glycerophosphate dehydrogenase, is not compelling, and the nature of these mediators is even more open to question and speculation. Also, it is not established that an extra- or intranuclear T_3 receptor functions within this general framework. The gap between the binding of T_3 to its receptor and the altered expression of the genome may be chronologically small but remains largely enigmatic.

Other models of the induction of RNA and protein synthesis are interesting to compare with the thyroid hormone model. The regenerating liver has been particularly well studied. After a partial hepatectomy in which approximately two-thirds of the liver is excised, the liver remnant passes through an orderly sequence of events to regenerate its original mass. A wave of DNA synthesis occurs at approximately 18 hours after hepatectomy, preceding mitosis by approximately 6 hours (Mayfield and Bonner, 1972). In the mouse, protein synthetic activity as measured by the *in vivo* incorporation of [^{14}C]leucine is 60% greater than nonregenerating liver 36 hours after hepatectomy (Scornik, 1974). RNA polymerase activity determined in isolated rat liver nuclei is increased twofold 18 hours after hepatectomy (Organtini *et al.*, 1975). Solubilized preparations of RNA polymerases I and II from the same animals retained increased activity on an exogenous template, indicating that either the polymerase molecules were more numerous or more active, but the native template itself need not have been altered significantly. Titration of nucleolar extracts of polymerase I (obtained 12–14 hours after hepatectomy) with increasing quantities of exogenous template demonstrated that the enzyme preparation from the hepatectomized rats were more active at all concentrations of added template, but reached a maximum activity at the same concentration as the control preparation (Schmid and Sekeris, 1975). These results implied that, at this time interval after hepatectomy, the polymerase I molecules were intrinsically more active but not more numerous. During this interval the nuclear T_3 receptor complement has been shown to decrease, although the relationship of this phenomenon to other concurrent nuclear events is as yet undefined (Dillmann *et al.*, 1978c). The earliest events noted after hepatectomy are increased rapidly labeled high molecular weight nuclear RNA, increased chromosomal RNA, and increased chromatin template activity, which are observed, respectively, at approximately 1, 2, and 3 hours after operation (Mayfield and Bonner, 1972). This sequence of events, allowing for the lack of studies directly comparing regenerating liver with that of T_3-stimulated liver, does not appear to be radically different from the chronology of T_3-stimulated events (excepting the DNA synthetic activity).

The T_3-induced increase in *in vitro* nuclear RNA polymerase activity observed 8–12 hours after T_3 treatment of hypothyroid animals is generally a slower response than that observed in other hormonal inductions of nuclear RNA polymerases. RNA polymerase I activity assayed in isolated normal or adrenalectomized rat liver nuclei or nucleoli is stimulated within 1½–3½ hours after the administration of hydrocortisone (Sajdel and Jacob, 1971; Todhunter *et al.*, 1978). RNA polymerases I and III are elevated in isolated rat uterine nuclei within 1½–3 hours of treatment with estrogen (Weil *et al.*, 1977). Growth hormone adminsitration to hypophysectomized and adrenalectomized rats induces increases in polymerases I and II within 1 hour (Spelsberg and Wilson, 1976). TSH administered to thyroid hormone-suppressed dogs induces elevations of RNA polymerases I, II, and III in isolated thyroid nuclei within 1½ hours (Kleiman de Pisarev *et al.*, 1979). RNA polymerase II activity in isolated chick oviduct chromatin is stimulated within ½ hour of treatment with estrogen (S. Y. Tsai *et al.*, 1975). Stimulation of RNA polymerase activity could be effected within 30 minutes in isolated oviduct nuclei of chicks withdrawn from estrogen by direct addition of purified estrogen–receptor complexes to the nuclear incubation medium (Taylor and Smith, 1979). Although the comparison of RNA polymerase induction intervals should be done with reservations when different hormones and different tissues are compared, thyroid hormone induction of generalized polymerase activity may in fact be relatively slow in onset. This observation, if correct, might imply that a more complicated sequence of events is involved in the interval between the entry of thyroid hormone into the cell and the generalized acceleration of RNA polymerase activity.

B. Synthesis of Specific mRNA's and Proteins

The generalized increases in total RNA and protein synthesis previously discussed may well be secondary events which are stimulated by agents also functional in nonthyroid hormone related metabolic stimuli. Several models of thyroid hormone-induced synthesis of specific mRNA's and proteins have also been described which are less likely to represent generalized responses. Ostensibly, the incremental increase in total RNA synthetic activity attributable to the augmented synthesis of one species of mRNA is usually too minor to be apparent.

α_{2u}-Globulin is a major urinary protein of male rats described in 1966 by Roy and Neuhaus (1966b). It is synthesized in the liver, secreted into the circulation where it is present in only small amounts, and concentrated in the urine (Roy and Neuhaus, 1966a). Its formation requires the simultaneous presence of glucocorticoid, growth hormone, thyroid hormone, and androgen, and is repressed by estrogen (Roy, 1973; Roy and Neuhaus, 1967). The function of α_{2u}-globulin is unknown, but its absence in prepubertal and in female rats suggests that it does not play a major role in the action of thyroid hormone. It is, nevertheless, a good example of an individual protein whose synthesis is directed by a specific mRNA in response to thyroid hormone. Specific mRNA activity was detected by recovery of an *in vitro* synthesized [^{3}H]leucine labeled protein which is immunoprecipitable with anti-α_{2u}-globulin antibody and comigrates with a known standard of α_{2u}-globulin in polyacrylamide gel electrophoresis. *In vitro* protein synthesis in a cell-free wheat germ translational system was directed by polyadenylated liver RNA thought to contain the spectrum of liver cell mRNA's. The appearance of specific α_{2u}-globulin mRNA after T_3 treatment of hypothyroid rats parallels the appearance of α_{2u}-globulin in the liver cytosol and in the serum (Kurtz *et al.*, 1976). The time course of this induction, however, did not suggest that the process was an early action of thyroid hormone, requiring 48 hours for the mRNA content to increase by 17.5% above control. Induction of α_{2u}-

globulin mRNA by glucocorticoids, however, required only 2 hours in a study by Chen and Feigelson (1978).

In cultured rat GH_1 cells, exposure to T_3 induces measurable increases in intracellular and extracellular growth hormone (GH) within 7 hours (Tsai and Samuels, 1974). Using an *in vitro* wheat germ translational system to measure the GH mRNA activity in polyadenylated RNA preparations, Shapiro *et al.* (1978) could demonstrate an increase in the amount of specific GH mRNA after 48–72 hours of exposure to T_3 and cortisol (Shapiro *et al.*, 1978). The lag period between exposure to T_3 and the synthesis of specific GH mRNA is considerably shorter than 48 hours in other *in vitro* systems. When [^{3}H]leucine was used as an intracellular marker of GH synthesis, Samuels and Shapiro (1976) could show that T_3 induced a 50% increase in GH synthesis within 1¼ hours. If the induction of [^{3}H]leucine incorporation into GH is directed by newly synthesized mRNA, a 1¼-hour response interval is quite consistent with the concept that specific mRNA induction is an early action of thyroid hormone, perhaps preceded only by the mechanism of T_3 receptor binding and function.

Seo *et al.* (1978) directly demonstrated induction of GH mRNA in isolated rat pituitaries, as early as 2 hours after *in vitro* exposure of pituitary tissue from hypothyroid rats to T_3 (Fig. 4). Extractable RNA preparations were enriched in GH mRNA activity as assayed in a rabbit reticulocyte lysate translational system. Prolactin mRNA content was unchanged at this same time interval.

Martial *et al.* (1977) were also able to show that, in cultured rat pituitary cells, T_3 and dexamethasone could induce specific GH mRNA activity in a cell-free translation system. Additionally, they could show that the appropriate GH mRNA nucleotide sequences were synthesized in parallel with the appearance of translational mRNA activity, as analyzed by the hybridization kinetics of extractable RNA with complementary DNA made from a known GH mRNA template. The postulation that T_3 functions by "activating" a preexisting translationally inactive mRNA precursor thereby seems unlikely. Were the latter mechanism true, the hybridization kinetics would have shown little if any change.

In rats treated daily with T_3 and high carbohydrate diet for 1 week, Towle *et al.* (1979b) could demonstrate an increase in the content of specific mRNA for malic enzyme in preparations of liver cytoplasmic polyadenylated RNA compared to those of untreated control animals. T_3 and/or high carbohydrate diets had already been shown to increase malic enzyme activity largely by increasing the cell content of the enzyme rather than by increasing the intrinsic efficiency of the enzyme itself (Isohashi *et al.*, 1971; Murphy and Walker, 1974). Thus, it appears that thyroid hormone ultimately controls this cytoplasmic response by effecting either the rate of specific mRNA synthesis or by activating the translational activity of a stored mRNA precursor. The study by Martial *et al.* (1977), previously discussed, would indicate that the former postulate is more likely.

Comparable mechanisms have been described in non-thyroid-related hormonal

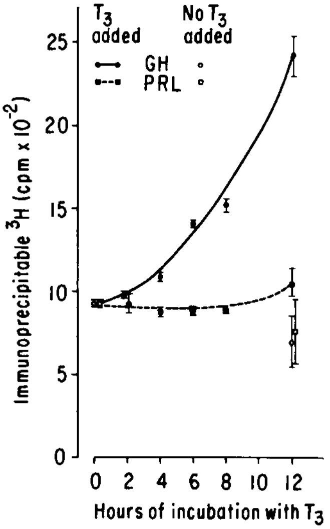

Fig. 4. T_3-induced stimulation of growth hormone (GH) mRNA in isolated rat pituitaries. Hypothyroid rat pituitaries were isolated and incubated for various times with or without 1.5×10^{-10} *M* T_3. After the incubation period pituitary cytoplasmic RNA was isolated and used to direct synthesis of ^{3}H-labeled proteins in a rabbit reticulocyte lysate translational system. *In vitro* synthesized labeled GH was isolated by specific immunoprecipitation with anti-GH antibody. *In vitro* synthesized labeled prolactin (PRL) was similarly isolated with anti-PRL antibody. Base line GH and PRL mRNA activities were determined by omission of T_3 from the incubation medium. Reprinted from Seo *et al.* (1978) with permission of the publisher and authors.

inductions of nuclear activity. Hydrocortisone, administered to rats 3 hours before sacrifice, has been shown to induce an increase in hepatic tryptophan oxygenase mRNA activity as determined in a cell-free Krebs ascites cell translation system (Schutz *et al.*, 1973). Assaying ovalbumin mRNA activity in immature chick oviduct polysomal RNA preparations, Means *et al.* (1972) have shown that ovalbumin mRNA activity was induced after 16 days of primary estrogen stimulation. Upon withdrawal of the estrogen, the ovalbumin mRNA content promptly decreased, but was rapidly reinduced by 24 hours after readministration of estrogen. In a subsequent study by the same group (Comstock *et al.*, 1972), it was shown that, after 4 days of primary estrogen stimulation, the ovalbumin mRNA content of oviduct polysomes was markedly elevated, while the ovalbumin content of the tissue was only minimally elevated. Of additional interest, the ribosomal translational capacity of the tissue, as assessed by the efficiency of the ribosomal preparations to translate poly(U) into [^{14}C]polyphenylalanine, was also elevated after 4 days of treatment. The augmentation of the ribosomal translational capacity was accompanied by an increase in the activity of the peptide chain initiation factors. Their impression was that mRNA synthesis and accumulation was the rate-limiting factor in the hormonal induction of ovalbumin and that cytoplasmic translational capacity may also have a role in hormonal protein induction. S. Y. Tsai *et al.* (1975) demonstrated that estrogen induction of ovalbumin mRNA was detectable within 30–60 minutes of secondary estrogen stimulation and was preceded by an increase in the number of nuclear estrogen receptor complexes and a doubling in the number of chromatin initiation sites for RNA polymerase. Sun and Holten (1978) reported that the refeeding of fasted rats induced a 20-fold increase in hepatic glucose 6-phosphate dehydrogenase activity and a twofold increase in the activity of its mRNA, implying some means of message amplification that they thought could be due to an increase in the efficiency of translation.

Although the evidence that thyroid hormone and other hormones induce the synthesis of specific proteins by increasing the transcription of the specific mRNA's, the processes that are temporally situated between mRNA precursor synthesis and mRNA translation are multiple and potentially regulatable by hormones. The hypothesis that high molecular weight heterogeneous RNA is transcribed as an mRNA precursor undergoes a variety of modifications including "cap" formation, polyadenylation, and "splicing," and associates with various proteins to form a transport and storage unit (ribonucleoprotein particles; informosomes) prior to its translation in the cytoplasm is widely held but not without criticism (Darnell *et al.*, 1976; Williamson, 1977; Revel and Groner, 1978; Ebelson, 1979).

How and if thyroid hormone is involved in the posttranscriptional modification of mRNA or RNP particles is as yet only speculation. Defer *et al.* (1977) have shown that T_3 binds to rat liver RNP particles with an affinity comparable to that of the T_3 receptor in an unfractionated rat liver nuclear extract. Other data also focus on the RNP particle as a possible locus of thyroid hormone action. Two rat liver nuclear proteins whose presence on polyacrylamide gel electrophoregrams appears to be dependent on the thyroid status of the animal have been reported by Bernal *et al.* (1978a) and characterized by Barsano *et al.*, (1980). Two larger cytoplasmic peptides appear to parallel the nuclear peptides in concentration (Fig. 5). One or both of the nuclear peptides may be components of rat liver RNP particles (Barsano *et al.*, 1980; Bernal and Rodriquez-Pena, 1979), on the basis of electrophoretic mobilities identical to known RNP components, although the significance of these findings is unknown and currently under investigation. The existence of a thyroid-responsive regulatory mechanism at the level of the informosome is appealing for its potential to govern at one locus the expression of multiple and diverse cell functions by control of the spectrum of cell mRNA's. The multiplicity of both T_3-induced and T_3-repressed peptides has recently been graphically illustrated on two-dimensional gel electrophoresis by Baxter and coworkers (J. D. Baxter, personal communication).

Exclusive control of cell function at a posttranscriptional level is, however,

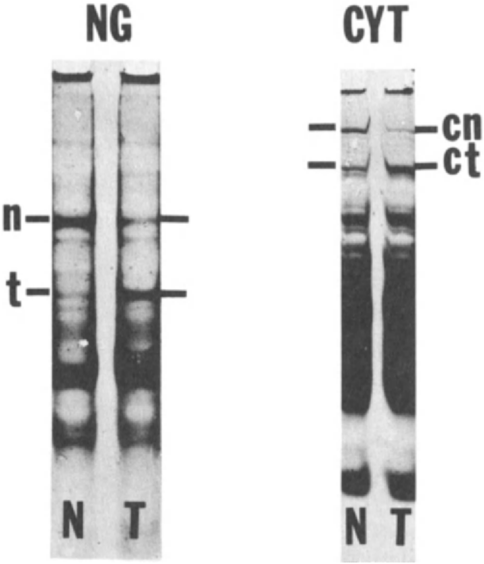

Fig. 5. Thyroid hormone-dependent nuclear and cytosolic proteins. Nuclear proteins (NG) were eluted from normal (N) and from thyroidectomized (T) rats with a buffer containing 0.14 *M* NaCl. The proteins were concentrated, denatured, and electrophoresed in 0.1% sodium dodecylsulfate–8% polyacrylamide gels. One peptide (n) was prominent in normal rats but faint in hypothyroidism. Another peptide (t) was inversely related in concentration to the n-peptide. Cytosolic peptides (CYT) were comparably denatured and electrophoresed. A large cytosolic peptide (cn) paralleled the nuclear n-peptide in intensity in both hypo- and euthyroidism, while a second cytosolic peptide (ct) paralleled the nuclear t-peptide in intensity under these same circumstances. Reprinted from Barsano *et al.*, (1980) with the permission of the publisher and authors.

difficult to reconcile with the data of other studies. Previously described hybridization studies by Martial *et al.* (1977) have shown that GH mRNA induction by T_3 is probably controlled by mRNA synthesis rather than by activation of preexisting mRNA sequences. Assuming that α-amanitin functions by inhibiting RNA polymerase II, the presumptive "long-lived intermediate" described by Dillmann *et al.* (1977) must be a pretranscriptional agent. Differences between normal and hypothyroid rat liver polyadenylated RNA also appear to be quantitative rather than qualitative, based on recent hybridization studies (Towle *et al.*, 1979a), although this work would not reveal differences in the availability of cell mRNA's imposed by the protein components of the RNP particles.

A number of gaps in our understanding of the mechanism of action of thyroid hormone at the nuclear level becomes apparent in even a very general interpretation of the above data. The T_3-induced gross increases in RNA and protein synthesis discussed in Section I appear, by their nature and magnitude, capable of directing the diverse and extensive changes involved in growth, maturation, calorigenesis, and other significant processes commanded by the thyroid hormones. These increases in mass macromolecular synthesis, however, are not observed early enough to assure that they are closely associated with the initial actions of thyroid hormone or the hormone–receptor complex. More importantly, these synthetic responses are observed in many other non-thyroid hormone-related situations and should probably be regarded as general or nonspecific cellular responses.

Thyroid hormone-induced synthesis of GH mRNA is observed to occur early enough after administration of hormone to be consistent with a very direct action of thyroid hormone or its receptor on the transcription apparatus (Seo *et al.*, 1978). Growth hormone synthesis and secretion, doubtless an important effect of thyroid hormone on the pituitary, cannot however be regarded as the mediator of the mass increases in RNA and protein synthesis which are apparent hours later and cannot account for all the gross physiological effects observed in the intact animal.

Induction of malic enzyme by thyroid hormone is similarly effected by induction of its mRNA. Towle *et al.* (1981) have recently shown that malic enzyme mRNA is detectable within 2 hours of T_3 administration. Kinetic analysis further indicates that if a mandatory lag period exists between the exposure to T_3 and the onset of malic enzyme mRNA induction it is considerably shorter than 2 hours. Induction of α_{2u}-globulin mRNA has been shown to occur at a time consistent with the general increase in total cellular RNA synthesis although it cannot be ruled out that more sensitive techniques may reduce this lag period considerably. Likely, other rapidly inducible mRNA's and proteins also exist and perhaps one or more may mediate the later enhancement of mass RNA synthesis.

C. Coordination of Nuclear and Mitochondrial RNA and Protein Synthesis

In a fashion comparable to the coordination of nuclear RNA synthesis and cytoplasmic protein synthesis, T_3-induced changes in mitochondrial respiratory function may be dependent on antecedent changes in mitochondrial RNA and protein synthesis. Mitochondria are known to possess their own ribosomes and tRNA complement, and some of their translation products are incorporated into the mitochondrial inner membrane as structural and enzymatic components. They also contain a primitive genome and a DNA-dependent RNA polymerase whose transcripts may include mitochondrial ribosomal RNA's and possibly some of the mRNA's translated by the mitochondrial protein synthetic machinery (Ashwell and Work, 1970; Rabinowitz, 1968). Although some components of the mitochondrial inner membrane may be locally synthesized the marked size limitation of the mitochondrial genome requires that many mitochondrial proteins be encoded within the nucleus, and probably translated outside the mitochondrion.

Mitochondrial O_2 consumption *in vitro* correlates well with the BMR of animals in different thyroid states (Bronk, 1963; Tata *et al.*, 1963). Further, hormone-related changes in mitochondrial O_2 consumption are likely manifestations of altered numbers of inner membrane respiratory assemblies rather than of changes in the intrinsic respiratory enzyme activities (Tata *et al.*, 1963). This raises the possibility that the products of mitochondrial RNA or protein synthesis, in addition to RNA and protein produced outside the mitochondrion, may be essential components for hormone-induced increases in O_2 consumption.

In vitro mitochondrial protein synthesis has, in fact, been shown to be depressed in hypothyroid animals and increased after initiation of treatment with T_3 (Freeman *et al.*, 1972; Gross, 1971). More recently, *in vitro* mitochondrial RNA synthesis has also been shown to be reduced in hypothyroidism and accelerated by T_3 treatment (Barsano *et al.*, 1977; Gadaleta *et al.*, 1972; Schimmelpfennig *et al.*, 1970). How T_3 ultimately increases the mitochondrial capacity to synthesize RNA and protein is unknown. If the T_3-induced augmentation of mitochondrial RNA synthetic activity involves an increase in the mitochondrial content of its RNA polymerase, changes in extramitochondrial macromolecular synthesis

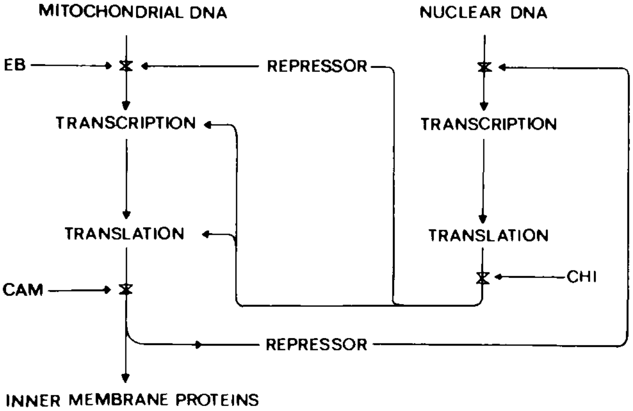

Fig. 6. Mutual repressor control of intra- and extramitochondrial gene products in *Neurospora crassa*. Cycloheximide (CHI), an inhibitor of extramitochondrial protein synthesis, stimulates the intramitochondrial synthesis of mitoribosomes and elongation factors (Barath and Kuntzel, 1972b) by inhibiting production of a hypothetical repressor. Ethidium bromide (EB) and chloramphenicol (CAM), inhibitors of mitochondrial RNA and protein synthesis, respectively, stimulate synthesis of mitochondrial RNA polymerase (coded for and synthesized outside the mitochondrion) by inhibiting production of a second hypothetical repressor. Reprinted from Barath and Kuntzel (1972a) with permission of the publisher and authors.

would be prerequisite. Mitochondrial RNA polymerase is coded for by nuclear cistrons and synthesized on extramitochondrial ribosomes as its activity is uninhibited (and in fact is enhanced) by inhibitors of mitochondrial RNA synthesis (ethidium bromide) and protein synthesis (chloramphenicol) (Barath and Kuntzel, 1972a,b). A hypothetical schema relating the interrelationship of nuclear and mitochondrial RNA and protein synthesis is depicted in Fig. 6.

It is questionable that thyroid hormones act directly at the level of mitochondrial RNA or protein synthesis, although the presence of the mitochondrial T_3 receptor would suggest mechanisms of T_3 action comparable to those hypothesized for the nuclear T_3 receptor on nuclear RNA transcription.

II. NUCLEAR AND CYTOPLASMIC THYROID HORMONE BINDING PROTEINS

Many early studies sought to localize the initial action of thyroid hormone retrospectively by identifying intracellular hormonal actions successively earlier than known hormonal effects. In such fashion, the calorigenic effects of thyroid hormone could be explained by antecedent increases in mitochondrial inner membrane respiratory assemblies. The identification of thyroid hormone receptors offered an alternative approach to understanding thyroid hormone action by presenting a starting point from which to seek, prospectively, the earliest effect of the hormone–receptor interaction and to link this effect to what are regarded as early hormonal actions by other criteria, e.g., the augmentation of nuclear RNA synthesis. The intracellular location of the thyroid hormone receptor might also have been anticipated to suggest the locus of the initial action(s) of thyroid hormones.

The current understanding of intracellular thyroid hormone binding proteins has not, unfortunately, resolved the uncertainties of thyroid hormone action and of the primacy of one or another subcellular organelle. One aspect of controversy involves the ubiquity of thyroid hormone binding proteins within the cell, specifically including components of the plasma membrane, mitochondria, cytosol, and nucleus. Another involves the difficulty in equating a protein with hormone binding activity to a true receptor, i.e., necessary *in vivo* intermediary in the hormone's action. Subsequent sections will include the evidence for intracellular binding proteins, their authenticity as true receptors, and what is speculated to be their function within the nucleus or cytoplasm.

A. Nuclear Binding Proteins

Of the intracellular T_3-binding proteins (T_3BP), the nuclear T_3BP has been the most intensively studied. It has been extensively reviewed in this volume and in several other sources (Baxter and Funder, 1979; DeGroot *et al.*, 1978; Oppenheimer *et al.*, 1979), and it will therefore not be discussed in great detail in this chapter. The nuclear T_3BP is a protein with a molecular weight of approximately 50,500 (Latham *et al.*, 1976), with a sedimentation rate constant of 3.4–4.5 S (Bernal and DeGroot, 1977; Latham *et al.*, 1976). On the basis of its nuclear extraction characteristics, it appears to be a nonhistone protein closely associated with the nuclear chromatin (DeGroot *et al., 1974; Surks et al.*, 1973). Recent studies by Eberhardt *et al.* (1979) suggest the hypothesis that the rat liver nuclear receptor is composed of a "core" molecule associated with certain histones. The "holo" receptor binds T_3 with greater affinity than T_4. After dissociation of the histones, the "core" receptor binds T_4 with an affinity comparable to that of the "holo" receptor, but binds T_3 significantly less avidly than T_4. Its equilibrium affinity constant (K_a) for T_3 is in the order of 5×10^9–5×10^{11} M^{-1} by Scatchard analysis (Bernal *et al.*, 1978; Oppenheimer *et al.*, 1974a). The K_a for T_4 is approximately 1/20–1/40 that of T_3 (Oppenheimer *et al.*, 1974a). These affinities are regarded as "high" by virtue of being at least one or two orders of magnitude greater than other nuclear proteins and are comparable to other hormone-binding protein complexes with assumedly physiologically "high" affinities, e.g., estrogen and its receptor (Aten *et al.*, 1978) and insulin and its receptor (Kahn *et al.*, 1974). The maximum T_3 binding capacity of the nuclear T_3BP in rat liver is low, perhaps only 4000–10,000 binding sites per rat liver nucleus (DeGroot and Strausser, 1974; Oppenheimer *et al.*, 1974a), and in the normal rat, these sites are approximately 40% occupied (Oppenheimer *et al.*, 1974b).

Several lines of evidence suggest, but do not prove, that the nuclear T_3BP is a physiologically important receptor or intermediary in the action of thyroid hormone. The T_3BP binds to various thyroid hormone analogs with affinities generally proportionate to the thyromimetic activity of the analogs, when the *in vivo* longevity of the analogs are taken into consideration (Jorgensen, 1978; Oppenheimer *et al.*, 1973; Samuels *et al.*, 1979). The T_3BP content of various tissues are also roughly proportionate to the alleged thyroid hormone responsiveness of the tissues (Oppenheimer *et al.*, 1974b). Variations in nuclear T_3BP content have been observed to occur in several disease states and stages of development, but for the most part tend to suggest potential roles for thyroid hormones in states previously thought to involve minimal, if any, aspects of thyroid deficiency at the tissue level, rather than correlate with obvious thyroid deficiency or excess. Specifically, nuclear T_3BP content in rat liver is diminished in starvation (Burman *et al.*, 1977; DeGroot *et al.*, 1977a; Dillmann *et al.*, 1978c; Schussler and Orlando, 1978), partial hepatectomy(Dillmann *et al.*, 1978c), and after glucagon administration (Dillmann and Oppenheimer, 1979; Dillmann *et al.*, 1978b), but it is not appreciably altered by hypothyroidism, or in some reports by hyperthyroidism (Bernal *et al.*, 1978b; Oppenheimer *et al.*, 1975; Spindler *et al.*, 1975). The physiological import of the nuclear T_3BP is perhaps most strongly implied by its apparent absence in a patient with the syndrome of peripheral resistance to thyroid hormone (Bernal *et al.*, 1976, 1978c). Heart rate and the reciprocals of serum cholesterol, CPK, and Achilles reflex times have also been shown to be linearly related to the estimated T_3BP occupancy in hyper- and hypothyroid patients (Bantle *et al.*, 1980). In other studies it appears that the T_3-induced synthetic rates of fatty acid synthetase, malic enzyme, 6-phosphogluconate dehydrogenase, and α-glycerophosphate dehydrogenase are maximal when the nuclear T_3BP complement is 95–100% saturated (Mariash *et al.*, 1980; Oppenheimer *et al.*, 1977). When T_3 is administered to hypothyroid rats in quantities sufficient to maintain supranormal degrees of T_3BP saturation in hypothyroid rats for 7 days, evidence of thyrotoxicosis, e.g., decreasing increments of weight gain, has been observed (DeGroot and Rue, 1979).

In short, it appears that the existence of a nuclear thyroid hormone binding protein is certain and its role as a physiological receptor or intermediary in the action of thyroid hormone is most probable, based on a variety of experimental and clinical data. The mechanism by which the T_3–T_3BP complexes function is still unknown, but ultimately probably relates to the induction, and perhaps, repression, of multiple gene products.

The mechanisms by which T_3 and T_3-binding proteins enter the nucleus are in many respects unknown. In contrast to the steroid hormones, T_3 and T_4 do not require complexing by a cytoplasmic receptor to enter the nucleus. That T_3 enters isolated nuclei in the absence of cytosol has been demonstrated in many laboratories. DeGroot and co-workers (1976) found that the addition of cytosol or serum proteins to the incubation medium actually reduced the uptake of T_3 by isolated nuclei (Table I). Inclusion of an excess of unlabeled T_3 with these proteins partially reversed this inhibition, presumably by preventing the loss of labeled T_3 to extranuclear protein binding. Tata (1975) and Defer *et al.* (1976) have reported similar findings. T_3–T_3BP complexes formed *in vitro* were virtually the same as those formed *in vivo* when Sephadex G-100 elution profiles, rates of nuclear release, maximal nuclear binding capacities, and salt-extraction characteristics were compared (Surks *et al.*, 1975). These studies indicate that authentic nuclear T_3–T_3BP complexes can be formed in the absence of cytoplasmic factors, but do not rule out the possibility that some of these complexes may have originated outside the nucleus. DeGroot and Rue (1980a), as discussed earlier, have shown that isolated nuclei do, in fact, have an avidity to take up T_3–T_3BP complexes, which is greater than their avidity for a variety of other protein hormones (Fig. 3). Admittedly, the phenomenon observed *in vitro* cannot a priori be assumed to occur *in vivo* in view of the unknown extent of compromise to the integrity of the nuclear membranes suffered during nuclear isolation and washing.

TABLE I

Effect of Cytosol on T_3 Binding to Rat Liver Nuclei[a]

Incubation conditions	T_3 bound/100 μg DNA
N nuclei	27
+ N cytosol, 5 mg	4.2
+ N serum, 4 mg	14.2
+ BSA, 5 mg	16.7
H nuclei	13.7
+ H cytosol, 1.4 mg	7.9
+ N cytosol, 1.0 mg	7.2
H(I) nuclei (T_3 75 ng)	11.9
+ H cytosol, 1.4 mg	7.6
+ N cytosol, 1.0 mg	5.7
N nuclei	8.7
+ H cytosol, 1.4 mg	4.5
+ H cytosol (heated), 1.4 mg	4.2
H nuclei	11.4
+ H cytosol, 1.6 mg	5.4
+ N cytosol, 1.6 mg	1.6
N nuclei	17
+ H cytosol, 1.6 mg	5.5
+ N cytosol, 1.6 mg	1.8
H(I) nuclei (T_3 200 ng)	12.6
+ H cytosol, 1.6 mg	3.4
+ N cytosol, 1.6 mg	1.1

[a] Nuclei isolated from normal (N), hypothryoid (H), or T_3-injected hypothyroid [H(I)] rats were assayed for *in vitro* T_3-binding capacity in the presence or absence of cytosol or bovine serum albumin (BSA). Reprinted from DeGroot *et al.* (1976) with permission of the publisher and authors.

It is inescapable that the nuclear T_3-binding protein is synthesized in the cytoplasm, but subsequent to that point virtually nothing is known of its route to the nucleus. It may be inactive while in the cytoplasm, perhaps unrecognized because of its greatly reduced affinity for T_3 when not complexed to histones (Eberhardt *et al.*, 1979). It is possible that the receptor is secluded within the membranes of the endoplasmic reticulum, arriving at the nucleus unexposed to the pool of cytoplasmic T_3. Alternatively, it may be complexed to T_3 soon after synthesis and enter the nucleus in the T_3-occupied state. As noted above, available data argue against the obligation that T_3 be protein bound to enter the nucleus, but do not rule out the possibility that some T_3 may enter the nucleus bound to its receptor, and do not by any means preclude that the T_3-binding protein itself may require binding to T_3 for nuclear entry. The finding of a normal liver nuclear T_3-binding protein content in hypothyroid rats suggests that unoccupied binding protein may also be admitted to the nucleus, but until this possibility is investigated in rats with undetectable, rather than simply subnormal, T_3 and T_4 levels, this question may be difficult to answer. The presence of unoccupied T_3-binding proteins in the nucleus may represent formerly occupied receptors in view of the generally short half-life of T_3–T_3BP complexes at 37°C (Jaffe and Means, 1977). Thus, their presence does not necessarily imply that unoccupied receptors can enter the nucleus. A reasonable judgment on when and how the T_3-binding protein enters the nucleus must await further studies.

The fate of the T_3-binding protein *in vivo* is virtually unknown. Whereas its locus of synthesis may comfortably be assumed to be extranuclear, its site of degradation cannot. Isolated nuclei have been shown to be very susceptible to receptor leakage in the absence of Ca^{2+} or in the presence of EDTA (Bernal and DeGroot, 1977), but their porosity *in vivo* is probably significantly less. The fact that little, if any, T_3-binding protein with the physical characteristics of the nuclear receptor can be recovered from the extranuclear compartments suggests that little leakage occurs *in vivo*, or that the receptor becomes inactive in the cytoplasm. In GH_1 pituitary cell cultures, Samuels *et al.* (1976, 1977, 1979) have demonstrated that exposure to T_3 induces a depletion of a fraction of the nuclear T_3 receptors, and that this phenomenon is linked to growth hormone synthesis. The actual mechanism for the loss of T_3 receptor activity in this model, however, is unclear.

B. Cytosol Binding Proteins

Cytosolic thyroid hormone binding proteins have been known for as long as their nuclear counterparts, although their physiological import has been less well established. The binding of labeled T_4 to intracellular proteins or extracted cytosol proteins has been reported as early as 20 years ago (Robbins and Rall, 1960; Tata, 1958). It was noted by Tata in 1958 that a cytosolic extract from rat skeletal muscle firmly bound $[^{131}I]T_4$. On paper electrophoresis, the bound $[^{131}I]T_4$ migrated further than free T_4 but not as far as labeled T_4 bound to the serum T_4-binding protein, thus indicating that the cytosol protein was not simply a plasma contaminant. Similarly, T_4-binding proteins from rabbit muscle and brain tissue were described by Mante-Bouscayrol *et al.* (1962). They migrated as β- or γ-globulins in contrast to the serum binding protein which appeared to be an α-globulin. Essentially the same findings were obtained by Grinberg (1964) in extracts of pituitary thyrotropic and mammotropic tumor cells.

Substantial investigation of cytosol thyroid hormone binding proteins has been undertaken by Hamada and co-workers. Liver cytosol incubated with $[^{131}I]T_3$ and $[^{131}I]T_4$ could be fractionated into three peaks of radioactivity by DEAE-cellulose column chromatography (Hamada *et al.*, 1970). One peak appeared to be a binding protein specific for T_3 while the other bound both T_3 and T_4. The molecular weight of these proteins was determined by Sephadex G-100 chromatography to be greater than 100,000; thus, neither protein could be regarded as the serum T_4 binding protein. In human liver cytosol, Hamada and Nanno (1975) demonstrated two major T_4-binding proteins on thin layer Pevikon electrophoresis. Addition of unlabeled T_4 to the cytosol resulted in displacement of the label from one protein to the other. Using sucrose gradient ultracentrifugation, they could show that the displacement was from an 8 S protein to a 4 S protein. The 8 S moiety, although of limited T_4-binding capacity, on the basis of the displacement experiments, had an affinity for T_4 which was 10-fold lower ($K_a = 2.4 \times 10^7 M^{-1}$) than that of the nuclear T_4-binding protein by Scatchard analysis. Unlabeled T_3 was not effective in displacing labeled T_4 from the cytosolic binding proteins. Diphenylhydantoin, salicylate, and heparin were able to displace labeled T_4 from the T_4-binding protein, but not able to displace labeled T_3 from the T_3-binding protein (Hamada and Fukase, 1976b). In contrast to the binding of T_4 by the cytosol protein, serum protein T_4 binding is not displaceable by heparin *in vitro* (Schatz *et al.*, 1969). The T_4 and T_3 liver cytosol binding proteins could additionally be distinguished from serum albumin, TBPA and TBG by their electrophoretic characteristics as β- and γ-globulins, respectively (Hamada and Fukase, 1976a). More recent studies by Hamada *et al.* (1977) show that human liver cytosol T_4 binding can be ascribed to 8.3 S and 4.7 S proteins on sucrose gradient ultracentrifugation, and that T_3 was ineffective in displacing T_4 from either protein. Cytosolic T_3 binding could be localized to a 4.2 S protein by the same technique. Binding of labeled T_3 by the 4.2 S protein, however, could be reversed by addition of T_4 to the medium.

In 1971, Spaulding and Davis described three T_4-binding proteins in rat liver cytosol. By polyacrylamide gel electrophoresis, the molecular weights were estimated to be 45,000, 95,000, and 130,000. The largest protein was present only in female rats, but could be induced in male rats by estrogen treatment. The 45,000 molecular weight protein had the same electrophoretic mobility as TBPA but, in contrast to TBPA, its T_4-binding ability was inhibitable by dilantin or D-T_4. Each of these proteins could also bind T_3. Interestingly, the 95,000 and 130,000 molecular weight proteins were not present in gut, muscle, or kidney cell cytosols. In cultured human fibroblasts, Refetoff *et al.* (1972) could identify electrophoretically different binding proteins for T_3 and T_4. Both proteins were also electrophoretically distinct from TBPA, albumin, and TBG. A protein that binds both T_4 and T_3 with high affinities (K_a's $= 1.4 \times 10^9 M^{-1}$ and $0.4 \times 10^9 M^{-1}$, respectively) was demonstrated in the cytosol of porcine anterior pituitary cells by Sufi *et al.* (1973). Posterior pituitary cell or frontal lobe brain cell cytosols also contained the binding protein, but in reduced quantities. The binding protein was distinct from serum T_4-binding proteins on thin layer chromatography. Two peaks of labeled T_4-binding activity were identified by Sephadex G-200 chromatography and by polyvinylchloride electrophoresis in rabbit liver cytosol by Liewendahl (1974). The T_4-binding capacity of the cytosol was relatively large (3.5–10.5 μg T_4/mg protein) but the binding affinities for T_4 were less than those of either human TBG or human TBPA.

The relative affinities (K_a's) of cytosol binding proteins for T_3 and T_4, as determined by Scatchard analysis, have generally been quite similar, differing by a factor of 10 or less. Samuels *et al.* (1974), in GH_1 pituitary cell cultures, and Sufi *et al.* (1973), in porcine anterior pituitary cells, found the affinity of the cytosol binding protein for T_4 to be greater than that for T_3. A similar relationship was found in dog kidney and liver (Davis *et al.*, 1974). Dillmann *et al.* (1974) and Defer *et al.* (1975) found rat liver cytosol binding proteins to have less affinity for T_4 than for T_3. Michelot *et al.* (1979) found the same relationship in human red blood cells. The affinity for T_3 of the nuclear binding protein is uniformly reported to be greater than that of the cytosol binding protein (Samuels *et al.*, 1974; Tata, 1975)—in one case by a 200-fold higher K_a (Dillmann *et al.*, 1974). The difference in affinities for T_4 between nuclear and cytosol binding proteins is less distinct, with both higher (Samuels *et al.*, 1974) and lower (Dillmann *et al.*, 1974) cytosol T_4 affinities reported.

Relative binding affinities for thyroid hormone analogs are also somewhat different between nuclear and cytoplasmic binding proteins. The nuclear T_3 binding protein generally binds triiodothyroacetic acid (triac) and isopropyldiiodothyronine (ip-T_2), as well as, or more strongly than, T_3 (DeGroot and Torresani, 1975; Oppenheimer *et al.*, 1973), whereas the cytosol T_3 binding protein binds T_3 with a substantially greater affinity than it binds triac or ip-T_2 (Defer *et al.*, 1975; Dillmann *et al.*, 1974).

The maximal thyroid hormone binding capacity of the cytosol binding protein is generally found to be significantly greater than that of the maximal nuclear hormone binding capacity (Davis *et al.*, 1974; Defer *et al.*, 1975; Dillmann *et al.*, 1974; Liewendahl, 1974). Dillmann *et al.* (1974) found the maximal cytosol T_3-binding capacity to be approximately 170 times that of the nuclear capacity and that less than 1% of the binding proteins were occupied, whereas 70% of the nuclear sites were occupied. Subsequent studies (Oppenheimer *et al.*, 1974b) show that nuclear binding protein occupancy is probably more in the order of 40–50%, but the latter values are still considerably greater than the cytosol estimate.

Molecular weight estimates of the cytosol thyroid hormone binding protein(s) are usually greater than the estimates for the nuclear protein. Whereas the nuclear binding protein is thought to have a molecular weight of approximately 50,000 (Latham *et al.*, 1976), cytosol binding protein estimates range from 45,000 to greater than 100,000 (Davis *et al.*, 1974; Hamada *et al.*, 1970; Spaulding and Davis, 1971). In contrast to the nuclear T_3-binding protein, the cytosol T_3-binding protein of bullfrog tadpoles described by Yoshizato *et al.* (1975) demonstrates significant dependence on the presence of divalent cations for T_3 binding.

Chelation of divalent cation with EDTA inhibits T_3 binding in a manner which could be overcome by the addition of excess Mn^{2+}, Ca^{2+}, Mg^{2+}, Ba^{2+}, Sr^{2+}, or Ca^{2+}. T_3 binding by rat liver cytosol, however, is abolished by *p*-hydroxymercuribenzoate (Defer *et al.*, 1975), as is nuclear rat liver T_3 binding (DeGroot *et al.*, 1974).

The content, and perhaps even the identify, of cytosol thyroid hormone binding proteins differ among the various tissues of individual animals. Yoshizato *et al.* (1975) described a T_3-binding cytosol protein in bullfrog tadpole tissues whose K_a's for T_3 were generally quite similar ($3.6–11.6 \times 10^6 M^{-1}$) in tail fin, muscle, and kidney, but was substantially higher ($68.0 \times 10^6 M^{-1}$) in liver. Conversely, the maximal binding capacities of the same three tissues were considerably greater (0.86–10.4 pmole/mg protein) than that of liver tissue (0.04 pmole/mg protein). In dog liver and kidney, Davis *et al.* (1974) found that the thyroid hormone cytosol binding proteins were electrophoretically identical. The K_a for rat liver cytosol T_3 binding ($0.88 \times 10^7 M^{-1}$) was also found to be essentially the same as that of rat cerebellar cytosol T_3 binding ($0.66 \times 10^7 M^{-1}$) by Geel (1977). The maximal binding capacity for T_3 of liver cytosol, however, was markedly greater (2.45 pmol/mg protein) than that of cerebellar tissue (0.75 pmol/mg protein).

The numerous reports cited above and summarized in Table II provide ample evidence that there are one or more proteins in the cytosol of liver and other tissues which bind T_4 and/or T_3 with high affinity, and are distinct from the serum-binding proteins. It is not uniformly established that there are separate binding proteins for T_4 and T_3 rather than more than one binding protein for either T_4 or T_3. It is still controversial if these binding proteins have a physiological role in the action of thyroid hormone, or even a potentially expendable role as an intracellular reservoir for thyroid hormones comparable to the serum thyroid hormone binding proteins. The generally large cytosol capacity for T_4 and T_3 relative to the nuclear hormone binding capacity may suggest a role as a reservoir rather than as a specific regulatory protein. Interestingly, Murthy *et al.* (1978) found that after injection of $[^{125}I]T_3$ into rats, relatively more label localized in the nuclei and less in the cytosol of hypothyroid rat liver than in the same compartments of normal rat liver. Their finding that hypothyroid rat liver has a subnormal cytosol T_3-binding capacity led them to speculate that decreased cytosol binding protein content in hypothyroid rat liver permits an elevated cytosol free T_3 content, which favors enhanced nuclear uptake and binding of T_3 to the nuclear receptors. The impression that cytosol binding protein content passively regulates the availability of cytoplasmic T_3 to the nuclei is supported by a similar but unpublished study by Geel in rat brain (S. Geel, personal communication).

The affinities of the cytosol proteins for thyroid hormones and their analogs differ somewhat from those determined for the nuclear receptor, but do not rule

TABLE II

Cytosolic Thyroid Hormone Binding Proteins

Reference	Hormone[a]		Source[b]
	T_4	T_3	
Tata (1958)	+		Rat skeletal muscle
Mante-Bouscayrol *et al.* (1962)	+		Rat muscle and brain
Grinberg (1964)	+		Pituitary tumor
Spaulding and Davis (1971)			
(i)[c]	+	+	Pituitary tumor (45,000)
(ii)	+	+	Pituitary tumor (95,000)
(iii)	+	+	Pituitary tumor (130,000)
Refetoff *et al.* (1972)			
(i)	+		Human fibroblasts
(ii)		+	Human fibroblasts
Liewendahl (1974)			
(i)	+		Rabbit liver
(ii)	+		Rabbit liver
Hamada *et al.* (1977)			
(i)	+		Human liver (8.3 S)
(ii)	+		Human liver (4.7 S)
(iii)		+	Human liver (4.2 S)
Sufi *et al.* (1973)	++	+	Pig anterior pituitary
Samuels *et al.* (1974)	++	+	Rat pituitary GH_1 cells
Davis *et al.* (1974)	++	+	Dog kidney and liver
Dillmann *et al.* (1974)	+	++	Rat liver
Defer *et al.* (1975)	+	++	Rat liver
Michelot *et al.* (1979)	+	++	Human erythrocytes

[a] (+) Demonstrable binding to T_4 or T_3; (++) bound with greater affinity.

[b] Sedimentation constant or molecular weight in parentheses.

[c] Biochemically distinct proteins within the same tissue are given separately.

out physiological significance. In several studies, T_4 is bound more firmly than T_3 (Davis *et al.*, 1974; Samuels *et al.*, 1974; Sufi *et al.*, 1973) in contrast to the nuclear binding protein, although T_3 is decidedly more potent as a hormone. At present, however, there is no reason to insist that the binding of a thyroid hormone to the cytosol receptor is so related to the initiation of hormone-affected cellular processes that equal binding of T_3 or T_4 should be followed by equal responses. That the cytosol binding proteins have a role in the regulation of the intracellular conversion of T_4 to the more active T_3 cannot be ruled out and may suggest that these proteins should bind T_4 much more strongly than T_3. Consistent with the affinity data obtained for nuclear binding proteins, and with thyromimetic activities of various analogs (Jorgensen, 1978), the cytosol binding proteins have no affinity for tyrosine, MIT, or DIT (Davis *et al.*, 1974; Defer *et*

TABLE III

Equilibrium Association Constants (K_a) and Maximal Binding Capacities (M) of Cerebellar and Liver Cytosolic T_3 Binding Proteins during Development

Tissue	Age (days)	K_a (liters/mol × 10^{-7})	M (pmol/mg protein)
Cerebellum	10	6.40	0.79
	20	1.25	0.87
	50	0.66	0.75
Liver	10	1.43	0.43
	20	1.25	1.16
	50	0.88	2.45

[a] Reprinted from Geel (1977) with permission of the publisher and author.

al., 1975; Michelot *et al.*, 1979), as would be expected from their lack of hormonal activity. The binding of the cytosol protein to reverse T_3 is also appropriately low (Dillmann *et al.*, 1974). Perhaps unexpectedly, D-T_4 was quite effective at displacing labeled L-T_4 from dog kidney and liver cytosol proteins (Davis *et al.*, 1974). This finding need not militate against a physiological role for the cytosol proteins, as D-T_4 is not without hormonal activity, and in the nucleus D-T_3 binds to the receptor almost as firmly as L-T_3 (DeGroot and Torresani, 1975; Oppenheimer *et al.*, 1973). The acetic acid derivative of T_3, triac, binds to the nuclear receptor as well as T_3. The reduced thyromimetic activity of triac compared to T_3 can be explained by its more rapid *in vivo* metabolism. Rat liver cytosol T_3 binding proteins, however, bind T_3 with significantly higher affinity than triac (Defer *et al.*, 1975; Dillmann *et al.*, 1974)., and thereby preclude the necessity for invoking the same argument required to explain the disproportionate binding and activity data for triac in the nucleus. The markedly reduced affinity of the rat liver cytosol binding protein for ip-T_2 (Dillmann *et al.*, 1974) does, however, cast a doubt on the proposition that the cytosol protein mediates the action of thyroid hormones, as this analog is the most potent thyroid hormone congener known, even more potent than T_3 (Jorgensen, 1978). Rat liver nuclear receptors bind ip-T_2 as well as T_3, and rat heart muscle nuclear receptors bind it even more firmly than T_3 (Oppenheimer *et al.*, 1973).

An interesting study by Geel (1977) demonstrated that rats, in the first 50 days of life, undergo a dramatic 10-fold reduction in the affinity of their cerebellar cytosol T_3-binding protein without a change in the MBC (Table III). Implied in these findings is the hypothesis that the decrease in the binding protein K_a results in the decreasing responsiveness of this tissue to thyroid hormone during maturation and correlates well with the observation that in rats the architecture of the cerebellar cortex is virtually completed within the first 3 weeks of life. Liver cytosol T_3-binding protein exhibited only a minimal decrease in K_a under the same circumstances, but underwent a marked increase in the MBC. Interestingly, the neonatal rat liver nuclear T_3 receptor also exhibits a marked increase in the MBC during this period of life, with a much less dramatic increase in its K_a for T_3 (DeGroot *et al.*, 1977b). Dozin-Van Roye and DeNayer (1978) have reported that the affinity of rat brain cytosol T_3 receptors is distinctly greater at 12–15 days of age than at 3 days, but declines to a very low level in adulthood. In this study, the maximal binding capacity of the cytosol T_3 receptor was essentially unchanged, whereas the liver cytosol maximal binding capacity markedly increased in the first 25 days of life.

Defer *et al.* (1976) found that the maximal binding capacity of rat liver cytosol T_3-binding proteins decreased in 2 weeks following thyroidectomy, and then began to increase to supranormal levels (Fig. 7). To some extent, this phenomenon was accompanied by parallel changes in the maximal binding capacity of the nuclear T_3-binding protein. The biological significance of this correlation is unknown, and consequently it is doubtful that it can be offered as strong evidence for the physiological relevance of the cytosol binding proteins.

The case for acknowledging that the cytosol binding proteins have a significant role as necessary mediators in the action of thyroid hormone is admittedly weak, but may be so only because of insufficient study. Studies relating thyroid hormone and analog binding affinities, cytosol maximal binding capacity, and degree of binding protein saturation to quantifiable hormonal effects would be most interesting. Similarly, further studies regarding binding protein affinities, maximal binding capacities and degree of hormonal occupancy in states of thyroid hormone excess or deficiency, and in tissues with varying degrees of hormone responsiveness, might prove very helpful in assessing the physiological

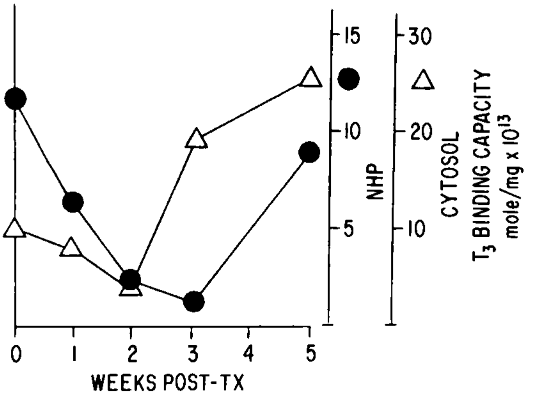

Fig. 7. Effect of thyroidectomy (TX) on rat liver nuclear and cytosol maximal T_3-binding capacities. NHP, nuclear nonhistone proteins. Binding capacities given in moles of T_3 per milligram of protein. Reprinted from Defer *et al.* (1976) with permission of the publisher and authors.

importance of the cytosol binding proteins. What is known of the cytosol binding proteins does not suggest an obvious active function in cell metabolism. The potential role of the cytosol binding proteins as an intracellular reservoir for thyroid hormones has already been discussed and until a more active function for these proteins is identified the "reservoir hypothesis" appears to be the most tenable.

C. Mitochondrial Binding Proteins

The binding of thyroid hormones to mitochondria and to other extranuclear organelles is not a recent finding (Robbins and Rall, 1960). The ubiquity and magnitude of nonspecific binding of tracer quantities of thyroid hormones demands, at a minimum, some demonstration of its saturability or limited presence within the cell compared to the bulk of other cellular constituents. This criterion is usually met by the demonstration that binding of a tracer quantity of labeled hormone can be reduced when coadministered with an excess of unlabeled hormone. The saturability of mitochondrial T_3 binding has been controversial for several years. Oppenheimer *et al.* (1972) could not show displaceable binding of *in vivo* administered labeled T_3 in rat liver mitochondrial fractions under conditions in which displaceable nuclear binding was readily demonstrable. Similarly, Grief and Sloane (1978) were unable to show displaceable T_3 binding in Triton X-100 extracts of sonicated mitochondria. More recently, Sterling has been able to demonstrate displaceable T_3 binding to a Triton X-100 extract of a mitochondrial membrane fraction after both *in vitro* (Sterling and Milch, 1975) and *in vivo* (Sterling *et al.*, 1979) exposure to labeled T_3. The relative binding affinities of the mitochondrial receptor to thyroid hormone analogs are generally comparable to those of the nuclear receptor. L-T_3 binds more strongly than L-T_4; L-T_4 binds more strongly than tetraiodothyroacetic acid (tetrac) or D-T_4; and 3′-isopropyl-T_2 (ip-T_2) binds more strongly than L-T_3 itself (Sterling, 1977). Notably, the equilibrium affinity constants (K_a's) of the mitochondrial binding protein have been reported to be over 100-fold higher than those of the nuclear binding protein for both T_3 and T_4 (Sterling, 1977).

After an initial fractionation of the mitochondrial membrane extract on Sephadex G-200, the mitochondrial binding proteins appear to have a molecular weight of approximately 150,000 (Sterling *et al.*, 1979). Sudan staining of the polyacrylamide gel electrophoregrams of the partially purified mitochondrial membrane fraction indicate the presence of lipoproteins in the extract (Sterling, 1977), though not necessarily as components of a binding protein. Similarly, affinity chromatography of a binding protein preparation reveals the presence of lecithin, phosphatidylethanolamine, and cardiolipin among other mitochondrial components (Sterling *et al.*, 1980b).

Evidence that mitochondrial T_3-binding proteins mediate an action of thyroid hormone *in vivo* is less compelling, at present, than that for the nuclear binding protein. Sterling *et al.* (1980a) have reported an increase in ATP production and O_2 consumption by isolated liver mitochondria from hypothyroid rats treated with as little as 3 ng T_3/100 g bw intravenously 30 minutes prior to sacrifice. This very rapid onset of action implies that RNA synthesis and cellular or mitochondrial protein synthesis are unlikely to have a role in the induction of these increases but rather that T_3 may directly affect the enzymatic apparatus responsible for these increases. Actinomycin D, cycloheximide, and chloramphenicol have accordingly been shown to be ineffective in the inhibition of these T_3-induced changes (Sterling *et al.*, 1980a). That the mitochondrial T_3-binding protein mediates the increased ATP production and O_2 consumption by isolated mitochondria, however, has not been directly established. The finding that the mitochondrial T_3-binding protein is present in tissues generally thought to be thyroid hormone responsive (liver, kidney, myocardium, skeletal muscle, intestinal mucosa, adipose, lung, and neonatal rat brain), but not in the alleged thyroid hormone unresponsive tissues (spleen, testis, and adult rat brain), indirectly supports a physiological role for this binding protein, just as similar findings support a physiological role for the nuclear T_3-binding protein (Sterling *et al.*, 1978).

The correlation of mitochondrial T_3-binding capacity, degree of saturation, or analog binding affinities with the capacity of mitochondria to produce ATP in response to T_3 or its analogs should be useful in establishing the relevance of the mitochondrial T_3-binding protein.

D. Plasma Membrane Binding Proteins

Pliam and Goldfine (1977) have reported high affinity, limited capacity T_3 binding to a rat liver plasma membrane preparation. D-T_3 and L-T_4 were also bound by the membrane fraction with affinities appropriately lower than that of L-T_3. Gharbi and Torresani (1979) have recently described specific binding of T_4 to rat liver plasma membranes. T_3 was less avidly bound by these receptors than T_4, while D-T_4 and rT_3 were bound even less avidly than T_3. Phospholipase A treatment considerably reduced T_4 binding to these receptors. A second membrane receptor, specific for T_3, was also described, although the affinity of the T_4 receptor for T_4 was distinctly greater than the affinity of the T_3 receptor for T_3.

Other studies (Goldfine *et al.*, 1975a,b, Segal *et al.*, 1977), which demonstrate T_3-induced increases in *in vitro* uptake of 2-deoxy-D-glucose and of nonmetabolizable amino acids by certain cells in culture, suggest that the plasma membrane may be a thyroid-responsive organelle. The incomplete inhibition of these plasma membrane functions by inhibitors of RNA and protein synthesis further suggests that thyroid hormone may in part affect the plasma membrane directly.

Extensive studies on the uptake of T_3 by isolated rat liver cells (Eckel *et al.*, 1979) support the concept of carrier-mediated translocation of T_3 across the plasma membrane. Cytosol T_3-binding proteins did not appear to play a role in the uptake of T_3. It is not known if the previously described plasma membrane T_3 receptors are components of the T_3 uptake mechanism or components of another intramembrane mechanism involved in the initiation of a separate thyroid hormone action.

III. SUMMARY AND SPECULATION

The uncertainty of the mechanics of thyroid hormone action both in the nucleus and in the cytoplasm render speculation on the interrelationships between the nuclear and cytoplasmic events highly vulnerable to the possibility of drastic change as new experimental data becomes available. Few, if any, aspects of nuclear–cytoplasmic interrelationships appear certain, although several concepts appear consistent with the body of data presently available. Aspects of growth, development and calorigenesis appear highly dependent on gross, cytoplasmic protein synthesis, which, in turn, appears closely related to the gross capacity of the nucleus to synthesize RNA's (messenger, ribosomal, and transfer). In the thyroid hormone-related stimulation of net nuclear RNA synthesis, the enhanced RNA synthetic capacity seems not to be a particularly early event after administration of T_3 to hypothyroid animals and appears primarily attributable to increased quantities (presumably newly synthesized) of available RNA polymerase rather than to augmented template activity. These same phenomena of sequential enhancement of RNA and protein synthetic capacity and cellular and whole animal responses are also observed in other metabolic situations not clearly related to thyroid hormone status. Consequently, these observations, however well documented and in detail enlarged on, may only represent an important cellular response set in motion by a wide variety of metabolic stimuli of which the amelioration of hypothyroidism is only one. The possibility remains that many stimuli to cellular growth and function are mediated by processes involving thyroid hormones, but this concept is wholly speculation that is difficult to actively rule out.

The induction of particular mRNA's by T_3 in certain tissues seems to be a specific action of thyroid hormone in the nucleus leading to a selective cytoplasmic response. The time course of this phenomenon in at least two cases is appropriately short, there being no need to suspect many steps within the interval

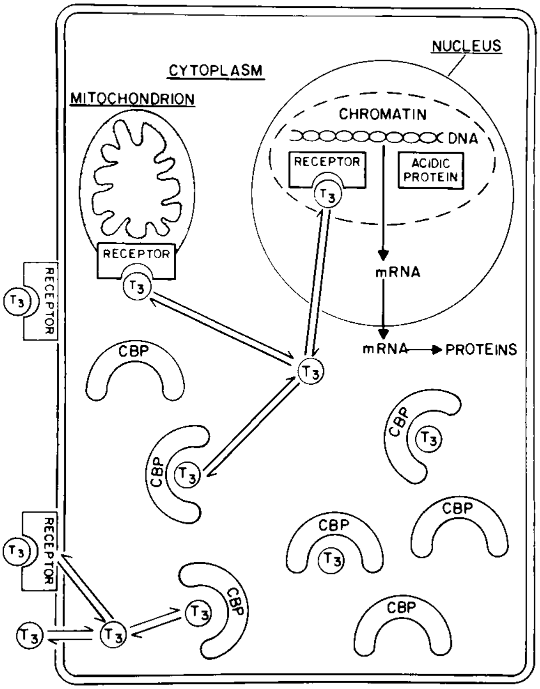

Fig. 8. Cellular T_3-binding proteins. Receptors for T_3 are indicated in the cytosol (CBP), plasma membrane, nucleus, and mitochondrion. The biochemically distinct receptors may mediate thyroid hormone activities in their respective cellular compartments or may facilitate the handling of T_3 en route to a single, primary locus of action. Reprinted by permission from *The New England Journal of Medicine*, with the consent of the author (Sterling, 1979).

in which T_3 enters the nucleus and leads to transcription of a specific mRNA. The nature of the gene products, e.g., growth hormone, α_{2u}-globulin, and malic enzyme, can hardly account for the variety of cell, tissue, organ, and whole animal effects that characterize the presence of thyroid hormone in the euthyroid organism, and probably represent precisely identified end products rather than key intermediates in the subsequent cascade of thyroid hormone effects. Perhaps the realm of thyroid-mediated effects on growth and development can be attributed to induction of a large number of different tissue-specific mRNA's and their translation products. This may well be the case, although the factors (if they are not the thyroid hormones or receptors themselves) which regulate when these mRNA's are turned on and off are at present totally enigmatic.

The coordination of nuclear and cytoplasmic events draws attention to the ribonucleoprotein particle or "informosome." The reports previously discussed concerning the binding of T_3 to these particles and their possible association with certain thyroid status-dependent nuclear proteins, suggest that this posttranscriptional, pretranslational site may be an important locus of thyroid hormone regulation.

The study of cellular T_3-binding proteins has surely raised more questions concerning the integration of thyroid-dependent nuclear and cytoplasmic events than it has answered, as illustrated in Fig. 8. Nuclear T_3-binding protein studies relating tissue content, degree of saturation, T_4 analog binding, and apparent human deficiency to various thyroid-related physiological parameters strongly imply a role in the mediation of thyroid hormone action. The nature of this role, however, is still unclear, although various published studies and studies in progress imply a role in the regulation of genomic expression. The mitochondrial, cytosol, and plasma membrane T_3-binding proteins share a number of important chemical and kinetic characteristics with the nuclear receptor, but have been less extensively studied. Certain important cellular phenomena, e.g., uptake of amino acids and deoxyglucose, and mitochondrial ATP synthesis, have been reported to occur rapidly after *in vitro* or *in vivo* exposure to T_3, but as yet there is little to substantiate that these effects are mediated by the described extranuclear T_3-binding proteins. Likewise, there is little evidence to argue against these proposed roles for the extranuclear T_3-binding proteins. *In vitro* and *in vivo* studies do not support an essential role for plasma membrane or cytosol binding proteins in the formation of nuclear T_3–T_3BP complexes in the fashion exemplified by the steroid hormones and their receptors, but do not exclude a regulatory role for this process *in vivo*. Metabolic states may exist in which T_3 access to the nucleus is restricted or facilitated by the extranuclear T_3-binding proteins. Alternatively, the hypothesis that thyroid hormones act through several unrelated pathways mediated by unrelated receptors cannot as yet be abandoned even if not particularly attractive to our preconceptions of the mechanics of hormone action.

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6

Effects of Thyroid Hormones on Cellular RNA Metabolism

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I. INTRODUCTION

Thyroid hormones are well known to elicit an amazingly diverse set of developmental and physiological responses in many different tissues of higher vertebrates (Wolff and Wolff, 1964). The underlying cellular mechanism(s) by which thyroid hormones affect this wide gamut of responses is unknown, withstanding

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several decades of intense investigation. Currently, the nuclear hypothesis of thyroid hormone action appears to be gaining the strongest experimental support (Samuels, 1978; Oppenheimer, 1979; Baxter *et al.*, 1979). According to this model, the first significant interaction of thyroid hormones in the cell is with receptor proteins located in the nucleus. This interaction is thought to lead to specific alterations in the production of nuclear RNA. These alterations in RNA synthesis result in changes in protein synthesis and subsequently lead to altered cell function. This scheme for thyroid hormone is quite analogous to current views of steroid hormone action (O'Malley and Means, 1974; Yamamoto and Alberts, 1976).

Specific nuclear sites capable of binding thyroid hormones in a limited capacity, high affinity manner have been identified in many target tissues (Oppenheimer *et al.*, 1972, 1974a; Samuels *et al.*, 1974; DeGroot and Torresani, 1975; Spindler *et al.*, 1975). Several lines of correlative evidence suggest the involvement of these binding sites with certain physiological actions of thyroid hormones (Samuels, 1978; Oppenheimer, 1979). The proposed causal relationship between hormone–receptor interaction and nuclear RNA production is presently untested. However, many efforts to correlate changes in thyroid hormone status with RNA metabolism have been reported. The purpose of this chapter will be to review these changes in RNA metabolism and to attempt to correlate the changes with the mechanism of action of thyroid hormones. The chapter will be divided into two parts. The first will deal with "general effects" on RNA populations, that is, measurements in which total RNA, or a large subgroup of total RNA, have been assessed without regard to the possible specificity of changes in individual species in the heterogeneous population. These studies have been performed almost exclusively on two systems: the rat liver and the amphibian tadpole. Since the results are largely analogous between the two systems, this review will deal primarily with the rat liver, the system with which I am most familiar. (For reviews on thyroid hormone action and amphibian metamorphosis, see Frieden and Just, 1970; Cohen, 1970; Tata, 1971.) The second part of the chapter will focus on studies on specific messenger RNA's and several potentially useful systems for analyzing the molecular mechanisms of thyroid hormone action.

II. GENERAL RNA EFFECTS

Before beginning a discussion of effects of thyroid hormone on RNA, it is worthwhile noting several important features in the design of such experiments. The importance of these aspects was first brought to the forefront by the pioneering work of J. R. Tata and his colleagues in the early and mid-1960's (Tata *et al.*, 1963; Tata and Widnell, 1966). In these experiments, the time course of events following administration of thyroid hormone to thyroidectomized rats was closely examined. The aim of this protocol was to identify the time required for each particular parameter to first respond to hormone administration (lag time). In this way, it was thought possible to differentiate between primary responses, those directly affected by hormone–receptor interaction, and secondary effects to an earlier intracellular hormonal response. Since the time required for thyroid hormone to equilibrate with nuclear binding sites is quite rapid (≥ 30 minutes) (Oppenheimer *et al.*, 1974b), one would expect primary responses to be detectable within the first few hours following hormone administration. In contrast, many of the commonly employed measurements of thyroid action in rat liver, such as tissue oxygen consumption and mitochondrial and microsomal enzyme activities, are affected only after 2–3 days of treatment (Tata *et al.*, 1963). Thus, these parameters are likely representative of secondary hormonal effects.

A second important feature of experiments on thyroid hormone action relates to the dose of hormone used. The effects of thyroid hormones can be dramatically different when administered at low physiological doses as opposed to high pharmacological doses used to induce hyperthyroidism. This is perhaps most simply illustrated by comparing the resumption of body growth associated with low doses of thyroid hormones given to hypothyroid rats and the severe tissue "wasting" accompanying prolonged hyperthyroidism. It is, thus, important to note the dose of hormone used in various studies. On a chronic basis, 0.3–0.4 μg 3,5,3′-triiodo-L-thyronine (T_3) per 100 g body weight daily is sufficient to normalize many parameters of the thyroidectomized rat such as body weight gain. Chronic administration of higher doses (e.g., 20 μg T_3/100 g body weight) will lead to hyperthyroidism and, in the extreme, thyrotoxicosis. Administration of thyroid hormone on an acute basis presents a more complex picture. For instance, Tata and co-workers frequently injected thyroidectomized rats with a single dose of 20 μg T_3/100 g body weight. This amount was chosen as the minimum dose of T_3 given acutely which could elevate the basal metabolic rate of thyroidectomized rats to the normal level. This normalization was reached at about 70 hours after injection by which time the serum T_3 levels had undoubtably returned to the hypothyroid range. However, during the first 24–36 hours after injection, the animals would be transiently hyperthyroid with regard to plasma T_3 levels. Thus, the effects of T_3 in such experiments are a complicated function of a constantly changing serum T_3 level varying between super- and subphysiological values. Analyzing dose–response relationships between receptor binding and hormonal effects is consequently difficult.

A. Nuclear RNA Polymerase Activities *in Vitro*

One of the earliest effects of thyroid hormone treatment on thyroidectomized rats noted by Tata and co-workers was in the RNA synthetic activity of isolated nuclei (Widnell and Tata, 1963; Tata and Widnell, 1966). After appropriate hormonal treatment of animals, nuclei were isolated from the liver and incubated *in vitro* in the presence of the four ribonucleotides, one of which was radioactively labeled. The ability of these nuclei to convert radiolabeled ribonucleotide to a trichloroacetic acid insoluble form was measured. By altering the reaction conditions, it was possible to assay predominantly RNA polymerase I activity, the enzyme responsible for ribosomal RNA synthesis, or RNA polymerase II activity, the enzyme that transcribes heterogeneous nuclear and messenger RNA. Following a single injection of 20 μg T_3/100 g body weight into thyroidectomized rats, an increase in the Mg-activated RNA polymerase I activity was first observed at approximately 10 hours. At 24–48 hours after injection, the response was maximal at a level 1.6-times greater than that of untreated thyroidectomized controls. This maximal level in T_3-treated thyroidectomized rats was virtually the same as that measured in euthyroid rats. For the Mn-activated RNA polymerase II activity, no change was detectable until 24 hours following treatment. The maximal response at 48–72 hours was 1.4- to 1.5-fold that of hypothyroid controls, again similar to the level observed in normal rat liver nuclei. These studies first led Tata and co-workers to postulate an early nuclear RNA event in the course of thyroid hormone action.

The observations of Tata have been confirmed and extended by a number of groups. Viarengo *et al.* (1975) used the mushroom toxin, α-amanitin, to differentiate between RNA polymerase I activity (amanitin-resistant) and RNA polymerase II activity (amanitin-sensitive). Similarly to the results of Tata, Viarengo *et al.* found a 50% increase in RNA polymerase I activity at 10 hours after hormone treatment (30 μg T_3/100 g body weight) and a 120% increase at 24 hours. RNA polymerase II activity was first significantly elevated at 24 hours. By separating nucleolar and nucleoplasmic compartments, these authors suggest a rise in RNA polymerase III activity (responsible for 5 S and tRNA synthesis) concomitant with the increase in RNA polymerase I activity. DeGroot *et al.* (1977) also measured significant increases in nuclear RNA polymerases following injection of T_3 (15 μg/100 g body weight) into thyroidectomized rats. In these studies, a small, but significant, increase in RNA polymerase II activity at 8–10 hours of treatment was found. Both Viarengo *et al.* (1975) and DeGroot *et al.* (1977) found no significant differences in the levels of endogenous ribonuclease activities in the isolated nuclear assays.

A very early enhancement of nuclear RNA polymerase II activity following T_3 treatment was reported by Jothy *et al.* (1975). These workers found a transient elevation of this polymerase activity occurring between 40 and 80 minutes when rats were injected with a low dose of 0.1 μg T_3/100 g body weight. The maximal response was twofold over hypothyroid values. No increase in RNA polymerase I activity in the same time range was observed. While such a rapid effect of a low dose of T_3 in nuclear RNA synthesis would indeed be interesting, this report awaits corroboration despite efforts in several laboratories (Viarengo *et al.*, 1975; DeGroot *et al.*, 1977; H. L. Schwartz and J. H. Oppenheimer, personal communication).

RNA synthesis in isolated nuclei is known to be largely due to the completion of RNA chains initiated *in vivo* and in the process of elongation at the time of nuclear isolation. Increases in RNA synthetic activity of nuclei can be potentially attributed to a number of different causes. One possibility is that a larger number of RNA polymerase molecules are actively engaged in RNA synthesis. Such an increase could be due to an increased number of RNA polymerase molecules present or an increase in the availability of template DNA sites for transcription. Alternatively, it is conceivable that differences in the inherent catalytic activity of RNA polymerase (moles nucleotide incorporated per minute) are responsible for altered levels of nuclear RNA synthesis.

Shields and Tata (1976) attempted to test the effect of T_3 on the total amount of RNA polymerase present in rat liver. Nuclear RNA polymerases were extracted and partially purified from rat liver prior to and 36 hours after treatment with T_3 (20 μg/100 g body weight). RNA polymerase levels were assayed using exogenous deproteinized DNA template to avoid potential changes in endogenous nuclear DNA availability. No difference was found between the total activity of extracted RNA polymerase I or II as a consequence of hormone treatment, despite the fact that at 36 hours of treatment whole nuclear RNA polymerase activities were significantly elevated. Thus, the increase in RNA polymerase activity of isolated nuclei cannot be explained by an increase in availability or catalytic activity of RNA polymerase molecules. These findings led Shield and Tata to suggest that a modification of the chromatin template may be involved in the stimulation of transcription in isolated nuclei.

Recently, this suggestion of altered template availability has been tested more directly by Baxter *et al.* (1979). These workers probed template availability by assessing the number of sites at which bacterial RNA polymerase could initiate RNA synthesis on isolated chromatin. In chromatin from cells treated for 24 hours with T_3, an increase of approximately 50% in the number of bacterial RNA polymerase binding sites over that of untreated cells occurred. It should be pointed out that these studies do not purport to probe true RNA initiation sites in the chromatin, as bacterial RNA polymerase probably does not recognize eukaryotic promoters. Nevertheless, the data suggest that T_3 can alter chromatin structure to increase the availability of binding sites for a protein with a molecular weight of approximately 500,000. The relationship between the altered chromatin structure and nuclear RNA synthesis is unknown. Several groups have reported steroid hormone induced alterations in binding of bacterial RNA polymerases to isolated chromatin (for review, see O'Malley *et al.*, 1977), again indicating possible parallels between the mechanisms of actions of these two classes of hormones.

In the course of studying the solubilized RNA polymerase activities, Shields and Tata noted that chromatography of nuclear extracts on DEAE-Sephadex to separate I and II forms of enzyme led to variable results. This variation was traced to a loss of enzymatic activity for RNA polymerases isolated from thyroidectomized rats compared to their T_3-treated compatriots. An earlier report of an increase in RNA polymerase enzyme following T_3 (Smuckler and Tata, 1971) was apparently due to this differential instability. The basis of the increased sensitivity of RNA polymerases from thyroidectomized rats to inactivation is unknown. One possibility is that a higher percentage of total RNA polymerase is engaged in RNA synthesis in the T_3-treated or euthyroid animals. The actively transcribing enzyme may be in a different form, due to the presence of other transcriptional factors or associated fragment of DNA, which is more stable. Other explanations, such as differential protease activity, can also be envisioned.

B. *In Vivo* RNA Synthesis

A second parameter of RNA metabolism monitored by Tata and co-workers was the rate of incorporation of precursor into nuclear RNA (Tata and Widnell, 1966). Radioactively labeled orotic acid was injected into appropriately treated rats and animals were killed 10 minutes later. During this short period, RNA degradation would be expected to be minimal and, thus, rates of precursor incorporation would largely reflect rates of nuclear RNA synthesis. Thyroidectomized rats were injected with 20 μg T_3/100 g body weight, and at varying times after hormone treatment, orotic acid incorporation was determined. The earliest change in the rat of incorporation of orotic acid into nuclear RNA (25% over the control value) was detected at 3–4 hours after treatment. By 11 hours, the rate of incorporation was twice that of thyroidectomized animals, and by 21 hours, the rate was three times higher, the maximal level observed. The increase seen at 3–4 hours was the earliest response to T_3 treatment observed by Tata and co-workers in their studies.

Tata and Widnell (1966) also performed labeling studies using [^{32}P]orthophosphate as precursor. With this label, longer periods of 10–16 hours were necessary to achieve significant incorporation. Since the majority of heterogeneous nuclear RNA turns over very rapidly, the predominant species that would be labeled at such periods would be ribosomal RNA, with a half-time of degradation of several days. In fact, the base composition of the ^{32}P-labeled RNA was similar to that of ribosomal RNA. Thyroidectomized animals injected with T_3 together with the ^{32}P-label were found to have an approximately threefold greater rate of incorporation than thyroidectomized controls. Thus, both heterogeneous nuclear RNA, the predominant species labeled in a 10-minute pulse, and ribosomal RNA were synthesized at a greater rate following T_3 treatment.

These results must be taken with some caution. Although not well appreciated at the time of Tata's studies, it is now understood that changes in the specific activity of the precursor pool, as well as changes in the rates of incorporation, can cause alterations in the specific activity of RNA in such labeling experiments. To obtain a true incorporation rate, therefore, it is necessary to assess the specific activity of the precursor pool during the labeling period.

In 1978, Dillmann *et al.* reexamined the effects of thyroid hormone on *in vivo* RNA synthesis. In these studies, the specific activity of the precursor uridine triphosphate pool was analyzed. Thyroid status was found to have a profound effect on the labeling of the hepatic UTP pool. In euthyroid animals, the specific activity of UTP after a 30-minute pulse label with orotic acid was twice that of thyroidectomized rats. Treatment of thyroidectomized rats with T_3 for 24 hours led to an increase of 50% in precursor specific activity. Thus, a portion of the change in incorporation observed by Tata could have been due to an alteration in precursor labeling. After correcting for this factor, Dillmann *et al.* found the rate of incorporation of orotic acid into nuclear RNA was still 1.5 times greater in euthyroid animals compared to thyroidectomized rats (Fig. 1). This same relative

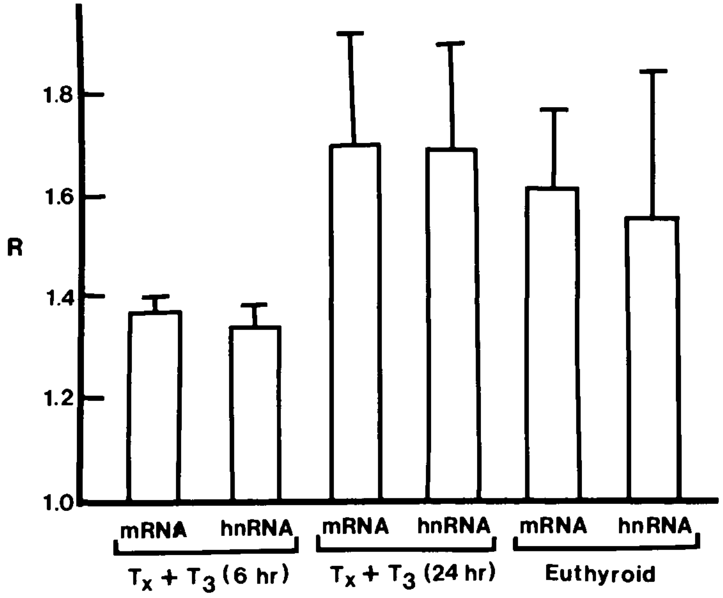

Fig. 1. Comparison of the incorporation of orotic acid into poly(A)-containing RNA of euthyroid or T_3-treated hypothyroid versus hypothyroid rat liver. Rats were injected with either 20 μCi [^{14}C]orotic acid or 200 μCi [^{3}H]orotic acid 30 minutes before sacrifice. Thyroidectomized controls were injected with the opposite isotope and equal weight portions of the two livers were combined. Nuclear poly(A)-containing RNA (hnRNA) or cytoplasmic polysomal poly(A)-containing RNA (mRNA) was extracted from the pooled sample and analyzed for the amount of incorporation. *R* is the ratio of incorporation of isotope into RNA for the euthyroid or T_3-treated sample, divided by the specific activity of the UMP pool for that group, to the same value for the thyroidectomized animal. Values represent means ($n \geq 4$) and standard deviations are indicated above bars. T_3 was injected at a dose of 1 mg/100 g body weight. Data adopted from Dillmann *et al.* (1978) with permission of authors.

difference could be observed after treatment of thyroidectomized rats with T_3 for 24 hours. A significant difference (34%) in RNA specific activity was also seen at 6 hours after T_3 treatment, the earliest time point measured in this study.

If thyroid hormone acts through a transcriptional route, mRNA represents a key intermediate in the chain of events leading to altered protein synthesis. Dillmann *et al.* (1978) also examined the rate of incorporation of orotic acid into poly(A)-containing polysomal RNA. Since the majority of mRNA species contain a 3′-stretch of polyadenylate, measurement of poly(A)-containing RNA is largely representative of the total mRNA population. The specific activity of poly(A)-containing RNA of euthyroid animals, corrected for precursor specific activity, was found to be about 60 to 70% greater than that of thyroidectomized controls (Fig. 1). No difference was found between labeling of free and membrane bound polysomal poly(A)-containing RNA. Injection of thyroidectomized rats with T_3 led to a detectable change at 6 hours and a level comparable to the euthyroid animal by 24 hours. Thus, the rate of formation of total cellular mRNA was stimulated by treatment with thyroid hormone.

C. Cellular Content of RNA

Thyroid hormone induced increases in nuclear RNA polymerase activities and rates of incorporation of precursor into RNA are consistent with an increased rate of RNA synthesis in these states. Increases in RNA synthesis might be expected to lead to higher cellular concentrations of RNA (assuming a concomitant increase in RNA degradation does not occur). Several reports confirm this expectation. As shown in Table I, the content of cytoplasmic RNA in livers of euthyroid rats, expressed per milligram DNA,* is increased 50–70% relative to thyroidectomized animals (Towle *et al.*, 1979). Polyribosomal mass is increased correspondingly (Dillmann *et al.*, 1978). Treatment of thyroidectomized rats with 1.5 μg thyroxine/100 g body weight/day for 10 days, a regimen known to normalize

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*It is difficult to know with certainty the best manner of expressing results when comparing thyroidectomized and euthyroid rat liver. Ideally, one would like to express information on RNA relative to the amount of DNA active in the synthesis of RNA. Clearly, expression of data per milligram of DNA is superior in this regard than expression on a tissue weight basis, which can be profoundly affected by alterations by unassociated factors, such as glycogen content. However, in dealing with the liver a complication arises with expressing results per milligram DNA. This complication is due to the progression of hepatocytes from diploidy to tetraploidy and higher ploidy states during normal development. Thyroidectomy has been shown to arrest this progression. Consequently, the average DNA content per cell of euthyroid rats can be markedly higher than thyroidectomized littermates. The question of concern is whether a tetraploid nuclei is twice as active in RNA synthesis as a diploid nuclei. Little sound information is available on this point, although modern advances in cell sorters and cytofluorimetry may make such measurements feasible. Nevertheless, if cells of higher ploidy are not proportionally active in RNA synthesis in their DNA content, the increased RNA content of euthyroid animal would be an underestimate of the actual difference.

TABLE I

Cytoplasmic RNA Content of Euthyroid, T_3-Treated Hypothyroid, and Hypothyroid Rat Liver[a]

Status	n	Total cytoplasmic RNA (mg/mg DNA)	Poly(A)-containing RNA in total RNA (%)	Poly(A)-containing RNA (μg/mg DNA)
T_x	10	1.27 (± 0.28)	2.00 (± 0.27)	23.8 (± 3.6)
$T_x + T_4$	10	2.10 (± 0.19)	1.88 (± 0.13)	39.8 (± 5.7)
Eu	18	2.29 (± 0.28)	1.93 (± 0.26)	42.8 (± 7.4)

[a] Total cytoplasmic RNA was extracted from the Triton treated postmitochondrial supernate of rat liver homogenate by precipitation with 4 *M* LiCl, 4 *M* urea. Total RNA content was determined by reading A_{260} of samples, assuming 1 mg/ml RNA yields an A_{260}/ml of 25. Poly(A) contents were measured as described in Fig. 2. Values represent means (± SD).

many thyroid responsive parameters, completely repaired the deficiency in RNA content (Simat *et al.*, 1980). Tata (1967a) reported that injection of a single dose of 15 μg T_3/100 g body weight to thyroidectomized rats resulted in a normalization of ribosomal RNA content at 48 hours. Considering the long estimated $t_{1/2}$ of ribosomal RNA (3–4 days), it is surprising that normalization would occur in such a short period. These results suggest that normal ribosomal RNA degradation might be altered in thyroidectomized animals following hormone administration. Experiments to test this proposition are difficult to perform in the whole animal due to the difficulty of effectively chasing radiolabeled nucleotide following the labeling period and the high degree of reutilization of nucleotide generated by RNA degradation.

The content of the cytoplasmic messenger RNA pool has also been determined in relation to thyroid hormone status (Towle *et al.*, 1979). Messenger RNA concentration was estimated by determining the poly(A) content of total cytoplasmic RNA. The protocol for this assay is outlined in Fig. 2. An aliquot of total cytoplasmic RNA was hybridized to an excess of [^{3}H]poly(dT) or [^{3}H]poly(U). After sufficient time for the hybridization to go to completion, the contents were treated with the single-strand specific S1 nuclease to degrade unhybridized nucleic acids. The amount of [^{3}H]poly(dT):poly(A)hybrid was then measured and compared to a standard curve made from parallel hybridization reactions containing known amounts of poly(A). Thus, the percentage of poly(A) in the total RNA could be directly measured. In order to determine the percentage of total RNA molecules which were poly(A)-containing RNA molecules, it is necessary to divide the former value by the average percentage of poly(A) in purified poly(A)-containing RNA. This value was estimated to be 7.5% (100 3′-A residues: 1350 nucleotide residues) for rat liver poly(A)-containing RNA and did not vary significantly with thyroidal status of the animal (Towle *et al.*, 1979). Utilizing

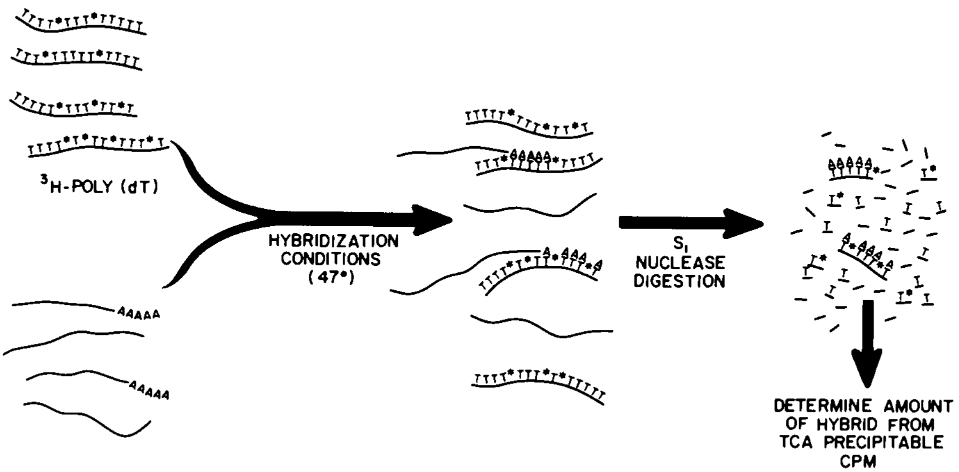
³H-POLY (dT)
HYBRIDIZATION CONDITIONS (47°)
S₁ NUCLEASE DIGESTION
DETERMINE AMOUNT OF HYBRID FROM TCA PRECIPITABLE CPM

Fig. 2. Protocol for determination of poly(A) content of RNA. Cytoplasmic RNA samples of 1–3 μg were mixed with [^{3}H]poly(dT) or [^{3}H]poly(U) in 0.01 *M* HEPES, pH 7.0, 0.4 *M* NaCl, 0.002 *M* EDTA, and allowed to incubate at 47°C for 30 minutes. The extent of hybridization was then analyzed by treatment with S1 nuclease, followed by precipitation with trichloroacetic acid, collection of precipitates on nitrocellulose filters, and counting. Standard curves were obtained by adding 1–5 ng of pure poly(A) to a set of hybridization reactions run in parallel (see Towle *et al.*, 1979, for details).

this technique, the proportion of poly(A) in total cytoplasmic RNA was found to be the same in thyroidectomized and euthyroid animals (Table I). Poly(A)-containing RNA was estimated to comprise 1.8–2% of cytoplasmic RNA molecules. Consequently, the increased mass of ribosomal RNA found in euthyroid animals is accompanied by an increased mass of poly(A)-containing RNA. Treatment of thyroidectomized rats with 1.5 μg T_4/100 g body weight/day for 10 days led to normalization of poly(A)-containing RNA content (Simat *et al.*, 1980). Thus, it is clear that thyroid hormones are necessary for maintaining normal levels of both ribosomal and messenger RNA in the rat liver.

From these content studies, it was estimated that euthyroid rat liver contains approximately 390,000 poly(A)-containing RNA molecules per diploid DNA equivalent, as opposed to 250,000 in the thyroidectomized rat (Towle *et al.*, 1979). The difference of about 140,000 molecules could represent the formation of new mRNA species not present in the hypothyroid animal, a generalized increase in the production of preexisting mRNA molecules, or a combination of these two factors. To attempt to differentiate between these possibilities, we have analyzed the sequence and frequency complexity of the total poly(A)-containing RNA population of euthyroid and hypothyroid rat liver. The technique utilizes an analysis of the kinetics of hybridization of poly(A)-containing RNA with a complementary DNA (cDNA) population transcribed from the RNA using the avian myeloblastosis virus RNA-dependent DNA polymerase. Under conditions of RNA excess, the rate of hybridization of cDNA to its template RNA is dependent only on the complexity of sequences in the RNA (Bishop *et al.*, 1974). By comparison to a RNA standard of known complexity, such as purified ovalbumin mRNA, it is possible to estimate the total sequence complexity of an unknown RNA population.

The analysis of liver poly(A)-containing RNA from either euthyroid or hypothyroid animals revealed the presence of at least three frequency classes of RNA (Table II). The estimated size and sequence complexity of each class of RNA were roughly the same in euthyroid and hypothyroid rat livers. The first cDNA to hybridize contained roughly 6–8 different mRNA species and 30% of the total cDNA; the second component contained 40% of the cDNA and 350–400 different sequences; and the last component to hybridize contained 30% of the cDNA and 10,000–11,000 different sequences. The total sequence complexity of the two RNA populations was similar. On the other hand, the number of copies per cell of each sequence is appreciably lower in the hypothyroid animal than the euthyroid animal. For instance, in the first hybridizing component, each of the 6–8 sequences is present roughly 8000 times per cell in the hypothyroid rat as compared to 18,400 copies per cell in the euthyroid animal. Thus, the increased content of mRNA in euthyroid animals represents a generalized increase in all frequency classes of mRNA rather than the *de novo* induction of a unique class of mRNA.

Sequence complexity analysis, which is designed for examining total RNA populations, is not sensitive to minor changes between two populations. Thus, a change of as little as 2% in the most complex component, which would be

TABLE II

Comparison of Sequence Complexity of Eu and T_x Poly(A)-RNA[a]

Source of liver	Component	Percentage of cDNA hybridized	Number of different sequences	Copies of each sequence per diploid DNA equivalent
Eu	1	27	5.7	18,400
	2	37	350	410
	3	29	11,300	10
T_x	1	27	8.5	7940
	2	40	400	250
	3	26	9800	7

[a] Analysis of hybridization kinetics of euthyroid or hypothyroid poly(A)-containing RNA with their respective cDNA copies. Curve fitting for determination of the number of components and $R_0t_{\frac{1}{2}}$ of each component were performed by computer analysis. Complexity of each class was estimated by comparison to $R_0t_{\frac{1}{2}}$ of purified ovalbumin mRNA (1.76×10^{-3} mole second liter^{-1}) and using an average size of 1400 nucleotides in liver poly(A)-containing RNA. Data from Towle *et al.* (1979).

difficult to detect, could represent a difference of as many as 750 sequences. A much more sensitive comparison can be obtained, however, by performing cross-hybridization experiments. In such experiments, cDNA transcribed from one population of poly(A)-containing RNA is hybridized to RNA from a different state. If RNA species are present in the first population of RNA (and thus its cDNA), which are absent in the second population, the final extent of hybridization will be lower in the cross-reaction than in the homologous hybridization. When this type of experiment was performed for hypothyroid and euthyroid poly(A)-containing RNA, no difference was detected in the final extents of hybridization. This is illustrated in Fig. 3 in which a comparison of the hybridization kinetics of cDNA prepared from euthyroid poly(A)-containing RNA to poly(A)-containing RNA of either homologous (euthyroid) or heterologous (hypothyroid) origin is shown. Only the uppermost portion of the hybridization curve and a scale linear with R_0t value is shown to emphasize the similarity of the two RNA populations. The hypothyroid liver appears to contain most of or all the same mRNA sequences present in the euthyroid liver. Again, it is worth mentioning that this method is limited in sensitivity due to the analysis of thousands of different RNA species on a scale of 0 to 100%. However, it is clear that the majority of the increase in poly(A)-containing RNA content must be accounted for by a generalized increase in mRNA species existing in the hypothyroid state.

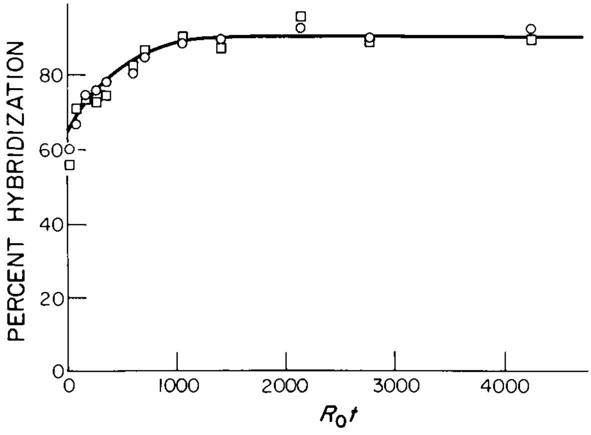

Fig. 3. Comparison of the extent of hybridization of euthyroid and hypothyroid mRNA with cDNA to euthyroid mRNA. Hybridization reactions were carried out in a volume of 50 μl and contained 1.5 ng of cDNA prepared from euthyroid poly(A)-containing RNA (1×10^7 CPM/μg), 0.01 *M* HEPES, pH 7.0, 0.5 *M* NaCl, 0.002 *M* EDTA, and varying amounts of either hypothyroid poly(A)-containing RNA (○) or euthyroid poly(A)-containing RNA (□). Incubation was at 68°C for times sufficient to yield the indicated R_0t values (up to 72 hours). Following incubation, reactions were analyzed for the extent of hybrid formation by S1 nuclease digestion. Background values (5–10%) of S1-resistant material in cDNA in the absence of added RNA were subtracted.

D. Relationship of Thyroid Hormone and Growth Hormone

Thyroid hormones are recognized to have profound effects on the pituitary production of growth hormone (Solomon and Greep, 1959; Hervas *et al.*, 1975). The thyroidectomized animal is not only deficient in thyroid hormones, but also in growth hormone. Injection of thyroid hormone to the thyroidectomized animal results also in increases in circulating growth hormone. Since the liver is a target organ for both hormones, it is important to assess whether changes measured following treatment are a direct action of thyroid hormone acting on the liver or occur indirectly due to stimulation of growth hormone.

One experimental approach to addressing this question is the use of hypophysectomized animals. Since the hypophysectomized animal does not produce thyrotropin, little endogenous production of thyroid hormones occurs. Treatment with either thyroid or growth hormone will consequently lead to a rise in the circulating level of only the administered hormone. The drawback to this approach is, of course, that the hypophysectomized animal is deficient in the production of many other hormones (e.g., glucocorticoids, androgens), which may be necessary for normal physiological action of thyroid or growth hormones.

Widnell and Tata (1966) examined the effects of growth hormone and thyroid hormone administration to hypophysectomized animals on levels of nuclear RNA polymerase activities. Using the maximally effective dose of hormone, both T_3 and growth hormone were found to be capable of stimulating the Mg-activated RNA polymerase I activity of hypophysectomized rat liver. The specificity of the responses was emphasized by a difference in the time courses of action. Growth hormone had a much earlier effect than T_3, with a maximal response at 3 hours following administration (as opposed to 24–48 hours for T_3). In experiments in which the two hormones were given together, the effects appeared to be almost totally additive. Interestingly, growth hormone administration did not lead to an elevation of Mn-activated RNA polymerase II activity, whereas T_3 did lead to a modest rise. In other studies, the enhanced incorporation of labeled precursors of RNA and protein following T_3 or growth hormone treatment of hypophysectomized animals was also found to be additive (Tata, 1970). The authors concluded that, although these hormones effect similar processes, the regulation of these effects in the liver appears to be mediated by independent pathways.

Recent studies in this laboratory on ribosomal and mRNA content have led to similar conclusions (Simat *et al.*, 1980). Hypophysectomized rats were injected with either a normal replacement dose of 1.5 μg thyroxine/100 g body weight/day or 0.2 IU ovine growth hormone/100 g body weight/day for 10 days. The content of cytoplasmic RNA and of poly(A)-containing RNA was found to increase to roughly the same extent after either treatment. Administration of both hormones resulted in an additive increase in RNA content. Thus, the two hormones appear to have independent effects on the liver. In the course of this study, it was noted that the level of RNA obtained when both hormones were given to hypophysectomized rats was still 20% less than that of normal animals. Administration of this treatment to thyroidectomized animals led to completely normal levels of RNA. Thus, the hypophysectomezed animal does not appear to be capable of responding to the same degree as the thyroidectomized rat. Perhaps other hormonal factors dependent on the pituitary are required for normal liver functions. In this regard, initial studies attempting to replace hypophysectomized animals with dihydrocortisone and testosterone in addition to growth and thyroid hormones were unsuccessful.

E. Protein Synthesis and Translational Efficiency

The increased mass of both ribosomal and messenger RNA in euthyroid or T_3-treated thyroidectomized rats compared to untreated thyroidectomized animals might be logically expected to result in a greater rate of protein synthesis. Measurements of *in vivo* rates of protein synthesis are complicated by possible effects of precursor specific activity and compartmentalization. Consequently, few studies have been performed attempting to relate thyroidal status with protein synthetic rates. Bernal *el al.* (1978) attempted to estimate protein synthetic rates in liver by following the incorporation of radioactively labeled leucine into protein. The level of incorporation into cytosolic and nuclear proteins, expressed as nanomoles leucine incorporated per milligram protein, was reduced by 50% in thyroidectomized animals in comparison to euthyroid controls. Injection of T_3 augmented isotopic incorporation within 5 hours and restored it to the normal rate by 24 hours. These changes did not appear to be simply a reflection of alterations in the specific activity of the leucine pool. Thus, the increased mass of ribosomes and mRNA may result in a higher rate of protein synthesis in the presence of physiological levels of thyroid hormones.

In addition to the reports of increased ribosomal and mRNA content in response to thyroid hormone, many reports have suggested an effect of thyroid hormone on the efficiency of the translational process. Perhaps the most convincing evidence comes from the laboratory of Haschemeyer and co-workers who have attempted to assess the effect of thyroid hormone status on translational efficiency using *in vivo* incorporation techniques (Mathews *et al.*, 1973). Animals were injected via the hepatic portal vein with radioactively labeled leucine and killed at very short time intervals thereafter (15–105 seconds). Incorporation into total protein and soluble (100,000 *g*) protein was measured at each time point. The appearance of radioactivity in the soluble protein pool, representative of completed protein chains, relative to total incorporation into protein is a function of the time for assembly and release of polypeptide chains. This ratio is independent of precursor specific activity (if it remains constant during the time period). For normal rats, the average polypeptide chain assembly time was estimated to be 1.16 ± 0.16 minutes. Thyroidectomized animals showed a markedly longer assembly time of 1.92 ± 0.22 minutes. Nearly complete recovery of protein synthetic rate in thyroidectomized rats was achieved by treatment with 20 μg T_3/100 g body weight/day for 2 days prior to assay. These results were interpreted as indicating that one or more reactions in chain elongation is subject to change in rate depending on thyroid hormone levels in the animal. Peavy *et al.* (1981) reported a study using similar methods to measure polypeptide chain elongation times. In this study, isolated hepatocytes from either thyroidectomized or normal rats were compared. In contrast to the results of Mathews *et al.* (1973), no difference was found in polypetide chain elongation time in the two states. The reason for the disparent results between these two studies is not clear.

A number of *in vitro* observations have also suggested an effect of thyroid hormones on translational efficiency. Several groups have reported that ribosomes isolated from thyroidectomized rats are less active in supporting amino acid incorporation than similarly prepared ribosomes of normal animals (Stein and Gross, 1962; Tata *et al.*, 1963; Garren *et al.*, 1967; Carter and Faas, 1979). This finding was true both for endogenous mRNA template and for incorporation of phenylalanine using the synthetic template, poly(U) (Tata and Widnell, 1966; Garren *et al.*, 1967). Injection of thyroid hormone to thyroidectomized rats was capable of rectifying the deficiency. The response to T_3, however, was relatively late, first being detectable at 26 hours and maximal at 40–50 hours following hormone administration (Tata *et al.*, 1963). Since then, Carter and Faas (1979) have suggested that an altered synthetic capacity was detectable as early as 3–6 hours following T_3 administration. This early effect on translation could be blocked by administration of α-amanitin, an inhibitor of RNA polymerase II. Thus, the effect of hormone was thought to represent an RNA mediated event rather than a direct action of T_3 on a component of the translational machinery. Assessment of the results from such *in vitro* experiments is not straightforward. Rates of incorporation in *in vitro* systems are several orders of magnitude less than estimated *in vivo* rates. Furthermore, the complexity of the system and the difficulty in reproducing *in vivo* conditions makes it impossible to pinpoint actual points of control.

Studies on polyribosome aggregates have indicated that the overall size of polyribosomes in euthyroid and hypothyroid animals is indistinguishable (Winkelman *et al.*, 1980; Peavy *et al.*, 1981). [An earlier report by Tata and Widnell (1966) had suggested a smaller overall size of polyribosomes from thyroidectomized rats; however, techniques for polysomes isolation and analysis have been greatly improved since that study.] Fractionation into free and membrane bound polysomes also failed to reveal any differences between polysome size of normal and thyroidectomized animals. Since the average size of mRNA does not change with thyroid hormone status, the packing ratio of ribosomes on the mRNA must be approximately the same. Evidence that the structure of the ribosome may be different in thyroidectomized animals has been reported. Correze *et al.* (1972) have found that the phosphorus content of ribosomal protein of thyroidectomized rats was considerably lower than that of normal rats. Jothy *et al.* (1976) have suggested an altered conformational state of ribosomes from thyroidectomized rats based on the reactivity of sulfhydryl groups of ribosomal protein. Whether these changes are related to the translational efficiency of ribosomes remains open to question.

In summary, two mechanisms, quite likely independent of each other, may be involved in increasing the protein synthetic capacity of euthyroid or T_3-treated thyroidectomized rats compared to the thyroidectomized animal. One mechanism involves the proliferation of the ribosomal and mRNA constituents involved in protein synthesis. In this regard, Tata (1967b, 1970) has shown a simultaneous proliferation of the membrane components presumably involved in forming the endoplasmic reticulum. It would be of interest to see whether other translational components, such as initiation and elongation factors or aminoacyl-tRNA's, are also increased during this transition. The second mechanism may involve an increased efficiency of translation (per ribosome) in the thyroid competent animal. The molecular nature of the change leading to increased translational efficiency is currently unknown. It is also left for further investigation to separate out the relative influences of these two mechanisms on cellular protein synthesis.

What is the final result of the increased protein synthetic capacity associated with thyroid hormone? It might seem curious that measurement of liver protein content per milligram DNA did not appear altered to any degree with thyroid hormone status (Schwartz *et al.*, 1980). This was true for all cellular fractions examined. One possibility to explain the apparent incongruity is that the synthesis of plasma proteins for secretion are increased selectively with T_3 treatment. To some degree, this suggestion may be true; for example, albumin production does seem to increase in the transition from hypo- to euthyroidism (Kekki, 1964; Morgan, 1969; Griffin and Miller, 1973). On the other hand, it also seems likely that protein degradation is increasing in concert with protein synthesis. Goldberg *et al.* (1980) observed that T_3 treatment of deficient animals results in a greater rate of protein turnover. Measurement of lysosomal cathepsins also indicated a higher activity in euthyroid animals than in thyroidectomized controls (DeMartino and Goldberg, 1978). Thus, it seems that a large proportion of the increased synthesis of protein in liver is represented by an increased turnover. In addition, thyroid hormone has a general growth effect on the liver. Both total liver weight (as a percentage of total body weight) and total liver DNA are higher in euthyroid animals than thyroidectomized animals. Restoration of normal liver weight and DNA is a very slow response to T_3, requiring several weeks of continued T_3 replacement. This long-term response may likely require the increased protein synthetic activity associated with the euthyroid liver.

F. Hyperthyroidism and General RNA Effects

The studies discussed to this point have attempted to define the transition from the hypothyroid to euthyroid state. At normal plasma thyroid hormone levels, approximately 50% of the nuclear receptor sites in the rat were estimated to be occupied by hormone, primarily T_3 (Oppenheimer, 1979). The hyperthyroid animal can be operationally defined as one which has greater than 50% receptor occupancy for an extended period of time. Studies on the transition from euthyroid to hyperthyroid states may also be illuminating in studying thyroid hormone action. However, the pitfalls of "pharmacological" effects of thyroid hormones at very high doses must be carefully considered.

Few studies have examined the effects of prolonged hyperthyroidism on RNA metabolism. Tata and co-workers (1963) induced hyperthyroidism by injection of normal rats with 20 μg thyroxine every fourth day for 35 days. They found that basal metabolic rate was elevated by 18% in treated animals relative to normal controls, but levels of total cellular RNA were unchanged. We have recently confirmed this finding for both cytoplasmic RNA and poly(A)-containing RNA fractions in rats made hyperthyroid by daily injection of 15 μg T_3/100 g bw for several days (Towle *et al.*, 1980). The increase in RNA content observed in the transition from the hypothyroid to euthyroid state does not apparently continue as animals are pushed into the hyperthyroid range. This leveling off of RNA content could be due to the absence of further increases in RNA production, or counterbalancing effects of elevated RNA degradation in hyperthyroidism. The data of Tata and Widnell (1966) would argue for the latter possibility. These workers found significant increases (27%) in Mg^{2+}-activated nuclear RNA polymerase I activity in chronically treated rats relative to normal controls. Furthermore, Venkataraman *et al* (1965) found that hyperthyroid rats had higher rates of incorporation of [^{32}P]orthophosphate into cellular RNA than normal animals. However, on the basis of this limited amount of experimental work, it is difficult to clearly assess the relation of RNA metabolism and chronic hyperthyroidism.

G. Mitochondrial RNA Metabolism and Thyroid Hormone

Due to the relationship among oxygen consumption, respiratory chain enzyme activities, and thyroid hormones, the mitochondrion has long been attractive as a potential site of primary hormone action. The direct addition of thyroid hormone to preparations of isolated mitochondria was shown many years ago to lead to dramatic increases in oxygen consumption (Lardy and Feldott, 1951; Hoch and Lipmann, 1954) and the rate of amino acid incorporation into protein (Buchanan and Tapley, 1966; Buchanan *et al.*, 1970). These changes occurred with essentially no lag time, suggesting a direct action of thyroid hormones independent of prior protein synthesis. This work, however, is currently thought to be largely artifactual (Gordon *et al*, 1973). Levels of thyroid hormone required to achieve these *in vitro* effects are several orders of magnitude greater than physiological levels. Direct addition of physiological doses of T_3 to isolated mitochondria was without effect on levels of protein synthesis (Roodyn *et al.*, 1965). In addition, the time of maximal "*in vitro*" effects (3–7 minutes) occurs well before observable changes "*in vivo*" following physiological doses of T_3 (36–45 hours) (Freeman *et al.*, 1963). Thus, the isolated mitochondria as on *in vitro* system for studying T_3 action has not proved useful to date.

By contrast, many studies have shown dramatic changes in RNA and protein synthesis of mitochondria following alterations of thyroid hormone plasma levels in the whole animal. Roodyn *et al.* (1965) found that the incorporation of amino acids into protein by isolated mitochondria of thyroidectomized livers was reduced compared to normal livers. Injection of animals with 20 μg T_3/100 g body weight led to an increase in the incorporation of amino acids by isolated mitochondria following a lag of 36 hours. The response was maximal at 2 days following treatment. More recently, Bouhnik *et al.* (1979) reported a decrease in the *in vivo* incorporation of [^{3}H]leucine into certain mitochondrially encoded peptides in the thyroidectomized animal. The same authors also found the *in vitro* incorporation by isolated mitochondria increased significantly as early as 10 hours following T_3 treatment (25 μg/100 g body weight) of the animal. Thus, the protein synthetic capacity of the mitochondria does appear to be influenced by thyroid hormone levels.

RNA polymerase activity of mitochondria also responds to thyroid status of the animal (Schimmelpfennig *et al.*, 1970; Gadaleta *et al.*, 1972; Barsano *et al.*, 1977). Mitochondrial RNA polymerase activity can be assayed in a fashion similar to whole nuclei using endogenous DNA of the isolated organelle. However, pretreatment of isolated mitochondria in phosphate buffer to allow intracistal swelling is necessary for efficient permeability of nucleoside triphosphate substrates. Following thyroidectomy, mitochondrial RNA polymerase activity fell to about 50–70% of normal levels. Treatment of thyroidectomized animals with 25 μg T_3/100 g body weight led to increases in mitochondrial polymerizing activity by 18 hours. Maximal levels close to those found in normal animals were found at 36 hours following the single injection. The time course and extent of changes seen in mitochondrial RNA polymerase activity are reminiscent of those seen with nuclear RNA polymerases.

As with whole nuclear RNA polymerase assays, changes in mitochondrial polymerase activity could be due to an increase in enzyme activity or template availability. Gadaleta *et al.* (1975) have attempted to address this question by solubilizing mitochondrial RNA polymerase and partially purifying it to a DNA-dependent stage. RNA polymerase extracted from animals 24 hours following treatment with 25 μg T_3/100 g body weight was found to have a twofold higher specific activity on exogenous DNA template than that of thyroidectomized animals. The extent of purification was not sufficient to allow a determination of whether this change represented an activation of preexisting RNA polymerase or an increased mass of enzyme. These studies do indicate that at least part of the increase in mitochondrial activity represents an enhancement of the RNA polymerase enzyme present.

As regards the mechanism by which T_3 affects mitochondrial protein synthesis, two hypotheses are possible. The first assumes that thyroid hormones exert direct effects on the mitochondria. Sterling and Milch (1975) reported the specific binding of T_3 to an inner membrane protein, but this finding has been repudiated by others (Greif and Sloane, 1978) and mitochondrial receptors remain, at best, elusive. The second hypothesis involves a secondary effect on the mitochondrial systems mediated thorugh an earlier direct action of T_3 at the nucleus. Mitochondrial synthesis of RNA and proteins is controlled by proteins synthesized from nuclear genes and translated in the cytoplasm. It is therefore possible that mitochondria are affected by hormone action secondary to the interaction of T_3 with its nuclear receptor. Efforts to differentiate between these possibilities would be enhanced by definition and detailed analysis of products encoded by mitochondrial DNA and the effect of T_3 on these proteins.

H. Summary: General RNA Effects and Thyroid Hormone Action

As can clearly be seen from the large amount of experimental data above, acute injection of thyroid hormone into thyroidectomized rats leads to a general enhancement of hepatic RNA and protein synthetic processes. Comparison of euthyroid animals with hypothyroid rats yields a similar conclusion. This shift is undoubtably associated with a more normally functioning animal, capable of growth and greater metabolic activity. Induction of chronic hyperthyroidism would appear to cause an imbalance between the normal synthetic and degradative pathways in the liver; however, the characteristics of these responses are much less studied.

Although undoubtably an essential feature of thyroid function, the proliferation of RNA and protein synthetic capabilities with thyroid hormone may not be associated directly with the initiation of hormone action. This conclusion is based on the time course with which these parameters change following hormone administration. In general, these responses occur with a long lag time on the order of 10–24 hours. It seems reasonable to conclude that the general enhancement of RNA and protein production is responding to an earlier, as yet undetermined signal, generated in response to thyroid hormone. In this regard, it should be noted that many other steroid and growth-promoting hormones are characterized by responses similar to those seen in the liver with thyroid hormone. For example, the induction of egg white proteins by estrogen in the chick oviduct is accompanied by a general proliferation of the ribosomal apparatus (Hamilton, 1968; Knowler and Smellie, 1971; Means and O'Malley, 1972). In rat liver, growth hormone also can cause similar RNA effects when given to hyposectomized animals (Tata, 1967b). Although it is impossible to rule out direct hormonal action at the present time, it seems more likely that the target tissues may be responding secondarily to the increased metabolic activity. The mechanism of such a coupling is entirely unknown and should provide an interesting area of research for the future.

It is also worth pointing out that the effects of thyroid hormone are highly tissue specific. A large portion of all work on RNA and thyroid hormones in the rat has focused on the liver. The response of other major target organs, such as kidneys or heart, is much less studied. However, the anterior pituitary and tumor-derived pituitary cell lines have been examined in some detail. In contrast to the liver, little effect of thyroid hormones on overall RNA or protein synthetic rates are observed (Samuels, 1978). In the pituitary, T_3 appears to act quite specifically to effect a very limited set of gene products (Ivarie *et al.*, 1980). It is interesting to speculate on the basis of this tissue specificity. By the best methods available, the T_3 nuclear receptor appears to be identical in the two tissues, although efforts to purify the receptor protein have met with little success to date. Both tissues also have a similar number of receptors present (Oppenheimer *et al.*, 1974a). Therefore, the interaction of the receptor with the tissue is probably mediated by cell-specific factors capable of effecting the site of action of the receptor. This area should be another interesting one for further study.

III. SPECIFIC MODEL SYSTEMS FOR STUDYING THYROID HORMONE ACTION

Although studies on general RNA effects of thyroid hormones have been important in forming the current hypotheses on hormone action, they are limited by the nonspecificity of the measurements. These measurements generally quantitate total RNA populations, often as trichloracetic acid insoluble material, without regard to the nature of the species being produced. Since RNA populations, especially messenger RNA, are tremendously diverse, changes in production of a small subset of RNA's in the total could be easily overlooked. Furthermore, focusing on the specific effects of thyroid hormones on individual processes is potentially much more useful in delineating the molecular events in thyroid hormone action. For these reasons, much attention in the past 5 years has been given to establishing specific model systems directly responsive to thyroid hormones. At the present time, three such systems have been developed and look promising for future studies. Undoubtably, other specific model systems will continue to be established for T_3 action in the next decade.

A. α_{2u}-Globulin

α_{2u}-Globulin is a protein with a molecular weight of 20,000, first found by Roy and Neuhaus (1966a) in the urine of adult male rats, but absent in the urine of female rats. This protein was shown to be synthesized in the livers of male rats, secreted into the serum and filtered through the kidneys to become the major protein in the urine of these rats (Roy and Neuhaus, 1966b). The function of α_{2u}-globulin is thus far unknown. The hepatic synthesis of this protein is under complex hormonal control: androgens, glucocorticoids, growth hormone, and thyroid hormone are all necessary for the hepatic synthesis of α_{2u}-globulin, and estrogen represses the synthesis. Since this multihormonal control has been recently reviewed (Kurtz and Feigelson, 1978), this discussion will focus only on the effects of thyroid hormone on the production of α_{2u}-globulin.

Roy (1973) first reported that thyroidectomy or hypophysectomy almost totally depressed the urinary output of α_{2u}-globulin. The effects of thyroidectomy on α_{2u}-globulin output could be reversed to normal by treatment with thyroxine. However, simultaneous treatment with growth hormone, corticosterone, testosterone, and thyroxine was required for complete reversal of the effect of hypophysectomy. This work suggested thyroid hormone was directly involved in α_{2u}-globulin production and that it was acting synergistically with as many as three other hormones.

In order to explore the mechanism of action of thyroid hormone on α_{2u}-globulin, the hepatic synthesis of this protein has been examined. Kurtz *et al.* (1976) found no *in vivo* incorporation of [^{3}H]leucine into immunoprecipitable α_{2u}-globulin in thyroidectomized male rats, whereas in intact males this protein accounted for approximately 1% of all hepatic protein synthesis. Administration of 100 μg T_3/100 g body weight/day for 4 days resulted in a partial restoration of the hepatic synthesis of this protein.

To determine if this modulation of hepatic synthesis of α_{2u}-globulin was the result of control of the functional level of its mRNA, hepatic poly(A)-containing RNA was extracted and translated in a cell-free protein synthesizing system derived from wheat germ (Kurtz *et al.*, 1976; Roy *et al.*, 1976). Radioactively labeled polypeptides synthesized *in vitro* were analyzed by immunoprecipitation for α_{2u}-globulin content. Thyroidectomized male rats had undetectable levels of functional α_{2u}-globulin mRNA activity in the translational assay. Again 4 days of treatment with thyroid hormone (10 μg thyroxine/100 g body weight) resulted in a partial restoration (20%) of α_{2u}-globulin mRNA activity. Treatment for 10 days resulted in normalization. A similar time course was observed for liver, serum, and urinary levels of α_{2u}-globulin protein, suggesting that the level of mRNA was responsible for controlling the appearance of this protein. These results were the first indication that thyroid hormones may influence the synthesis of specific proteins by regulating production of the mRNA coding for that protein.

One of the potential drawbacks of *in vitro* translational assay for mRNA activity is the necessarily indirect nature of such measurements. Thus, the presence of functionally inactive α_{2u}-globulin mRNA sequences in the thyroidectomized rat livers could not be ruled out. However, the isolation of a complementary DNA specific for α_{2u}-globulin mRNA by Kurtz and Feigelson (1977) made the direct measurement of mRNA sequences by hybridization techniques possible. The isolation of this cDNA was aided by the relatively high concentration of this specific mRNA in intact males, an indirect immunoprecipitation of α_{2u}-globulin polysomes, and a preparative hybridization step utilizing mRNA from female rat livers, which contains no α_{2u}-globulin sequences, to adsorb out cross-hybridizing species in the male mRNA. Using the specific cDNA probe, the results of the earlier translational studies of α_{2u}-globulin mRNA were substantiated. Thus, hepatic mRNA from thyroidectomized rats had less than one copy of α_{2u}-globulin mRNA per cell, compared to the level in intact males of 1% of the total mRNA. Treatment of thyroidectomized males with thyroxine for 10 days led to normalization of α_{2u}-globulin mRNA levels as determined by specific hybridization.

The characteristics of the response of α_{2u}-globulin mRNA to thyroid hormone have not been well studied. The dose–response relationship of T_3 and α_{2u}-globulin synthesis has not been explored. The time course of induction of α_{2u}-globulin mRNA after treatment with thyroid hormone is not fully defined especially with respect to the early time points after hormone treatment. The estimates of 10 days of thyroxine treatment required for normalizing α_{2u}-globulin synthesis seem quite long; however, since no $t_{1/2}$ of the mRNA is known, it is difficult to assess this question. It seems clear that thyroid hormones influence α_{2u}-globulin production and do so by controlling the levels of mRNA coding for this protein. Whether this represents a primary effect of thyroid hormone, or occurs secondarily to some earlier action, remains to be seen. Also the interaction between thyroid hormones and the other hormonal factors influencing α_{2u}-globulin production should provide interesting insights into multihormonal regulation.

B. Growth Hormone

Thyroid hormones are known to influence the production and secretion of growth hormone in the rat adenohypophysis *in vivo* (Solomon and Greep, 1959; Hervas *et al.*, 1975). The presence of several growth hormone producing cell lines derived from rat pituitary tumors provided the opportunity to study this regulation under *in vitro* conditions (Tashjian *et al.*, 1968). The ability to manipulate and maintain defined hormone concentrations makes such *in vitro* model systems highly attractive for studying hormone action. Tsai and Samuels (1974) first demonstrated that the production of growth hormone in the GH_1 cell line is responsive to thyroid hormone levels in the media. Addition of T_3 to cells grown in thyroid hormone-depleted media resulted in a four- to sixfold increase in growth hormone found in the cells and secreted into the media. To examine the mechanism of this response, Samuels and Shapiro (1976) measured the rate of synthesis of growth hormone in the presence and absence of T_3. Although T_3 did not alter the rate of incorporation of [^{3}H]leucine into total proteins, it did induce a four-fold increase in the rate of synthesis of growth hormone. Thus, the increased level of growth production is correlated with an elevated synthetic rate for this specific protein.

Samuels and co-workers have studied the characteristics of the response of GH_1 cells to T_3 in some detail (Samuels, 1978). T_3 stimulated the rate of growth hormone synthesis by twofold in 2.5 hours and by a maximal fourfold by 8.5 hours after addition of T_3 to thyroid hormone depleted media. The lag time between significant T_3 binding to nuclear sites and a detectable increase in growth hormone synthesis was 45–60 minutes. This short lag time is one of the earliest reported responses to thyroid hormones and is strongly suggestive of a direct hormonal action on the production of growth hormone in these cells. In addition to the kinetics of the response, Samuels has also studied the dose–response relationship between T_3 concentration and growth hormone synthesis in GH_1 cells. The free T_3 concentration required for half-maximal response of the growth hormone synthesis was 2.2×10^{-10} *M*. Half-maximal saturation of the putative nuclear receptor occurred at 3×10^{-10} *M*. Thus the T_3-binding curve and the growth hormone response do not appear to be linearly related to each other, but they are affected in the same range of T_3 concentrations. Furthermore, the relative effectiveness of various hormonal analogs of T_3 in eliciting the growth hormone response in GH_1 cells is related to their affinity for the nuclear binding sites. These correlations suggest that T_3 is affecting the synthetic rate of growth hormone directly through interaction at the nuclear receptor.

An increase in mRNA coding for growth hormone in response to T_3 has been reported in several related rat pituitary tumor cell lines. In the GH_1 (Shapiro *et al.*, 1978), GH_3 (Seo *et al.*, 1977), and GC (Martial *et al.*, 1977a) cell lines, levels of growth hormone mRNA activity, measured in a cell-free protein synthesizing system, all responded to T_3 in parallel with increased production of growth hormone. This increase in specific mRNA has also been confirmed using hybridization to a cDNA probe prepared from enriched growth hormone mRNA (Martial *et al.*, 1977a). Studies on intact rats have confirmed the correlation between pituitary levels of growth hormone mRNA and thyroid hormone (Seo *et al.*, 1979). Thyroidectomized rats were found to have less than 2% of the pituitary content of growth hormone mRNA of normal rats; this deficiency could be normalized by one week of replacement with low levels of thyroxine (1.75 μg/100 g body weight/day). These data strongly indicate that thyroid hormones are acting to increase the production of growth hormone mRNA. One potential site of regulation is the synthesis of the mRNA; however, on the basis of current experimental evidence it is not yet possible to pinpoint which step in the production of mRNA is affected by hormone.

In addition to the effects of thyroid hormone, growth hormone production in pituitary tumor cell lines is also influenced by glucocorticoids. Tushinski *et al.* (1977) found that addition of the glucocorticoid dexamethasone to GH_3 cells cultured in normal serum (containing thyroid hormones) resulted in a 5- to 15-fold increase in growth hormone mRNA. When medium depleted of thyroid hormones was used for cell culture, however, little effect of dexamethasone on growth hormone mRNA levels was observed (Martial *et al.*, 1977b). Addition of both hormones to these T_3-depleted cells resulted in 45-fold stimulation of growth hormone mRNA levels, whereas T_3 alone gave a 17-fold induction. Samuels *et al.* (1979) have studied the interaction of the hormones in GH_1 cells maintained in serum-free conditions. In the absence of glucocorticoids, T_3 induced a four- to sixfold increase in growth hormone synthesis within 24 hours. In the absence of thyroid hormones, dexamethasone induced only a small growth hormone response. In contrast, the growth homone response of cells incubated with both T_3 and glucocorticoid was two- to fivefold greater than T_3 alone. Thus, it appears that growth hormone production can be regulated synergistically by thyroid hormone and glucocorticoid. The possible interaction of the nuclear receptors for these two hormones in the regulation should be extremely interesting in relation to hormone action.

C. Malic Enzyme

A third potential model system for studying the action of thyroid hormone on a specific gene product is malic enzyme [L-malate:NADP$^+$ oxidoreductase (decarboxylating)]. The cytosolic form of this enzyme in certain tissues, most notably liver, responds markedly to the level of circulating thyroid hormones (Tepperman and Tepperman, 1964; Wise and Ball, 1964; Ruegamer *et al.*, 1965). In rat liver, for example, malic enzyme activity increases over 20-fold in the transition from the hypothyroid to extremely hyperthyroid states (Oppenheimer *et al.*, 1977). Hepatic malic enzyme has consequently been used as a marker of thyroidal status in the rat. In addition to thyroid hormone levels, this enzyme also responds to the diet of the animal. Starvation leads to substantial reduction in enzyme activity, whereas feeding a high carbodydrate, fat-free diet causes marked elevation (6- to 10-fold) over enzyme levels found in rats fed standard laboratory chow (Fitch and Chaikoff, 1960; Pande *et al.*, 1964; Tarentino *et al.*, 1966). In this regard, malic enzyme behaves similarly to a set of enzymes involved in lipogenesis, including fatty acid synthetase, acetyl-CoA carboxylase, and the hexose monophosphate shunt dehydrogenases. Thus, the primary function of cytosolic malic enzyme in liver is thought to be generation of NADPH for supporting fatty acid synthesis.

The alterations in enzyme activity observed following hormonal or dietary manipulation of animals have been shown immunologically to be due to changes in the mass of enzyme protein (Isohashi *et al.*, 1971). Furthermore, measurements of rates of enzyme synthesis and degradation indicate that alterations in the synthetic rate of malic enzyme are responsible for changes in enzyme mass (Silpanata and Goodridge, 1971; Gibson *et al.*, 1972; Murphy and Walker, 1974; Li *et al.*, 1975). Thus, the increased enzyme activity found in liver following administration of thyroid hormone or feeding a high carbohydrate, fat-free diet is due to an increase in the *de novo* synthesis of new enzyme.

Goodridge and co-workers have developed the use of primary hepatocyte culture from chick embryos for the study of malic enzyme regulation (Goodridge, 1975). Hepatocytes obtained from 17- to 19-day-old chick embryos were maintained in a chemically defined (serum-free) medium. Addition of T_3 to this medium for 3 days led to a 23-fold increase in malic enzyme activity (Goodridge and Adelman, 1976). The thyroid hormone levels required for the stimulation were well within the physiologically significant range; the estimated free T_3 level that produced 50% of the maximal response was 4×10^{-11} *M*. As with studies in the rat, the major effect of T_3 was on the synthetic rate for malic enzyme and not on its degradation. The relative rate of synthesis of malic enzyme was increased 4.5-fold by 3 hours after addition of T_3 and by 25 hours had reached an apparent plateau 20 times that of control cells. Extrapolation of the time course of increases in the relative synthetic rate to base line indicates almost no lag time for response. Because thyroid hormones *in vivo* are known to affect levels and responsivity of many other hormonal systems, these *in vitro* results are important for demonstrating the direct action of thyroid hormones on the liver cells in stimulating malic enzyme. In addition, the rapid response of the system following hormone addition strongly suggests that this response may be a primary effect of thyroid hormone.

We have recently assessed the mRNA of rat liver to determine whether the increased rate of enzyme synthesis in response to T_3 can be accounted for by an induction of mRNA coding for malic enzyme (Towle *et al.*, 1980, 1981). For this purpose, total mRNA was isolated from livers of rats after various treatments. This mRNA was translated in the mRNA-dependent reticulocyte lysate assay system and the radioactively labeled polypeptides synthesized were analyzed for malic enzyme. Because mRNA for malic enzyme constitutes only a minor proportion of total messages, the detection of labeled malic enzyme in the translational products required an extremely sensitive procedure. Total translational products were first chromatographed on N^6-(6-aminohexyl)adenosine 2′,5′-diphosphate-agarose, a biospecific affinity resin for NADP-dependent proteins. Products that were eluted with NADP were subjected to immunoprecipitation using specific antibody to purified malic enzyme and protein A-bearing *Staphlococcus aureus* cell walls as an immunoadsorbent. Finally, immunoprecipitated products were electrophoresed on sodium dodecyl sulfate–polyacrylamide gels and the labeled product that comigrated with authentic malic enzyme was quantitated. This procedure allowed detection of products constituting as little as 0.003% of the total incorporation, the level observed for normal euthyroid rats.

To examine the effects of T_3 on mRNA coding for malic enzyme, euthyroid animals were injected daily with from 2 μg to 50 μg T_3/100 g body weight for 7 days. Livers were then removed and both malic enzyme activity and mRNA were measured (Table III). A proportional increase in enzyme activity and mRNA activity occurred over the dose range of T_3 tested. Thus, at the highest dose tested, malic enzyme activity had increased 14.5-fold over euthyroid values, and mRNA activity for malic enzyme had increased approximately 12-fold. The parallel response of malic enzyme activity and its specific mRNA to T_3 indicates

TABLE III

Effect of Thyroid Hormone Administration to Normal Rats on Malic Enzyme Activity and mRNA[a]

Dose of T_3 (μg)	Malic enzyme activity (units/mg DNA)	% mRNA coding for malic enzyme
0	241	0.003%
2	803	0.0086%
5	1663	0.0156%
15	2462	0.0216%
50	3476	0.0344%

[a] Normal rats were injected with the indicated dose of T_3 daily for a period of 7 days. Livers were then removed and assayed for malic enzyme activity by the method of Hsu and Lardy (1967) or for malic enzyme mRNA activity by the method of Towle *et al.* (1981).

that enzyme induction is due exclusively to hormonal action on mRNA production. A similar conclusion has been reached by Siddiqui *et al.* (1981) for regulation of malic enzyme in the avian liver.

The time course of accumulation of malic enzyme mRNA activity following T_3 administration was also measured. Little change was observed for the first few hours following hormone injection, but by 6 hours the mRNA was found to rise significantly. The half-maximal response was obtained after 14 hours and by 60 hours the level of malic enzyme mRNA appeared to plateau. Extrapolation of the accumulation curve back to the euthyroid level of mRNA led to an estimate of a 2 hour lag prior to the earliest T_3 effect. This time course is very similar to that observed for the increase in the relative rate of synthesis of malic enzyme following T_3 (Mariash *et al.*, 1980), and supports the concept that an increased production of mRNA coding for malic enzyme may be a primary response to thyroid hormone.

Levels of mRNA are determined by both rates of mRNA formation (a multistep process involving synthesis, processing and transport from the nucleus) and rates of mRNA degradation. Alterations in levels of mRNA can be potentially affected at any of the sites involved in determining mRNA levels. To attempt to determine whether thyroid hormone affected the degradation of malic enzyme mRNA, estimates were made of its $t_{1/2}$ in the presence or absence of hormone. Based on the accumulation curve for malic enzyme mRNA in the presence of T_3, an estimate of 12 hours for the $t_{1/2}$ of malic enzyme mRNA can be obtained. This estimate assumes that the change in the rate of production of mRNA is rapid relative to the overall time course of mRNA accumulation. When animals were stimulated with T_3 and then withdrawn from hormone, malic enzyme mRNA activity declined with first-order kinetics. Based on this curve at times when thyroid hormone levels are extremely low, a $t_{1/2}$ of 10 hours can be obtained for malic enzyme mRNA. The $t_{1/2}$'s estimated from the rates of appearance and disappearance are, thus, not remarkably different, suggesting that thyroid hormone does not affect mRNA stability. The major effect of thyroid hormones must be exerted on the rate of cytoplasmic mRNA formation. The exact step in this process which is affected is unknown.

It is interesting to note that the induction of malic enzyme by diets high in carbohydrate and low in fat is also accompanied by a parallel increase in mRNA activity for malic enzyme (Towle *et al.*, 1980). Furthermore, the regulation of malic enzyme activity by thyroid hormone and diet appear to interact at some level. The amount of T_3 required to achieve 50% of maximal malic enzyme induction for animals on high carbohydrate, fat-free diet is five- to eightfold less than for animals on standard laboratory chow (Mariash *et al.*, 1980). This shift in the dose–response curve would not be expected if the two controls were completely independent. The level at which the dietary and hormonal regulations of malic enzyme interact is presumably at some step leading to production of mRNA. However, since the proximal signal for altered mRNA production is not known for the dietary control and the site of regulation for mRNA production is not known for either control, the elucidation of this interaction must await further experimentation.

D. Pleiotrophic Effects of T_3 on Rat Liver mRNA

We have recently initiated a study to more accurately assess the effects of T_3 on the overall mRNA population of rat liver (Seelig *et al.*, 1981). Total hepatic poly(A)-containing RNA was isolated from rats subjected to various hormonal treatments. These mRNA populations were translated into their corresponding polypeptides in the mRNA-dependent rabbit reticulocyte lysate system. The ^{35}S-labeled translational products were then separated by high resolution, two-dimensional gel electrophoresis. By this technique, the translational products of 200–250 different mRNA species could be resolved and quantitated. Although this is still a small portion of the total sequence complexity of rat liver mRNA, it does provide a powerful means of assessing a large number of mRNA species simultaneously.

Comparison of mRNA populations of hypothyroid, euthyroid, and hyperthyroid rat livers revealed that 19 products out of 230 visualized were influenced by T_3. Of these, 11 mRNA species were augmented with increasing T_3 levels, whereas 7 were attenuated. One product displayed a biphasic response, increasing in the hypothyroid to euthyroid transition, but decreasing in the euthyroid to hyperthyroid transition. These 19 mRNA species constitute a "T_3 domain" of rat liver mRNA.

Further evidence of the heterogeneity of response to T_3 was observed in the time course of T_3 action (Liaw *et al.*, 1982). Two of the responsive mRNA species were found to increase within the first 4 hours after administration of T_3 to hypothyroid animals. Several others had lag times varying between 4 and 12 hours, whereas a few mRNA species did not show any response until 12–24 hours of treatment. One particular mRNA of interest, which encoded a polypeptide with a M_r of 17,500 and an approximate pI of 5.0, was found to increase by fourfold as early as 1.5 hours following T_3 administration. Whereas the nature of this protein is unknown, this response is one of the earliest yet detected for T_3 acting on an hepatic process.

Since T_3 is known to stimulate pituitary production of growth hormone, it is possible that many of the hepatic mRNA species responding to T_3 treatment of hypothyroid animals are actually responding to growth hormone. To test this possibility, hypothyroid animals were treated with ovine growth hormone. Six of the 19 products that had responded to T_3 were influenced by the growth hormone treatment. In several cases, growth hormone administration resulted in normalization of the response and these mRNA species do not appear to be affected by T_3, other than through its effect on growth hormone production. Two of the products only showed a partial response to the growth hormone treatment and it is likely that these mRNA species require both growth hormone and T_3 for full expression.

A similar study has been performed to assess the effect of high carbohydrate, fat-free diet on rat liver mRNA. For malic enzyme, it has been shown that both T_3 and high carbohydrate, fat-free diet act through a mechanism leading to increased levels of mRNA (Towle *et al.*, 1980). Interestingly, 10 of the mRNA species affected by T_3 were also influenced by the change in diet. In every case, the direction of the response was the same as with T_3. These studies clearly demonstrate the complexity of regulation in the rat liver. Of the products that respond to T_3, the majority are also responsive to either growth hormone or high carbohydrate, fat-free diet. As many other hormonal and nutritional factors may also play a role in hepatic function, one must consider this only part of the story. Studying the interrelationship between these various regulators may help clarify the role of T_3 in the process.

E. Other Potential Model Systems

The three specific model systems discussed above were selected because of the relatively more advanced state of experimental work on their control by thyroid hormones. These three products represent the only cases so far tested in which the cellular content of protein and specific mRNA coding for that protein have been shown to be responsive to thyroid hormone. Many other possible model systems for studying T_3 action are currently under investigation. For example, Edelman and co-workers have shown the content and synthetic rate of the Na^+,K^+-ATPase of kidney cortex is stimulated by T_3 treatment (Lo and Edelman, 1976; Lo and Lo, 1980). Vonderhaar (1975, 1977) reported that thyroid hormones enhance the production of α-lactalbumin in mammary explants. This effect required the simultaneous presence of insulin, hydrocortisone, and prolactin. Recently, two groups have simultaneously reported that thyroid hormones can increase the number of β-adrenergic receptors in cardiac tissue (Tsai and Chen, 1977; Williams *et al.*, 1977). In addition, thyroid hormones are known to effect the activities of a large number of enzymes in various tissues (Pitot and Yatvin, 1973). Thus, there are a large number of cellular responses to thyroid hormone which may be suitable for studying the molecular mechanisms involved in hormone action.

IV. CONCLUSIONS

The hypothesis that thyroid hormone action is initiated through interaction with a nuclear receptor and subsequent alteration of cellular RNA production is certainly a plausible one today. The demonstration of high affinity, low capacity binding sites for thyroid hormones in chromatin places the hormone–receptor complex in a favorable subcellular location for affecting RNA production. The rapid and specific stimulation of messenger RNA production for certain thyroid responsive proteins strengthens the concept of a direct action of hormone on gene expression. There remains, however, many fundamental questions on this hypothesis of thyroid hormone action.

One important question is whether all the diverse effects of thyroid hormone are initiated through the nuclear route. The transport of certain metabolites into cells, as studied through the use of sugar and amino acid analogs, has been reported to be stimulated by thyroid hormones (Goldfine *et al.*, 1975; Segal and Gordon, 1977). These effects occur within minutes of hormone administration and are not blocked by inhibitors of protein synthesis. Alternate binding sites for thyroid hormones, such as the inner mitochondrial membrane, have also been suggested (Sterling and Milch, 1975). The physiological significance of these extranuclear sites has not been demonstrated; nevertheless, it remains conceivable that hormone action is mediated through multiple pathways in any given tissue.

A second important question is the nature of the interaction of thyroid hormone with other hormonal and metabolic controls. One common feature of many T_3 responses is that they also respond to other physiological regulators. The three potential model systems discussed in this chapter illustrate this point: growth hormone production is affected by glucocorticoid as well as thyroid hormone; α_{2u}-globulin synthesis requires the simultaneous presence of four hormones; malic enzyme levels are synergetically affected by T_3 and an unknown dietary signal. Thus, many regulated products are subject to multiple input signals in setting the level of production. The molecular mechanisms of such interactions are largely unexplored.

Perhaps the most fundamental question of all concerns the mechanism by which binding of hormone to a receptor can transduce a signal leading to altered RNA production. Efforts to address this question are hampered by the lack of knowledge on the mechanism of gene expression and structure of chromatin in eukaryotes. In fact, in this regard, thyroid hormone-responsive products provide a potentially useful tool for probing these processes. Since the thyroid hormone receptor is a chromosomal nonhistone protein presumably involved in the regulation of gene expression, it may be an excellent candidate for probing eukaryotic transcriptional processes. At the present time, speculation on the mechanism of the regulation by hormone receptor would be largely fanciful. Undoubtedly the next decade will see the elucidation of a much clearer picture of thyroid hormone action.

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7

Role of Thyroid Hormone in the Expression of α_{2u}-Globulin and Other Multihormonally Regulated Genes

ARUN K. ROY

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MOLECULAR BASIS OF
THYROID HORMONE ACTION

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I. INTRODUCTION

The basic information for the processes of development, differentiation, morphogenesis, and growth is coded within the genetic apparatus, and the systematic unfolding of these events is generally guided by various chemical signals among which hormonal mediators are known to play a predominant role. In addition, hormones are also required to maintain the metabolic homeostasis in the adult. Many of the biochemical steps involving sequential and coordinated changes that lead to the ultimate expression of the phenotype are generally controlled by more than one hormone. At present we have a number of well-documented examples of the regulation of both single and functionally regulated clusters of genes through multiple hormonal interactions (Roy, 1973a; Goodridge and Adelman, 1976; Baxter *et al.*, 1979; Gebhardt and Mecke, 1979; Samuels *et al.*, 1979; Sassa *et al.*, 1979; McGuire *et al.*, 1980; Rosen *et al.*, 1980). In many of these multihormonal regulatory systems the thyroid hormones are involved in the ultimate expression of the target genes. Long before the era of molecular biology and the availability of molecular probes for the study of gene expression, classical endocrinological approaches such as endocrine ablations and hormone supplementations have resulted in the development of the interesting concept of the multiple hormone interactions in the development and differentiation of the mammary gland. Many of these early findings have withstood the test of time and molecular probings.

Because of the historical reason it is appropriate to begin this chapter with a brief descripton of the multiple endocrine interactions in mammogenesis and lactation. Involvement of multiple hormonal factors in the control of mammary growth and lactation was clearly established by Lyons *et al.* (1958). Endocrine ablations followed by hormone supplementations showed that more than one hormone is required in the development of mammary gland and lactation in the rat. Figure 1 summarizes the findings of Lyons *et al.* (1958) which show that at least six hormones including progesterone, glucocorticoid, estrogen, thyroxine, growth hormone, and prolactin are involved in the development of the mammary gland and the initiation of lactation. Later studies also implicated the role of insulin in this process (Topper, 1970).

Although the mammary model provided an excellent example of multiple endocrine interactions in development, differentiation, and organogenesis at the cellular level, the subcellular mechanism of the cooperative effect of various hormones was not explored until the mid-1960's. This question was first investigated in the laboratory of Tata who examined the interacting influence of the growth and developmental hormones on the protein and RNA synthesis in both amphibian and mammalian liver (Widnell and Tata, 1966; Tata and Williams-Ashman, 1967; Tata, 1967). Since several growth and developmental hormones are known to stimulate RNA and protein synthesis in the hepatic tissue, Tata and

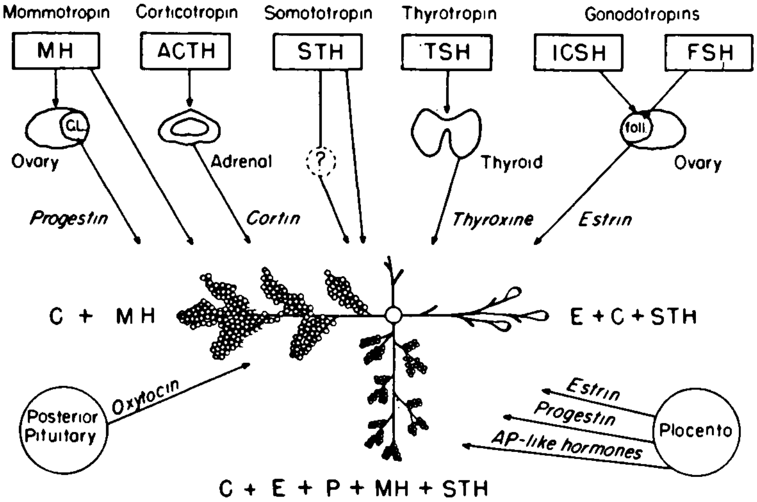

Mommotropin
Corticotropin
Somototropin
Thyrotropin
Gonodotropins
MH
ACTH
STH
TSH
ICSH
FSH
CL
Ovory
Adrenol
?
Thyroid
foll
Ovory
Progestin
Cortin
Thyroxine
Estrin
C + MH
E + C + STH
Posterior Pituitory
Oxytocin
Estrin
Progestin
AP-like hormones
Plocento
C + E + P + MH + STH

Fig. 1. Schema showing some of the hormones that influence mammary growth and lactation. In the mammary diagram: upper, rudimentary gland; right, prepubertal to pubertal gland; lower, gland of pregnancy (prolactational); left, lactating gland. From Lyons *et al.* (1958).

his associates examined the role of testosterone, thyroxine, and growth hormone administered alone or in combinations on the activity of nuclear RNA polymerase I and RNA polymerase II, the two enzymes responsible for synthesizing the ribosomal and messenger RNA, respectively. Results of these experiments indicated that the effects of these hormones were additive and the sites of action of these three hormones on the stimulation of nuclear RNA polymerase activity were each different from one another. The effect of hypophysectomy and the additive influence of growth hormone and thyroxine on the amino acid incorporation by free and microsome bound polysomes in the liver were also examined by these investigators (Tata and Williams-Ashman, 1967). The capacity of the polysomal incorporation of amino acids was markedly depressed in the hypophysectomized rats and the deficiency was more than corrected by combined treatment with growth hormone and triiodothyronine. All these results pointed toward an important physiological role of endocrine–endocrine interactions in the regulation of gene expression and protein synthesis for the maintenance of metabolic homeostasis.

The mammary model revealed the role of multiple hormones in development, differentiation, and organogenesis while the studies on the regulation of hepatic RNA and protein synthesis by several growth and developmental hormones underscored the general importance of the cooperative influence of various hormones in the regulation of protein synthesis. Subsequently the interacting influence of various growth and developmental hormones in the regulation of a single gene product was clearly established in the case of α_{2u}-globulin (Roy, 1973a). The following text shows that the cooperative effect of different hormones known to be involved in the regulation of α_{2u}-globulin synthesis takes place through endocrine interactions at both systemic and cellular levels. Although I will primarily review the multihormonal regulation of α_{2u}-globulin gene expression, the relationship of this model system to other genes under the regulatory influence of multiple hormones will also be considered.

II. α_{2u}-GLOBULIN AND ITS HEPATIC SYNTHESIS

A. Identification, Isolation, and Characterization of α_{2u}-Globulin

Normal male rats excrete high amount of protein in their urine. Investigation of the various proteins in the normal male rat urine showed that approximately half of the total urinary protein is due to one protein moiety with the electrophoretic mobility of α_2-globulins. This urinary α_2-globulin was immunochemically different from the α_2 globulins of the rat serum and therefore it was designated as α_{2u}-globulin, with the subscript "u" to denote its abundance in urine (Roy and Neuhaus, 1966a). α_{2u}-Globulin was purified to homogeneity from the urine of male rats and was found to have the following physiochemical properties. It is a single chain polypeptide, coded by a multigene family (Roy *et al.*, 1966; Kurtz, 1981). Minor differences in the structural genes lead to at least three distinct forms of α_{2u}-globulin differing in their isoelectric points, and, in addition to these charge variants, the products of α_{2u} gene can be separated into two distinct polypeptides differing in approximately five amino acid residues (Chatterjee *et al.*, 1982) (Fig. 2).

B. Synthesis of α_{2u}-Globulin by Hepatic Tissue *in Vivo* and Cultured Hepatocytes *in Vitro*

The origin of the urinary α_{2u}-globulin was initially screened by analyzing various tissue extracts for their immunochemical reactivity with the rabbit antiserum prepared against pure α_{2u}-globulin. These tests identified liver, kidney, and the salivary gland as containing appreciable concentrations of this protein. Labeled amino acid incorporation, cell fractionation, tissue uptake of externally supplied α_{2u}-globulin, and immunofluorescent studies proved liver to be the site of synthesis while both kidney and the salivary gland concentrate this protein from the circulation (Roy and Neuhaus, 1966b; Roy and Raber, 1972; Roy and Byrd, 1976). These experiments also indicated that, primarily because of its low

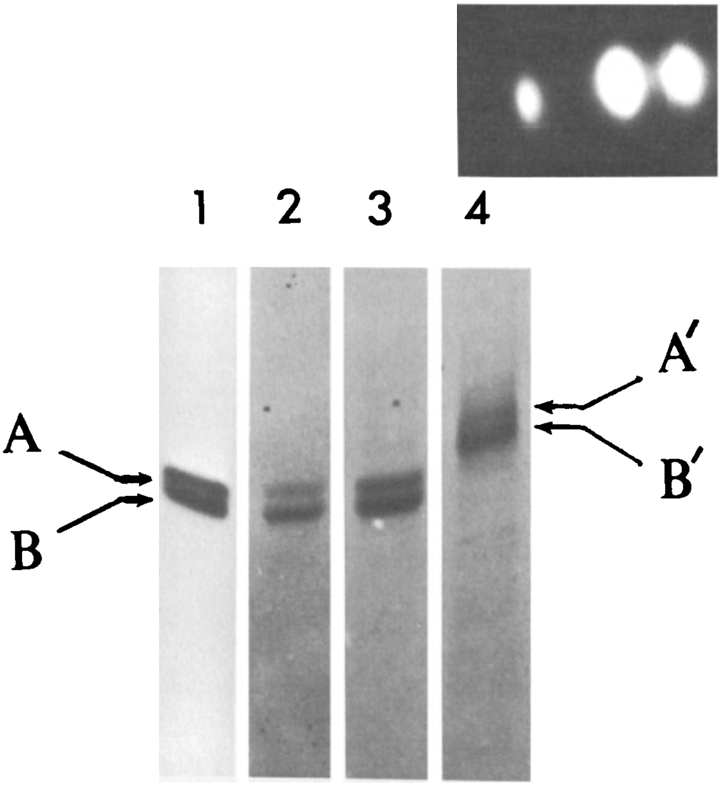
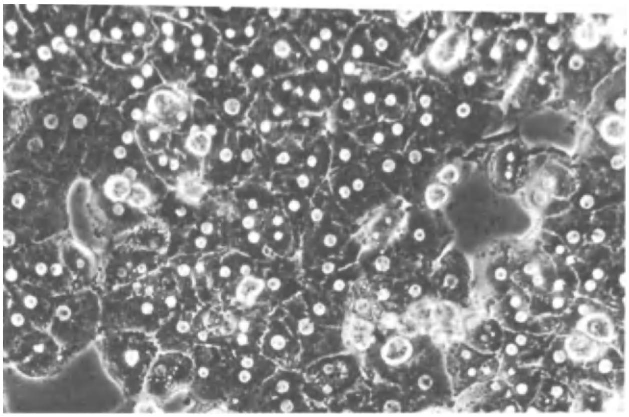


Fig. 2. Two molecular forms of α_{2u}-globulin and their *in vitro* synthesis from rat liver mRNA. Autoradiographic patterns of the SDS-polyacrylamide slab gel shown in the different lanes: Lane 1, ^{125}I-labeled α_{2u}-globulin purified from male rat urine. The two electrophoretically distinct components are marked A and B. Lane 2, immunoprecipitated ^{35}S-labeled α_{2u}-globulin synthesized in the *Xenopus* oocytes after microinjection of rat liver mRNA. Lane 3, immunoprecipitated ^{35}S-labeled α_{2u}-globulin synthesized in the rabbit reticulocyte lysate in the presence of microsomal membrane from dog pancreas. Lane 4, immunoprecipitated ^{35}S-labeled α_{2u}-globulin synthesized in the rabbit reticulocyte lysate in the absence of dog pancreas membrane. The two preprotein forms of α_{2u}-globulin are marked as A' and B'. M_r of the different forms of α_{2u}-globulin were found to be as follows: $\alpha_{2u}A = 18{,}800$; $\alpha_{2u}B = 18{,}100$; $\alpha_{2u}A' = 20{,}300$; $\alpha_{2u}B' = 19{,}600$. The inset shows the three isoelectric variants of α_{2u}-globulin separated by isoelectric focusing. From Chatterjee *et al.* (1982) and unpublished data.

molecular weight, α_{2u}-globulin is rapidly filtered through the kidneys into the urine and therefore only very low amount of this protein can normally be detected in the serum. Studies with primary cultures of rat hepatocytes confirmed the results of the *in vivo* experiments and showed that hepatocytes isolated from normal male rats are capable of synthesizing and secreting α_{2u}-globulin (Fig. 3).



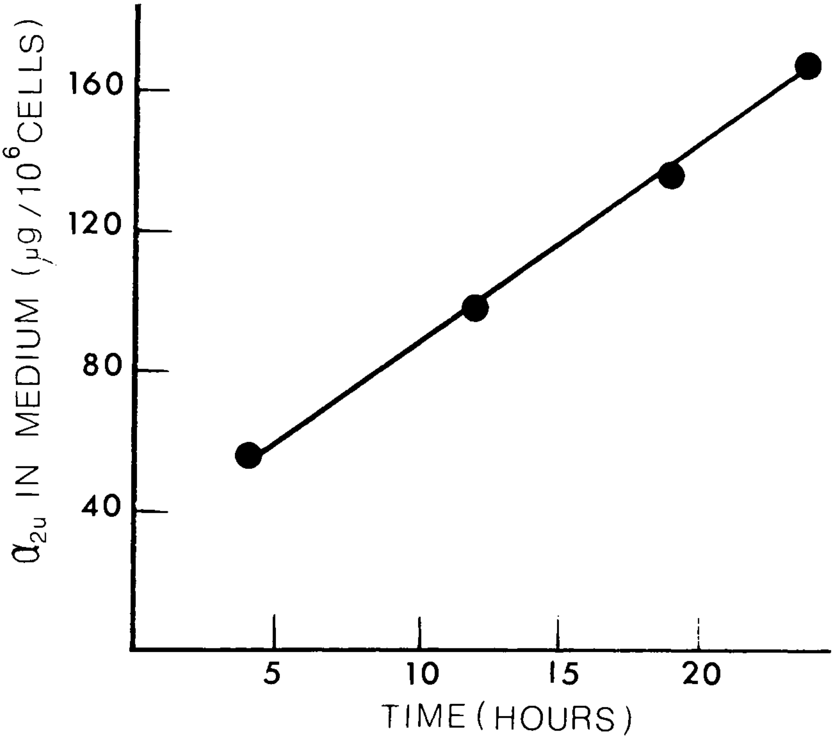


Fig. 3. Synthesis and secretion of α_{2u}-globulin by monolayer cultures of normal rat hepatocytes. At the top of the figure is a photomicrograph of a 24-hour hepatocyte culture, taken under phase contrast illumination. The graph at the bottom shows linear rate of secretion of α_{2u}-globulin by the cultured hepatocytes within the experimental period.

III. PURIFICATION OF THE MESSENGER RNA FOR α_{2u}-GLOBULIN AND CLONING OF THE DOUBLE-STRANDED cDNA

A. Partial Purification of the Messenger RNA for α_{2u}-Globulin and Synthesis of a cDNA Probe

α_{2u}-Globulin constitutes about 1% of the total mRNA population in the liver of mature male rats. Electrophoretic fractionation of the poly(A)-containing hepatic mRNA from mature male rats in the agarose–urea gel showed that the messenger for α_{2u}-globulin migrates as a single band in the 14 S region (Chatterjee and Roy, 1980). This procedure also allowed about 18-fold purification of the mRNA for α_{2u}-globulin. The purity of the cDNA obtained by the reverse transcription of the partially purified α_{2u}-mRNA reflected the heterogeneity of the mRNA preparation. However, by judicious choice of a proper restriction endonuclease (*Hae*III) it is possible to cause selective site-specific fragmentation of the major cDNA species within the heterogeneous mixture and random degradation of the minor cDNA populations (Seeburg *et al.*, 1977). Action of this restriction endonuclease (*Hae*III) resulted in the generation of two discrete fragments from α_{2u}-cDNA. One of these fragments containing 410 nucleotide residues was isolated and used as a probe for α_{2u}-mRNA (Chatterjee and Roy 1980).

B. Cloning of the Double-Stranded cDNA for α_{2u}-Globulin

Molecular hybridization analysis of the cDNA isolated by the restriction cleavage procedure showed that the probe still contained about 15% cross contamination with other sequences. In order to obtain a pure cDNA for α_{2u}-globulin we have cloned the double-stranded cDNA synthesized from the total poly(A)-containing hepatic mRNA of male rat liver and then used the *Hae*III fragment of the α_{2u}-cDNA as a probe to identify the recombinant clones carrying the α_{2u}-cDNA insert. Total poly(A)-containing hepatic mRNA was initially fractionated on a sucrose density gradient and the mRNA's of approximately 10–16 S range were used as the template for reverse transcriptase. The single-stranded cDNA synthesized from this mRNA preparation was converted to the double-stranded form with DNA polymerase. These enzymatic treatments generally result in the generation of a hairpin loop at one end of the cDNA and a relatively short single-stranded stretch at the other end. Both of these ends can be uniformly blunted with a single-strand specific (S1) nuclease. The blunt ended double-stranded cDNA was then tailed at the 3′ end with oligo$(dC)_{20}$. The complementary nucleotide dG was used to introduce an oligo$(dG)_{20}$ tail to the linearized plasmid (pBR322) DNA. The C-tailed cDNA and G-tailed plasmid were then annealed to obtain the chimeric plasmid which was used to transform *E. coli* strain HB101.

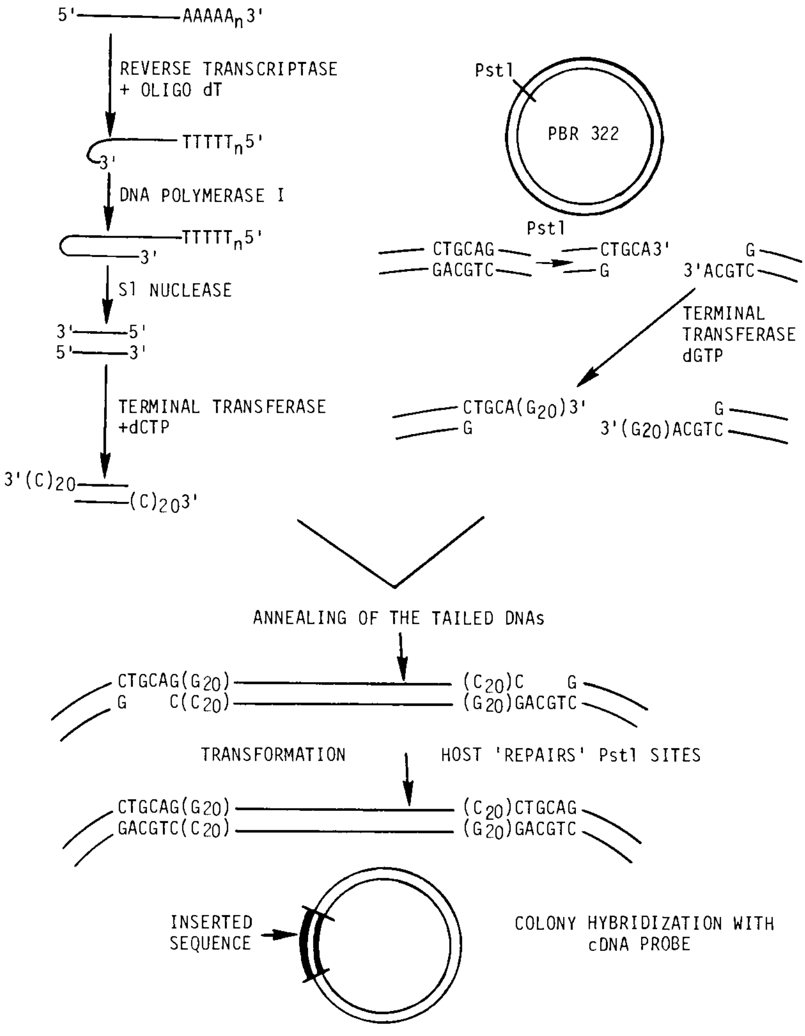

5'——AAAAA$_n$3'
REVERSE TRANSCRIPTASE
+ OLIGO dT
TTTTT$_n$5'
3'
DNA POLYMERASE I
TTTTT$_n$5'
3'
S1 NUCLEASE
3'——5'
5'——3'
TERMINAL TRANSFERASE
+dCTP
3'(C)20
(C)20 3'
PstI
PBR 322
PstI
CTGCAG
GACGTC
CTGCA3'
G
G
3'ACGTC
TERMINAL
TRANSFERASE
dGTP
CTGCA(G20)3'
G
G
3'(G20)ACGTC
ANNEALING OF THE TAILED DNAs
CTGCAG(G20)
G C(C20)
(C20)C G
(G20)GACGTC
TRANSFORMATION
HOST 'REPAIRS' PstI SITES
CTGCAG(G20)
GACGTC(C20)
(C20)CTGCAG
(G20)GACGTC
INSERTED
SEQUENCE
COLONY HYBRIDIZATION WITH
cDNA PROBE

Fig. 4. Scheme showing the steps involved in cloning the double-stranded cDNA for α_{2u}-globulin in the bacterial plasmid pBR322.

The complete procedure is outlined in Fig. 4. The *E. coli* carrying the recombinant plasmid were selected by their tetracycline resistance and ampicillin sensitivity. The recombinant colonies were then replated on gridded agar plates for identification of the individual colonies. A filter paper imprint of the agar plate containing 100 recombinant colonies were hybridized with ^{32}P-labeled α_{2u}-cDNA probe prepared by the *Hae*III restriction procedure. Those recombinant colonies that contained plasmids carrying the α_{2u}-cDNA insert hybridized with the radioactive probe. The filter paper when autoradiographed on an X-ray plate

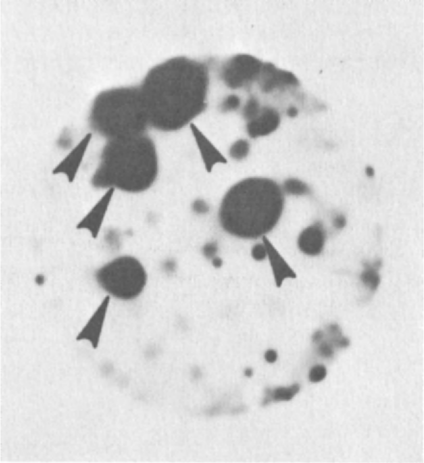


Fig. 5. Screening of the recombinant clones containing α_{2u}-cDNA inserts. *E. coli* colonies harboring the recombinant plasmids were imprinted on a nitrocellulose filter paper and hybridized with ^{32}P-labeled α_{2u}-cDNA probe. The arrows point to the colonies selectively hybridized to the labeled probe.

generated the picture shown in Fig. 5. Out of 100 recombinant colonies, five (pointed with arrows) showed a high degree of specific hybridization with the ^{32}P-labeled α_{2u}-cDNA probe. These positive clones were further characterized and authenticated for harboring the α_{2u}-plasmid by a procedure which uses selective retention of the α_{2u}-mRNA by the plasmid DNA immobilized on a nitrocellulose filter. Plasmid DNA from one of the above clones was isolated and subsequently digested with a restriction enzyme (*Hha*I) which chews up the plasmid DNA into small pieces leaving the inserted α_{2u}-cDNA as the largest DNA fragment. The α_{2u}-cDNA fragment released from the recombinant plasmid can then be separated from the smaller fragments of plasmid DNA by agarose gel electrophoresis. The cloned α_{2u}-cDNA band thus isolated from the plasmid can be identified under ultraviolet light after ethidium bromide treatment. The DNA fragment is labeled by "nick translation" and used as a pure probe for studying the hormonal regulation of the mRNA for α_{2u}-globulin.

IV. ROLE OF ANDROGENIC AND ESTROGENIC STEROIDS IN THE HEPATIC SYNTHESIS OF α_{2u}-GLOBULIN

A. Androgenic Induction and Estrogenic Suppression

α_{2u}-Globulin is normally synthesized by mature male rats and it is absent in the immature male and female rats of all ages (Roy and Neuhaus, 1967). Figure 6

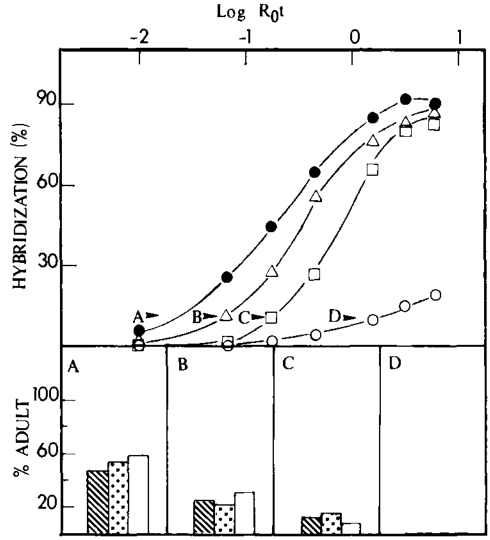


Fig. 6. Correlation between hybridizable and translatable α_{2u}-mRNA and the hepatic concentrations of α_{2u}-globulin in maturing male rats. The upper frame shows the hybridization kinetics of α_{2u} cDNA to a vast excess of poly(A)$^+$ hepatic RNA from rats of different ages: A=60 days; B=50 days; C=45 days; D=35 days. The lower frame shows the relative concentration of hybridizable α_{2u}-mRNA as estimated from $R_0t_{1/2}$ of hybridization (hatched bars), translatable α_{2u}-mRNA as estimated by cell-free translation followed by specific immunoprecipitation, and SDS-polyacrylamide gel electrophoresis (dotted bars) and α_{2u}-globulin in the hepatic cytosol (open bars) at different days of age as shown in the upper frame. From Chatterjee and Roy (1980).

shows the age-dependent appearance of α_{2u}-globulin and its corresponding mRNA in maturing male rats. From the data presented in this figure, it can be seen that the appearance of α_{2u}-globulin and its mRNA in the hepatic tissue of the maturing male rat coincides with the maturation of the testes which takes place at about 6 weeks of age. Although normal female rats do not synthesize α_{2u}-globulin, ovariectomy followed by androgen treatment is known to induce α_{2u}-globulin in female rats. On the other hand, daily treatments of mature male rats with estradiol result in gradual decrease and, within 5–7 days, complete inhibition of α_{2u} synthesis and disappearance of the mRNA for this protein from the hepatic tissue (Roy *et al.*, 1977). An additional and interesting observation with respect to the androgenic and estrogenic regulation of α_{2u}-globulin is that in the spayed female rat at the early stage of hormone action both androgenic and estrogenic steroids were able to induce this protein (Fig. 7). However, continued exposure to androgenic hormones resulted in enhanced response to the final injection of the androgen whereas similar pretreatments with estrogen resulted in a gradual decrease and, within 3 days, complete loss of the estrogenic induction of α_{2u}-globulin (Roy, 1977).

B. Role of the Hepatic Androgen Binding Protein in the Regulation of α_{2u}-Globulin Synthesis

The apparently contradictory result of both androgenic and estrogenic induction of α_{2u}-globulin can be reconciled if one assumes a physiological role of the bifunctional androgen–estrogen binding protein first reported from our laboratory and confirmed by several other groups of investigators (Milin and Roy, 1973; Roy *et al.*, 1974; Dickson *et al.*, 1978). This hepatic binder is found to be absent in the testicular feminized male, immature male, and senescent male rat, all of which are insensitive to the androgen induction of α_{2u}-globulin (Roy, 1973b; Roy *et al.*, 1974; Chatterjee *et al.*, 1981). In addition, certain synthetic antiandrogens such as cyproterone acetate, which do not bind to this hepatic binder, fail to inhibit the androgen dependent synthesis of α_{2u}-globulin in the mature male rat (Roy, 1976). More interestingly, the hepatic concentration of this sex hormone binding protein is also under hormonal regulation, induced by the androgen and repressed by the estrogen (Roy *et al.*, 1974). Thus, if this binding protein plays the postulated physiological role in the regulation of the genes of

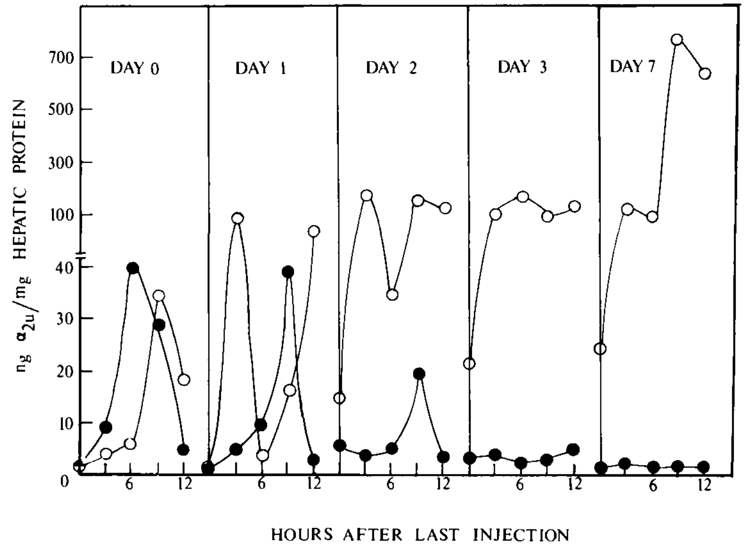


Fig. 7. Effect of daily pretreatments with estradiol or dihydrotestosterone on the hepatic levels of α_{2u}-globulin in castrated female rats after a single final injection of the same hormone. The animals on day 0 did not receive any pretreatment and received only single injections of either estradiol (●) or dihydrotestosterone (○). Animals on days 1, 2, 3, and 7 received 1, 2, 3, and 7 daily pretreatments with either estradiol (●) or dihydrotestosterone (○) followed 24 hours later by a single injection of the same hormone. Doses per injection were 5 μg estradiol/100 g body weight and 30 μg dihydrotestosterone/100 g body weight. The animals were sacrificed at various times (hours) after the final injection. From Roy (1977).

α_{2u}-globulin (Roy *et al.*, 1980a), it can explain both the androgenic and estrogenic induction as an early event in the steroid action (because of its ability to bind either the androgen or the estrogen) as well as the enhancement of the androgen response and decrement of the estrogenic induction after continued exposure to either one of these hormones (due to corresponding changes in hepatic concentration of this binding protein). This hepatic sex hormone binding protein, however, is not translocated into the nucleus (Roy, 1979). Therefore, any effect of this ''receptor'' has to be mediated through some other ''mediator'' molecules capable of directly interacting with the nuclear structures.

V. MULTIHORMONAL REGULATION OF α_{2u}-GLOBULIN

A. Multiple Hormone Requirements for the Hepatic Synthesis of α_{2u}-Globulin in the Hypophysectomized Rat

Prior to the development of the radioimmunoassay (RIA) for the quantitation of α_{2u}-globulin, we used a simple single-diffusion immunoassay several orders of magnitude less sensitive than the RIA procedure (Roy and Neuhaus, 1966b; Roy, 1977). Initial studies on the androgenic induction of α_{2u}-globulin in the spayed female rat showed an apparent lag period of about 4 days before any detectable increase in the urinary output of this protein could be measured (Roy and Neuhaus, 1967). Since steroid hormones are known to cause rapid induction of their target proteins, at the beginning of our investigation the possibility of an indirect effect of the androgen through other neuroendocrine adjustments seemed to be an attractive hypothesis worthy of experimental verification. With this idea in mind, we first looked into the possible indirect effect of the androgenic hormones mediated through the pituitary gland. Hypophysectomy of the male rat completely abolished the hepatic synthesis of α_{2u}-globulin. Hypophysectomized and spayed female rats also failed to synthesize α_{2u}-globulin after androgen administration (Kumar *et al.*, 1969). These observations led us to explore the possibility for an unknown pituitary factor responsible for mediating the androgenic induction of α_{2u}-globulin in rat liver. However, through experimentations with various combinations of known pituitary hormones, we were able to show that the hepatic synthesis of α_{2u}-globulin is under multihormonal regulation and that, besides the androgen, the hypophysectomized rat required glucocorticoid, growth hormone, and thyroxine for optimal synthesis of this protein (Roy, 1973a). Although the apparent long lag period led us to the discovery of the multihormonal regulation of α_{2u}-globulin, we know today that the lag is unreal, for the sensitive RIA is able to detect increased level of α_{2u}-globulin in the liver of spayed female rats within 1 hour after androgen administration.

Figure 8 shows the effect of various hormone supplementations on the hepatic synthesis of α_{2u}-globulin as measured by the urinary output of this protein.

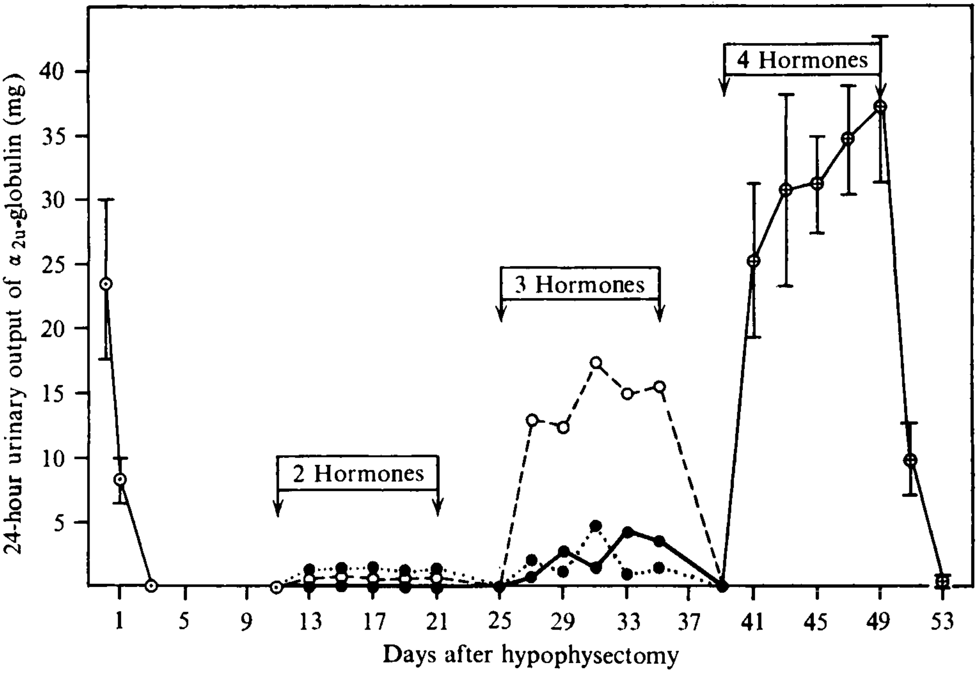


Fig. 8. The effect of hypophysectomy and various hormone treatments on the daily urinary output of α_{2u}-globulin in adult male rats. Nine animals were hypophysectomized at day 0 and their daily urinary output of α_{2u}-globulin was assayed for 10 days (⊙——⊙). These nine animals were divided into three groups (three rats in each group), and each group received daily injections of two hormones for 10 days: (●——●) androgen + thyroxine; (●···●) androgen + corticosterone; (○- - -○) androgen + growth hormone. After 4 days of rest, these animals received daily injections of three hormones for another 10 days: (●——●) androgen + thyroxine + growth hormone; (●···●) androgen + corticosterone + thyroxine; (○- - -○) androgen + growth hormone + corticosterone. After another 4 days of rest, all nine animals received daily injections of four hormones for 10 days: (⊕——⊕) androgen + thyroxine + growth hormone + corticosterone. The periods of hormone treatments are marked with arrows. From Roy (1973a).

Hypophysectomy of mature male rats within 3 days caused complete inhibition of the synthesis of α_{2u}-globulin. These animals were then subjected to either two, three, or four hormone treatments, androgen being the common principal in all hormone combinations. Other three hormones included corticosterone, thyroxine, and growth hormone. It can be seen that a combination of two hormones (i.e., testosterone + thyroxine, testosterone + growth hormone, and testosterone + corticosterone) had very little effect on the synthesis of α_{2u}-globulin in the hypophysectomized rats. On the other hand, three hormone regimens (testosterone + thyroxine + growth hormone, testosterone + corticosterone + thyroxine, and testosterone + growth hormone + corticosterone) fared better than the two hormone combinations and the last combination (i.e., testosterone + growth hormone + corticosterone) brought back the urinary α_{2u}-globulin level to more than 50% of the intact control. Finally, treatment of the animals with a combination of all four of these hormones led to a complete reversal of the effect of hypophysectomy on the synthesis of α_{2u}-globulin. These results, therefore, clearly establish the role of multihormones in the regulation of α_{2u}-globulin.

B. Specific Requirements for Thyroxine, Glucocorticoids, Growth Hormone, and Insulin

Once the multihormonal requirements for the synthesis of α_{2u}-globulin in the hypophysectomized rat were established, the individual role of these hormones on the process was examined in animals with a single endocrine deficiency *in vivo* and in cultured hepatocytes *in vitro*. Thyroidectomy resulted in an approximately 90% reduction in the hepatic synthesis of α_{2u}-globulin which reverted to normal after thyroxine treatment (Fig. 9). Similar degrees of inhibition were also observed after bilateral adrenalectomy or induction of experimental diabetes in mature male rats and these were reversed by glucocorticoid and insulin treatments, respectively (Irwin *et al.*, 1971; Roy, 1973a; Roy and Leonard, 1973).

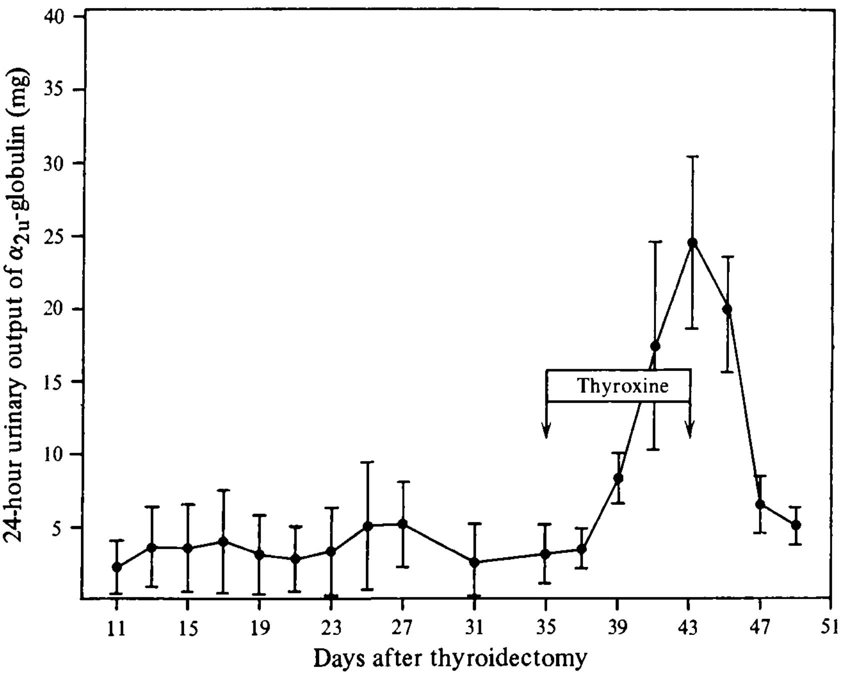


Fig. 9. The effect of thyroxine on the daily urinary output of α_{2u}-globulin in thyroidectomized adult male rats. The duration of daily thyroxine treatment is indicated with arrows. From Roy (1973a).

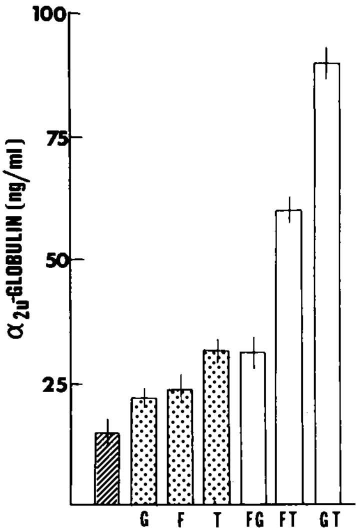


Fig. 10. Effect of GH, dexamethasone, and T_3, individually and as combinations of two hormones, on the accumulation of α_{2u}-globulin in the media of explant-imprinted hepatocytes. All hormones were added from the beginning of the culture, and the media were changed on the fourth day. The cultures were allowed to continue for another 4 days, and the samples of the media were assayed for α_{2u}-globulin. Each value is an average of four experiments ± SEM, corrected for 5×10^3 cells/flask. The different hormones are represented by letter symbols, i.e., G, GH; F, dexamethasone; T, T_3. The hatched bar is the corresponding value for control hepatocytes in the minimal maintenance medium. From Motwani *et al.* (1980).

The individual requirements for these hormones were further confirmed with cultured hepatocytes *in vitro*. A nonenzymatic procedure for the isolation and culture of rat hepatocytes developed in our laboratory was found to maintain the ability of these cells to synthesize and secrete α_{2u}-globulin for more than 2 weeks (Motwani *et al.*, 1980). The results presented in Fig. 10 show that the addition of growth hormone, glucocorticoid and thyroid hormone added to the culture medium, individually or in combinations, significantly stimulates synthesis and secretion of α_{2u}-globulin by the cultured cells. These results substantiate the observed effect of the above hormones *in vivo* and also show that they exert a direct effect on the liver cells in regulating the synthesis of α_{2u}-globulin.

C. Mechanism of the Multihormonal Regulation of α_{2u}-Globulin

1. *Action of Androgen, Estrogen, and Glucocorticoid*

The molecular basis of the steroid mediated regulation of gene expression has been the subject of extensive investigation in recent years and a consensus seems to be emerging with regard to the direct interaction between the activated steroid hormone receptor and the target gene on the chromosome (Chan and O'Malley, 1976). However, it should be noted that much remains to be known especially at the level of postreceptor events, before a clear scheme can be formulated with precision (Mueller, 1980). Both androgenic induction and estrogenic inhibition of the hepatic synthesis of α_{2u}-globulin are associated with corresponding changes in the hepatic concentration of α_{2u}-globulin synthesis by controlling the hepatic level of the mRNA for this protein (Sippel *et al.*, 1975; Roy *et al.*, 1976b; 1977). These results are consistent with the observed changes in other steroid sensitive model systems where these hormones are known to regulate specific protein synthesis through corresponding changes in gene transcription. Moreover, transcription of isolated liver nuclei from male and female rats in the presence of mercurated CTP has also indicated that androgen-dependent synthesis of α_{2u}-globulin is due to increased rate of gene transcription (Chan *et al.*, 1978). However, at this stage it seems appropriate to introduce two additional points that may be considered somewhat of a departure from the main theme of the molecular mechanism of steroid hormone action. First, it has already been mentioned under Section IV that there is a high degree of correlation between the presence of a cytosol androgen–estrogen binding protein and the ability of the liver to respond to the androgenic induction of α_{2u}-globulin. Since this hepatic binder does not translocate into the nucleus, its physiological role in the regulation of α_{2u}-globulin gene expression may provide a new and additional insight into the basic mechanism of the steroidal regulation of gene expression. Second, we have shown that actinomycin D, a well-known inhibitor of DNA-dependent RNA synthesis, can cause superinduction of α_{2u}-globulin, which is associated with a corresponding increase in the hepatic concentration of its messenger RNA (Chatterjee *et al.*, 1979). We have interpreted these results to indicate that, although the primary action of the steroid hormones is at the level of gene transcription, minor influence of the steroids in the regulation of mRNA stability cannot at present be completely overlooked. Glucocorticoids, another class of steroid hormone which are known to regulate α_{2u}-globulin synthesis, are also found to exert their influence through changes in the hepatic concentration of the messenger RNA (Sippel *et al.*, 1975).

2. *Action of Insulin*

Experimental induction of diabetes by either alloxan or streptozotocin treatment is generally associated with multiple metabolic alterations. In order to minimize the complications of chronic and severe diabetes the role of insulin in the synthesis of α_{2u}-globulin was examined in mildly diabetic rats undergoing insulin deficiency for a relatively short duration (Roy *et al.*, 1980b). The mildness of diabetes was ascertained by the modest increase in the level of blood glucose (250–350 mg/dl) after streptozotocin treatment and minor changes in the

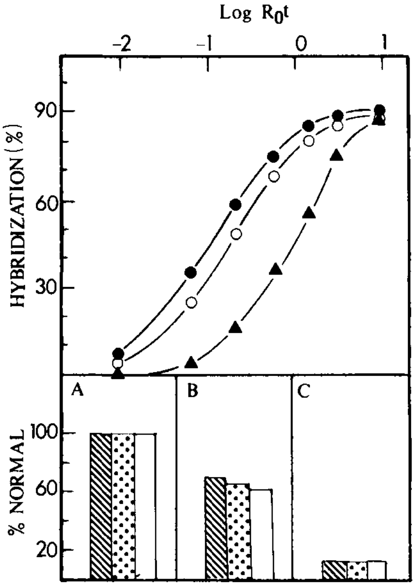


Fig. 11. Correlation of hepatic α_{2u}-globulin and both translatable and hybridizable mRNA for α_{2u}-globulin in normal and diabetic rats with and without insulin supplementation. The upper frame shows the kinetics of hybridization of ^{32}P-labeled cDNA fragment complementary to α_{2u}-mRNA in the presence of a vast excess of poly(A)$^+$ hepatic RNA from normal (●), diabetic with insulin supplementation for 4 days (○), and diabetic without insulin supplementation (▲). The lower frame shows composite data expressed as percent normal of hepatic concentration of α_{2u}-globulin (hatched bar), translatable mRNA for α_{2u}-globulin (dotted bar), and the open bar showing hybridizable α_{2u} sequence derived from the relative $R_0t_{1/2}$ values of the R_0t curves shown in the upper frame: (A) normal; (B) diabetic with insulin treatment for 4 days; (C) diabetic without insulin supplementation. From Roy *et al.* (1980b).

hepatic polysomal aggregate and stacking of the rough endoplasmic reticulum in these animals. Even this mild degree of diabetes caused more than 80% reduction in the hepatic synthesis of α_{2u}-globulin, which could be reversed to 80% of the normal within 4 days after insulin supplementation from osmotic minipumps (0.1 unit/hour). The degree of the hepatic synthesis of α_{2u}-globulin under insulin deficiency and after insulin supplementation was found to correlate with hepatic concentration of the specific mRNA for α_{2u}-globulin (Fig. 11). These studies clearly showed that insulin, a peptide hormone, is capable of contributing to the regulation of a specific gene expression.

3. *Action of Growth Hormone and Thyroxine*

As mentioned earlier, for a complete restoration of the hepatic synthesis of α_{2u}-globulin in hypophysectomized rats, growth hormone needs to be added to the multihormonal combination including androgen, glucocorticoid, and thyrox-

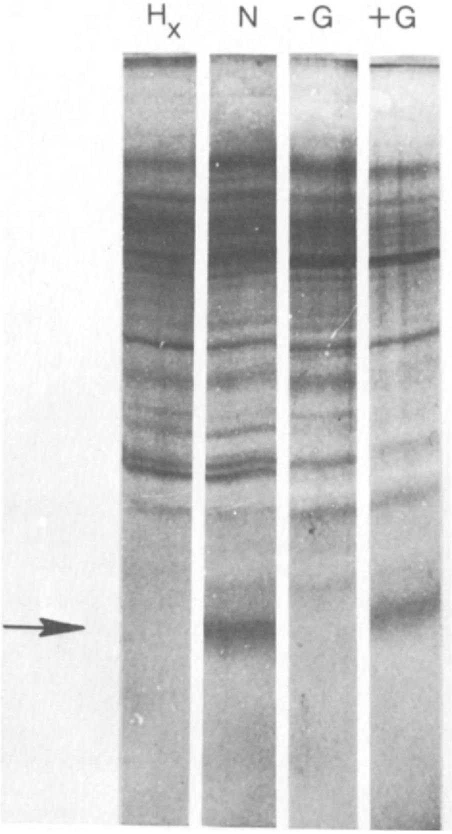


Fig. 12. Autoradiogram of the electrophoretically separated translation products of hepatic mRNA obtained from rats of different endocrine status. Hepatic mRNA was translated in the rabbit reticulocyte lysate system in the presence of [^{35}S]methionine. Translation products containing labeled proteins were electrophoresed on a SDS–polyacrylamide slab gel. The arrow shows the position of α_{2u}-globulin in the gel. Each lane contains translation products primed with pooled hepatic mRNA from three animals. Hx, hypophysectomized male rats without any hormone supplementation; N, unoperated normal male; −G, hypophysectomized male rats that received eight daily injections of DHT, corticosterone and thyroxine; +G, hypophysectomized male rats that received eight daily injections of DHT, corticosterone, thyroxine, and growth hormone. From Roy *et al.* (1982).

ine. The molecular mechanism of growth hormone action was therefore investigated in the hypophysectomized male rats, which received either a three hormone combination (androgen, glucocorticoid and thyroxine) or a complete four hormone regimen, which included the above three hormones plus growth hormone (Roy and Dowbenko, 1977; Roy *et al.*, 1982). Figure 12 shows an electrophoretic autoradiogram of the *in vitro* translation products of the hepatic mRNA obtained from the hypophysectomized rats treated with or without growth hormone as compared to the controls. In the absence of growth hormone, very little translatable mRNA for α_{2u}-globulin can be detected in the liver of the hypophysectomized rats. Molecular hybridization of the same RNA preparations with a cloned cDNA probe for α_{2u}-globulin also substantiated these results indicating that growth hormone is required for the maintenance of α_{2u}-mRNA sequences within the liver cells. The correlation of the translatable α_{2u}-mRNA, hybridizable α_{2u}-mRNA sequences, and hepatic α_{2u}-globulin in the hypophysectomized rats treated with and without growth hormone is summarized in Fig. 13. In the absence of growth hormone, there is a specific decrease in the hepatic concentration of α_{2u}-mRNA, which can be corrected with growth hormone supplementation. This finding is substantiated by experiments with cultured hepatocytes obtained from hypophysectomized rats which were treated *in vivo* with either a three hormone (androgen, glucocorticoid, and thyroxine) or a four hormone (androgen, glucocorticoid, thyroxine, and growth hormone) combination. Hepatocytes isolated from these animals were then cultured for a period of 24 hours in the presence of all four hormones. Figure 14 shows an autoradiograph of the labeled cellular proteins separated by the O'Farrell technique

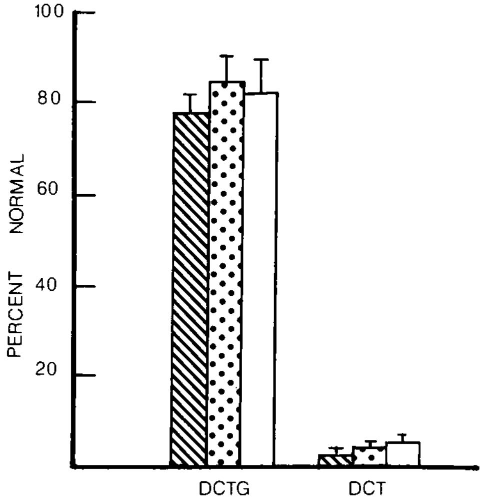


Fig. 13. Hybridizable α_{2u}-mRNA sequence, translatable α_{2u}-mRNA and cytoplasmic α_{2u}-globulin in the hypophysectomized rats with and without growth hormone supplementation. Hatched bars, α_{2u}-globulin mRNA sequences as determined by liquid hybridization with a cloned α_{2u}-globulin cDNA probe; dotted bars, translatable α_{2u}-globulin mRNA as determined by *in vitro* translation followed by specific immunoprecipitation and SDS-polyacrylamide gel electrophoresis; open bars, cytoplasmic α_{2u}-globulin determined by radioimmunoassay. Animals received eight daily treatments of either DHT, corticosterone, thyroxine, and growth hormone (DCTG) or DHT, corticosterone, and thyroxine (DCT). From Roy *et al.* (1982).

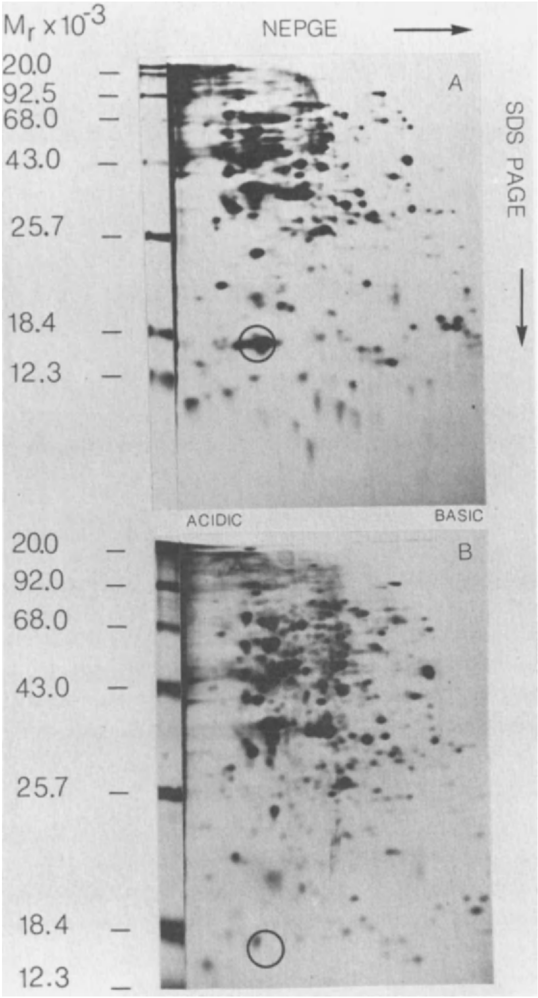


Fig. 14. Autoradiogram of the [^{35}S]methionine-labeled proteins synthesized by isolated rat hepatocytes and separated by two-dimensional gel electrophoresis. (A) Proteins synthesized by hepatocytes derived from hypophysectomized rats that received eight daily treatments of DHT, corticosterone, thyroxine, and growth hormone. (B) Proteins synthesized by hepatocytes derived from hypophysectomized rats that received eight daily treatments of DHT, corticosterone, and thyroxine. In both cases, the hepatocytes were cultured *in vitro* in the presence of DHT, corticosterone, thyroxine, and growth hormone. The position of pure α_{2u}-globulin in the two-dimensional gel is circled. Nonequilibrium pH gradient gel electrophoresis (NEPGE) was first performed from left (acidic) to right (basic). SDS-polyacrylamide slab gel electrophoresis (SDS-PAGE) was then run from top to bottom. Positions of the molecular weight marker proteins are indicated on the left. From Roy *et al.* (1982).

(O'Farrell *et al.*, 1977). Results show that only the hepatocytes derived from the hypophysectomized rat which received growth hormone *in vivo* (Fig. 14A) were able to synthesize α_{2u}-globulin *in vitro* and that exposure of growth hormone *in vitro* (Fig. 14B) failed to initiate the synthesis of this protein. All these results fail to support the conclusion concerning the translation regulation of α_{2u}-globulin (Kurtz *et al.*, 1978) and conclusively prove that growth hormone is required for maintaining the hepatic concentration of the messenger RNA for this protein.

The decreased synthesis of α_{2u}-globulin in hypothyroid rats and its reversal after thyroid hormone supplementation also showed a strong correlation with the corresponding changes in hepatic concentrations of the messenger RNA for α_{2u}-globulin (Roy *et al.*, 1976a). Similar results were also reported by Kurtz *et al.*

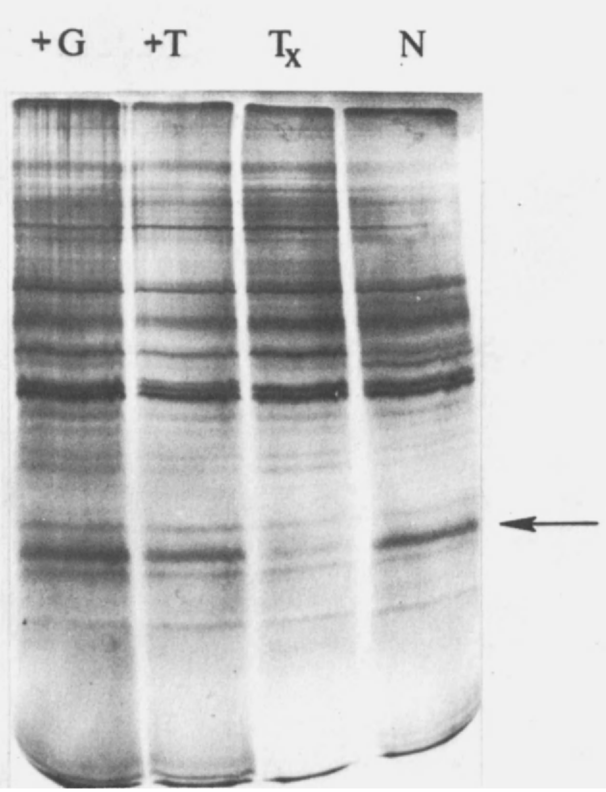


Fig. 15. Effect of growth hormone and thyroxine on the hepatic mRNA for α_{2u}-globulin. The picture shows the electrophoretic autoradiogram of the *in vitro* translation products of male rat liver mRNA obtained from normal (N), thyroidectomized (T_x), thyroidectomized treated with thyroxine (+T), and thyroidectomized treated with growth hormone (+G) animals. The arrow points to the translation product of the mRNA for α_{2u}-globulin.

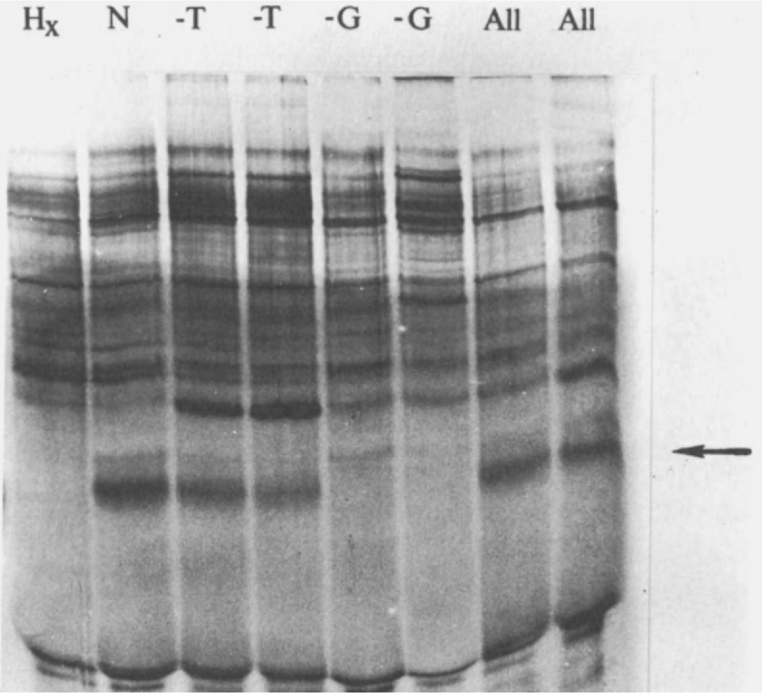


Fig. 16. Effect of growth hormone on the translatable mRNA for α_{2u}-globulin. The picture shows the electrophoretic autoradiogram of the *in vitro* translation products of the male rat liver mRNA obtained from normal (N), hypophysectomized (H_x), and hypophysectomized rats which received the following multihormone treatments: androgen, glucocorticoid, thyroxine and growth hormone (All): androgen, glucocorticoid, and thyroxine (−G); androgen, glucocorticoid, and growth hormone (−T). The arrow points to the translation product of the mRNA for α_{2u}-globulin.

(1976). Since all these studies were performed on whole animals, the report of Hervas *et al.* (1975) showing a drastic reduction in the content of growth hormone in the thyroidectomized rats led us to examine the interacting influence of thyroxine and growth hormone in the regulation of α_{2u}-globulin. Figure 15 shows the electrophoretic autoradiogram of the *in vitro* translation products of hepatic mRNA obtained from thyroidectomized male rats which received either growth hormone or thyroxine. Growth hormone was almost equally as effective as thyroxine in reversing the effect of thyroidectomy on the hepatic concentration of the messenger RNA for α_{2u}-globulin. These results suggested that some of the effects of thyroid hormone deficiency on α_{2u}-globulin synthesis may be indirectly mediated through pituitary growth hormone. The question of the predominant role of either growth hormone or thyroxine in the regulation of the hepatic concentration of α_{2u}-mRNA was more clearly established in the hypophysectomized male rats that received a three hormone treatment lacking in either thyroxine or growth hormone. Results of such an experiment shown in Fig. 16

definitely indicated that growth hormone plays a predominant role in the regulation of the messenger RNA for α_{2u}-globulin. In the absence of growth hormone, thyroxine was almost ineffective in increasing the hepatic concentration of the RNA for α_{2u}-globulin. On the other hand, growth hormone in the absence of thyroxine was capable of restoring the hepatic α_{2u}-mRNA to a level comparable to the normal control.

Although these results indicated that the predominant effect of thyroidectomy on the hepatic concentration of α_{2u}-mRNA is indirectly mediated through pituitary growth hormone, the results of *in vivo* pulse labeling of α_{2u} globulin with [^{35}S]methionine consistently show that in spite of reversal of hepatic concentration of α_{2u}-mRNA in the hypothyroid rat by growth hormone, the rate of synthesis of α_{2u}-globulin still remained at approximately 50% of the normal control. Composite correlation of the biologically active and chemically defined α_{2u}-mRNA, rate of labeling of α_{2u}-globulin after a pulse of labeled amino acid and the hepatic content of α_{2u}-globulin is summarized in the data presented in Fig. 17. These results suggest that although growth hormone alone can engineer the increase in the hepatic content of α_{2u}-mRNA to the level of normal control, complete reversal of the hepatic synthesis of α_{2u}-globulin still requires the help of thyroxine.

The ultrastructural features of the hepatic parenchymal cells of the hypothyroid rats with either thyroxine or growth hormone supplementation as presented in Fig. 18 show a general paucity of the rough endoplasmic reticulum in the

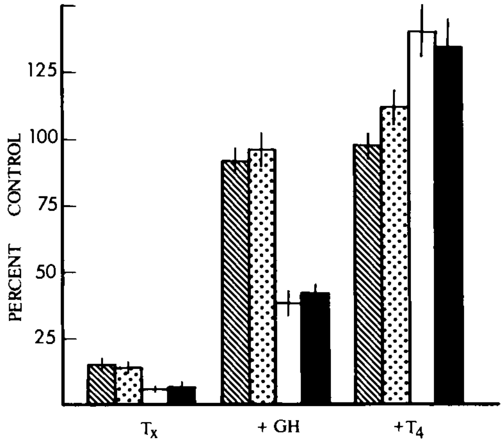
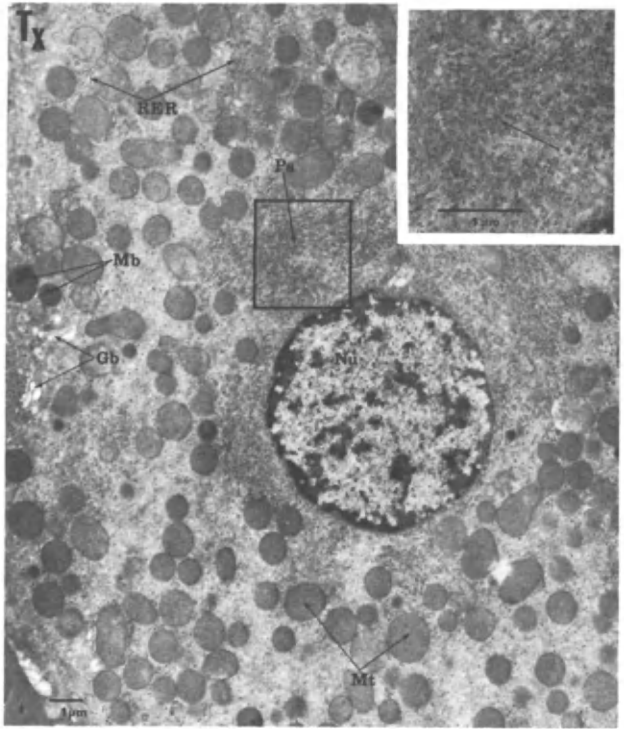


Fig. 17. Quantitative relationship among hybridizable α_{2u}-mRNA sequence, translationally active α_{2u}-mRNA, hepatic α_{2u}-globulin, and its rate of synthesis in thyroidectomized (T_x) rats receiving either growth hormone (+GH) or thyroxine (+T_4) supplementations. Hatched bar, hybridizable α_{2u}-mRNA sequence; dotted bar, translationally active α_{2u}-mRNA; open bar, radioimmunoassayable α_{2u}-globulin; shaded bar, incorporation of [^{35}S]methionine into α_{2u}-globulin after an *in vivo* pulse of 18 minutes.


Tx
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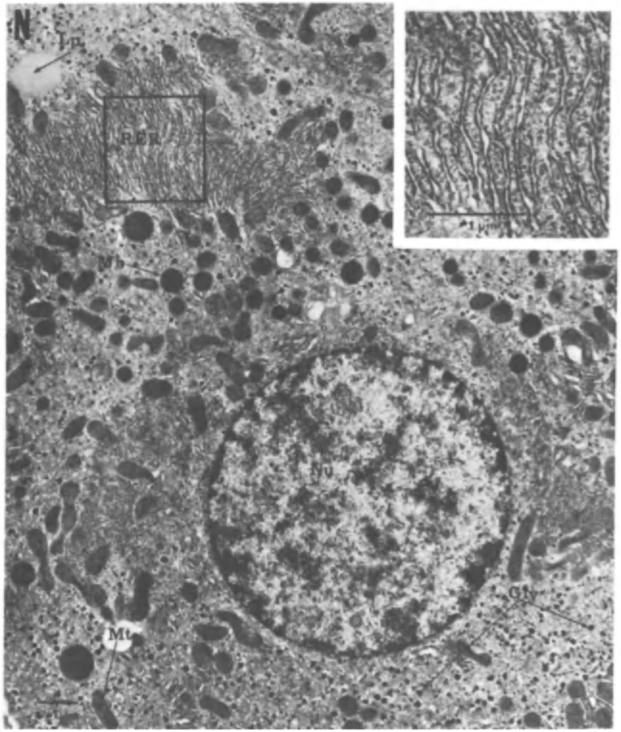
N
Lp
RER
Mb
Nu
Gly
Mt
1 µm

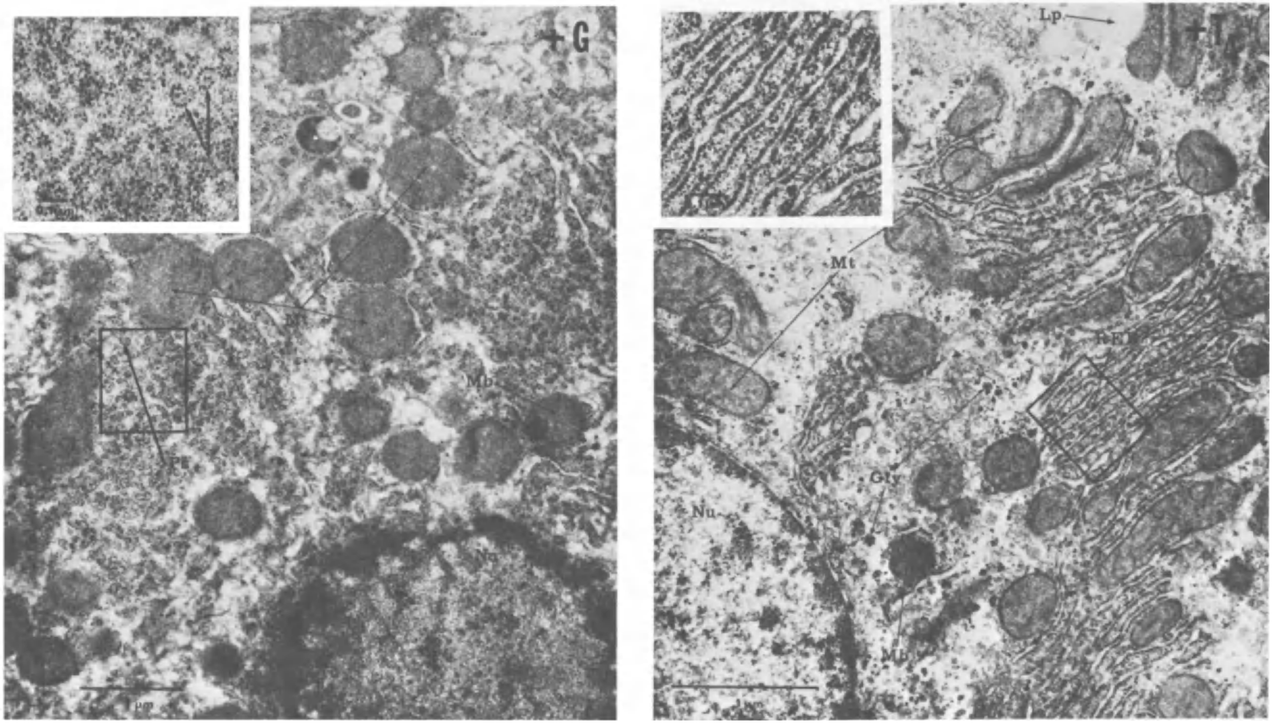

Fig. 18. Alterations in the rough endoplasmic reticulum of the liver cells after thyroidectomy and either growth hormone or thyroxine treatment. The liver samples were obtained from thyroidectomized (T_x), normal (N), thyroidectomized treated with growth hormone (+G), and thyroidectomized treated with thyroxine ($+T_4$). Some of the subcellular structures are labeled as follows: RER, rough endoplasmic reticulum; Ps, polysomes; Mt, mitochondria; Gly, glycogen; Nu, nucleus. The insets show the enlarged areas as outlined in each of the photomicrographs.

hypothyroid state, where most of the polysomes are found to be present in the free form, i.e., not bound to any membranous structure. Treatment of these animals with thyroxine but not with growth hormone can reverse this unusual ultrastructural feature to the normal state where most of the polysomes are attached to stacks of rough endoplasmic reticulum. These results indicate the specific importance of the thyroid hormone in the proliferation and maintenance of the endoplasmic reticulum of the hepatocytes. α_{2u}-Globulin, like other secretory proteins, contains a hydrophobic signal sequence which is responsible for guiding it into the lumen of the rough endoplasmic reticulum (Drickamer *et al.*, 1981; Chatterjee *et al.*, 1982). In spite of an adequate amount of the messenger RNA for α_{2u}-globulin, the paucity of the endoplasmic reticulum is expected to prevent the cotranslational removal of the signal sequence resulting in the unusual folding of this protein with the possibility of rapid lysosomal degradation. This may explain the lower level of α_{2u}-globulin in the hypothyroid rats treated with growth hormone which nevertheless contained normal level of α_{2u}-mRNA. It is of interest to note that a similar noncoordinated increase in hepatic level of albumin mRNA and albumin synthesis in the hypothyroid rat has also been reported (Peavy *et al.*, 1981).

VI. INVOLVEMENT OF THYROID HORMONE IN THE REGULATION OF SEVERAL OTHER MULTIHORMONALLY REGULATED GENES

Synthesis and secretion of growth hormone by the somatotrophs is one of the most important multihormonally regulated physiological processes where the thyroid hormone is known to play a critical role. Since this subject is covered in considerable detail in Chapter 3, only a gist of the multiple endocrine involvement in the regulation of growth hormone synthesis will be mentioned here. By utilizing the powerful method of radioimmunoassay (RIA), it was conclusively demonstrated that acute hypothyroidism is associated with a marked deficiency in both pituitary and circulating levels of growth hormone (Peake *et al.*, 1973; Hervas *et al.*, 1975). These results were subsequently confirmed *in vitro* (Samuels *et al.*, 1973; Martial *et al.*, 1977; Seo *et al.*, 1977). In addition to the thyroid hormone, the expression of growth hormone gene is also regulated by several other hormones including glucocorticoid, insulin, and epidermal growth factor. Glucocorticoid is known to act synergistically with the thyroid hormone to stimulate the synthesis of growth hormone mRNA. On the other hand, insulin inhibits the glucocorticoid response while EGF interferes with the stimulatory effect of the thyroid hormone (Baxter *et al.*, 1979; Samuels *et al.*, 1973). Transcriptional studies with nuclei isolated from a growth hormone secreting pituitary cell (GH_3)

line indicate that both thyroid and glucocorticoid hormones increase the number of RNA polymerase molecules involved in the transcription of the growth hormone gene (Eberhardt *et al.*, 1980).

Genes for several hepatic enzymes are known to be regulated by multiple endocrine interactions. One of the most well-explored examples is the multihormonal regulation of the malic enzyme, which has been investigated both *in vivo* and in cultured hepatocytes *in vitro* (Towle *et al.*, 1981; Siddiqui *et al.*, 1981). In the cultured hepatocytes the mRNA for this enzyme is stimulated nearly 100-fold in the presence of insulin and T_3. Glucagon antagonizes the stimulatory effect of the thyroid hormone. The molecular biology of this system will be described in further detail in Chapters 8 and 9.

Multihormonal regulation of glutamine synthetase in cultured rat liver cells has been described by Gebhardt and Mecke (1979). Although addition of either dexamethasone or growth hormone alone did not significantly stimulate the enzyme activity, addition of both of these hormones during the second day of primary culture increased the level of glutamine synthetase to 60–70% above the control. Supplementation of the culture medium with triiodothyronine caused a further augmentation of the inductive response by growth hormone and dexamethasone. Another hepatic enzyme δ-aminolevulinic-acid (ALA) synthetase, involved in the porphyrin biosynthesis, is also under multihormonal regulation. Insulin, glucocorticoid, and thyroxine are known to provide cooperative permissive effect in the regulation of this enzyme (Sassa *et al.*, 1979). Unlike other multihormonally regulated hepatic enzymes described above, histidase is antagonistically influenced by the thyroid hormone. Estrogen, glucocorticoid, and glucagon can induce this enzyme, whereas both hypophysectomy and treatment with T_3 inhibit its synthesis (Neufeld *et al.*, 1971; Armstrong and Feigelson, 1980). Involvement of the same growth and developmental hormones in the regulation of these hepatic enzymes and α_{2u}-globulin is indicative of a common regulatory mechanism mediating hormonal induction of the hepatic genes. Because the molecular biology of none of the above systems has been studied, the mechanism of the thyroidal regulation of these enzymes remains to be established. In their original scheme for the multihormonal regulation of mammogenesis and lactation, Lyons *et al.* (1958) suggested a role for the thyroid hormone in the process. Later studies of Vondarhaar (1977) also showed that when added to cultured mammary explants, physiological concentrations of T_3 can cause 10-fold enhancement of the prolactin mediated induction of α-lactalbumin. However, most of the recent reports on the molecular endocrinology of the mammary gland have only emphasized the roles of steroid and peptide hormones, and little progress seems to have been made in the elucidation of the contributory influence of thyroxine in the overall regulation of mammary gene expression.

VII. CONCLUDING REMARKS

The role of endocrine interactions in the regulation of mammary growth and lactation was a landmark discovery in the evolution of the concept of multihormonal regulation of a specific physiological process. This early concept in physiological endocrinology was carefully extended into biochemical endocrinology by studies in the laboratories of Tata and Williams-Ashman who showed the interacting and additive influences of various growth and developmental hormones in the hepatic protein synthesis (Tata and Williams-Ashman, 1967; Tata, 1967). Many of these effects were found to be mediated through increased synthesis of ribosomes and topographical redistribution of the polyribosomes to the cytoplasmic membranes. The findings of Lyons *et al.* (1958) on the role of multiple hormonal requirements for mammogenesis and lactation have been brought into the area of subcellular and molecular endocrinology by the efforts of many investigators in this country and abroad including Topper (1970), Turkington *et al.* (1973), Houdebine (1980), and Rosen *et al.* (1980). Although our studies on the multihormonal regulation of α_{2u}-globulin started serendipitously to uncover the reason for the apparent lag period for androgenic induction, they have subsequently been influenced by the elegant studies of the mammary group. We do not yet fully understand the extent of the complex multihormonal interactions in the actual regulation of α_{2u} gene expression and have not been able to pinpoint the exact molecular events regulated by each of the participating hormones. However, based on the work in our laboratory and that of others, the following tentative conclusions seem to emerge. Androgen, the primary inducer for α_{2u}-globulin, may be involved in changing the chromatin structure which can facilitate specific transcription of the genes for α_{2u}-globulin. The estrogenic hormones seem to act directly by inhibiting testicular androgen synthesis and acting directly on the liver by depleting the hepatic androgen binding protein (Roy *et al.*, 1974; 1975). Another steroid hormone glucocorticoid is also found to stimulate synthesis of the mRNA for α_{2u}-globulin. However, glucocorticoid is only effective when the androgen has already acted on the hepatocytes to make the genes accessible to other endocrine regulators. Of the nonsteroidal hormones, both insulin and growth hormone are effective in regulating the hepatic concentration of the messenger RNA for this protein and most likely this is achieved through increased gene transcription. The possibility that insulin's effect is mediated through changes in the hepatic receptors for other hormones is still open. Growth hormone and thyroxine play an intimate interacting role both at the physiological and the biochemical level. At the physiological level, thyroid hormone is responsible for maintaining the normal concentration of circulating growth hormone by acting directly on the pituitary gland. At the biochemical level, thyroxine seems to be required for maintaining an adequate amount of the endoplasmic reticulum on which hepatic proteins are synthesized and processed.

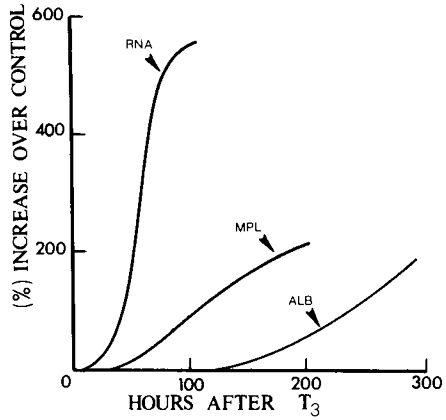

Fig. 19. Sequential changes in the rates of hepatic RNA and phospholipid synthesis in relation to the increase in the level of serum albumin after precocious induction of metamorphosis in *Rana catesbiana* tadpoles with triiodothyronine. The curves are marked as follows: RNA, rapidly labeled nuclear RNA; MPL, microsomal phopholipid; ALB, serum albumin. Redrawn from Tata (1967).

Discovery of the nuclear receptor for the thyroid hormone, its localization on the chromatin structures, and the observed correlation between fractional nuclear occupancy and certain biological responses have provided strong circumstantial evidence for a direct role of thyroid hormone in the regulation of gene expression (Oppenheimer, 1979). Studies on the thyroidal regulation of the growth hormone mRNA in the pituitary (Baxter *et al.*, 1979), and the malic enzyme mRNA in the liver (Towle *et al.*, 1981) also support such a hypothesis for a direct effect of thyroxine on specific gene transcription. However, recent results in our laboratory underscore the importance of the pleiotropic effect of the thyroid hormone, especially its effect on membrane synthesis and the resulting influence of this process in the regulation of synthesis and secretion of secretory proteins. This aspect of thyroid hormone action was first indicated from studies of Tata on the metamorphosis of the bullfrog tadpoles with thyroxine. The profound effect of the thyroid hormones on various aspects of gene expression is most dramatically exemplified in amphibian metamorphosis. Figure 19, which is adapted from Tata (1967), shows the temporal relationship between increased hepatic RNA and phospholipid synthesis and the appearance of serum albumin in the blood of *Rana catesbiana* tadpoles. In this system, the thyroxine-dependent increase in microsomal phospholipid synthesis is clearly found to precede the synthesis and secretion of serum albumin. These observations on the effect of thyroxine in the synthesis and secretion of the hepatic proteins emphasize the importance of regulatory influence of the thyroid hormone on gene expression mediated through intracellular membrane organization.

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8

Regulation of Malic Enzyme in Hepatocytes in Culture: A Model System for Analyzing the Mechanism of Action of Thyroid Hormone

ALAN G. GOODRIDGE

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I. INTRODUCTION

Malic enzyme (EC 1.1.1.40) catalyzes the NADP-dependent oxidative decarboxylation of malate to pyruvate and CO_2 (Ochoa *et al.*, 1947). The activity of this enzyme is positively correlated with the rate of *de novo* fatty acid biosynthesis in different tissue types of the same animal and in the same tissue type of animals in different hormonal and nutritional conditions (reviewed in Frenkel, 1975). An important functional role of malic enzyme, therefore, is the production of NADPH which is utilized for the synthesis of long chain saturated fatty acids from malonyl-CoA. Malic enzyme is localized in the cytosol in cells specialized for lipogenesis and, together with the cytosolic form of malic dehydrogenase (EC 1.1.1.37), converts NADH formed during glycolysis to NADPH which can be used for fatty acid synthesis (Wise and Ball, 1964; Young *et al.*, 1964; Pande *et al.*, 1964).

The action of thyroid hormone on liver malic enzyme activity was first reported by Tepperman and Tepperman (1964). Subsequent studies have established that malic enzyme activity is an indicator of thyroid status (Tarentino *et al.*, 1966). Because these were the results of *in vivo* studies, it was unclear whether thyroid hormone directly interacted with the hepatocyte to stimulate malic enzyme activity or directly interacted with another tissue to produce a product that subsequently interacted with the hepatocyte to stimulate malic enzyme activity. The results of the tissue culture experiments described below indicate a direct effect of thyroid hormone on the hepatocytes.

II. REGULATION OF MALIC ENZYME ACTIVITY IN CULTURE

A. Experiments Using Serum-Containing Medium

Our initial interest in the regulation of malic enzyme involved the rapid induction of enzyme activity that occurred when neonatal chicks were fed (Goodridge, 1968a,b). In order to study the mechanisms involved in this response we developed a tissue culture system so that we could examine regulation of enzyme activity under defined conditions (Goodridge *et al.*, 1974). Isolated cells prepared from the livers of prenatal or unfed neonatal chicks have very low malic enzyme activities. Incubation for 4 days in a culture medium containing 15% horse serum and 2.5% fetal calf serum caused more than a 100-fold increase in malic enzyme activity (Fig. 1). Addition of glucagon blocked this effect whereas addition of triiodothyronine (T_3) resulted in a further increase to 290 times the initial activity. The induction of malic enzyme was associated with a large increase in *de novo* synthesis of fatty acids and increases in the activities of

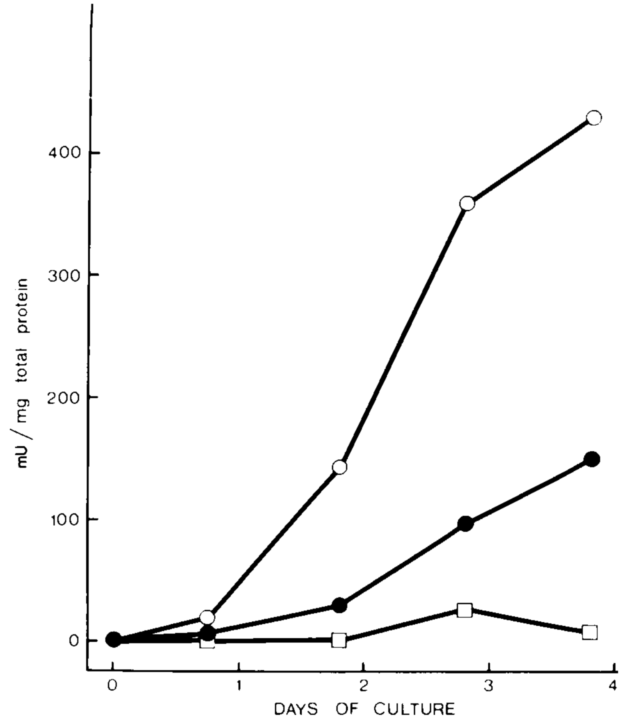


Fig. 1. Malic enzyme activity in hepatocytes in culture. Cells were isolated from 18-day-old chick embryos as previously described (Goodridge, 1973a) and incubated in Nutrient Mixture F-10 containing 15% horse serum, 2.5% fetal calf serum, and 17 m*M* glucose. At the designated intervals, the cells were harvested and malic enzyme assayed in the 105,000 *g* for 1 hour supernatant. The results are expressed as milliunits of enzyme activity per milligram total cellular protein. (●) Cells incubated without any added hormone; (○) incubated with L-triiodothyronine (2 μg/ml); (□) incubated with glucagon (1 μg/ml). Other experimental details are as described in Goodridge *et al.* (1974).

several of the lipogenic enzymes (Goodridge *et al.*, 1974). Other enzymes not associated with lipogenesis either did not change or decreased in activity (Goodridge *et al.*, 1974).

Upon further analysis, thyroid hormone in the added serum was found to be a major cause of the induction which occurred in the absence of added hormones. Addition of the thyroid hormone-binding proteins, bovine serum albumin, human thyroxine-binding prealbumin and anti-thyroxine rabbit serum, markedly inhibited the induction (Table I). Addition of exogenous T_3 overcame the inhibition caused by the hormone-binding proteins.

In addition to inhibition by glucagon and stimulation by thyroid hormone, the induction of malic enzyme in culture was stimulated by a dialyzable serum factor (Goodridge *et al.*, 1974). We chose to circumvent difficulties imposed by the

TABLE I

Effects of Thyroxine-Binding Proteins on the Total Activity of Malic Enzyme in Hepatocytes in Culture[a]

Addition	Malic enzyme activity
T_3	290 ± 20 (6)
Albumin (20 mg/ml)	25 ± 6 (4)
Albumin (20 mg/ml plus T_3)	270 ± 40 (2)
Prealbumin (0.2 mg/ml)	28 ± 12 (2)
Prealbumin (0.2 mg/ml plus T_3)	340 ± 140 (2)
Nonimmune rabbit serum (0.02 ml/ml)	109 ± 5 (2)
Anti-thyroxine rabbit serum (0.02 ml/ml)	34 ± 6 (2)

[a] Cells were isolated from the livers of 18- or 19-day-old chick embryos and incubated for 3 days in Nutrient Mixture F-10 as described in the legend to Fig. 1. The results are expressed as percentage of the activity in cells incubated without added hormones or binding proteins ± SE. The number of experiments is given in the parentheses. The T_3 concentration was 1 or 2 μg/ml.

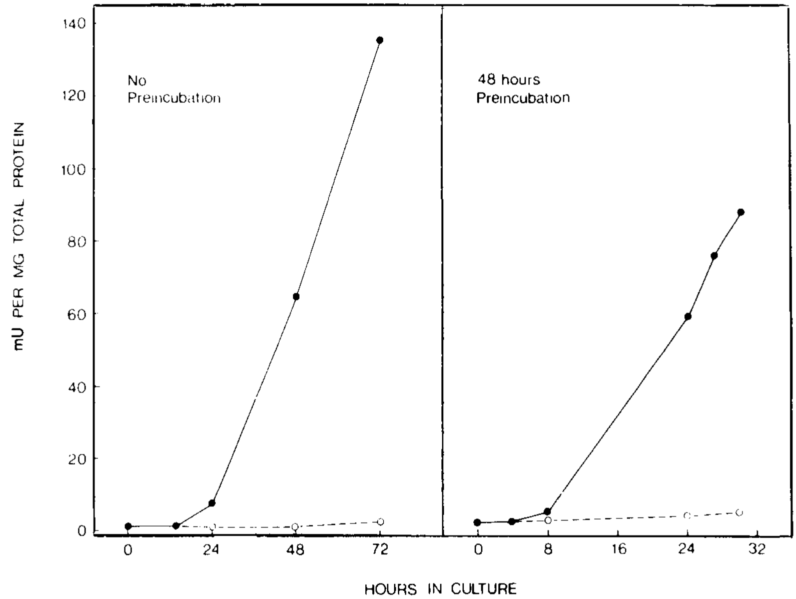


Fig. 2. The effect of preincubation on the stimulation of malic enzyme activity caused by triiodothyronine in liver cells incubated in serum-free Waymouth medium MD 705/1 with insulin (1 μg/ml) in the presence of triiodothyronine (1 μg/ml) (●) or in the absence of triiodothyronine (○). In the left panel, triiodothyronine was added at the time the cells were first put into culture. In the right panel, the cells were preincubated for 48 hours without triiodothyronine; the medium was then removed and replaced with medium containing triiodothyronine (1 μg/ml). The results are expressed as milliunits of malic enzyme per milligram total cellular protein. Reprinted from *Fed. Proc.* **34,** 117–123, 1975.

complex nature of serum by using serum-free incubation medium. When freshly prepared hepatocytes from 17- to 19-day-old chick embryos were incubated for 3 days in Waymouth medium MD 705/1, no change in the activity of malic enzyme was observed. Addition of insulin stimulated enzyme activity slightly whereas addition of T_3 caused a 23-fold increase in enzyme activity (Goodridge and Adelman, 1976). The combination of insulin and T_3 stimulated malic enzyme activity more than 75-fold (Fig. 2). In addition to amplifying the response to T_3, preincubation of the cells with insulin alone led to a progressive increase in the relative stimulation of enzyme activity at 24 hours after adding T_3 (Fig. 2). These effects of insulin may be related to the ability of this hormone to stimulate general protein synthesis in hepatocytes in culture (Goodridge *et al.*, 1974; Goodridge and Adelman, 1976). Thyroid hormones had no effect on total protein synthesis.

B. Experiments in Serum-Free Medium

Since the response to T_3 was more rapid in cells preincubated with insulin we used the preincubation protocol to determine the response of malic enzyme to

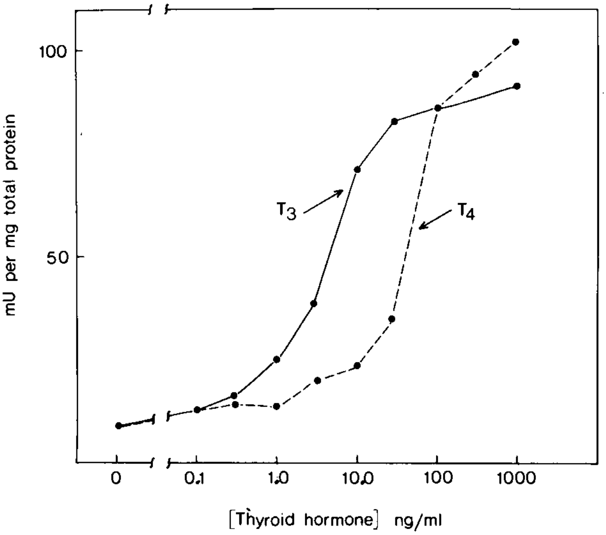


Fig. 3. Dose–response curves for triiodothyronine and thyroxine. Cells were preincubated for 2 days in serum-free Waymouth medium MD 705/1 containing insulin (1 μg/ml). The medium was then changed to one containing albumin (10 mg/ml) and the indicated concentrations of L-triiodothyronine or L-thyroxine. After an additional 48 hours of incubation the cells were harvested and assayed for malic enzyme (Goodridge *et al.*, 1974). Total concentrations of triiodothyronine or thyroxine are as shown. In separate equilibrium dialysis experiments, the concentration of hormone that was free, as opposed to bound to albumin, was determined to be 0.8 and 0.4% of the total for triiodothyronine and thyroxine, respectively. The results are expressed as milliunits of malic enzyme activity per milligram total cellular protein. Reprinted from Goodridge (1975).

different concentrations of T_3 and thyroxine (T_4). Bovine serum albumin (10 mg/ml) was added to the medium to protect the hormones from degradation. [Even in the absence of cells, T_3 and T_4 are rapidly deiodinated by incubation in Waymouth medium without a binding protein (A. G. Goodridge, unpublished results).] In separate equilbrium dialysis experiments albumin was found to bind 99.2 and 99.6%, respectively, of T_3 and T_4. The concentrations of unbound hormone which elicited 50% stimulations of malic enzyme were 4×10^{-11} M and 2×10^{-10} M for T_3 and T_4, respectively (Fig. 3). These concentrations are similar to the concentrations of unbound hormones in the sera of many animals (Refetoff *et al.*, 1970).

III. TURNOVER OF MALIC ENZYME IN HEPATOCYTES IN CULTURE

A. Regulation of Enzyme Concentration

Having established that the response to thyroid hormone was rapid, selective, and elicited by physiological concentrations of the hormones, we next turned to an analysis of the mechanisms involved in the increase in enzyme activity. Based on the results of our *in vivo* experiments (Silpananta and Goodridge, 1971), we anticipated that changes in activity would be due to changes in the concentration of enzyme protein. This prediction was verified by performing quantitative precipitation reactions between a specific rabbit anti-malic enzyme antiserum (Silpananta and Goodridge, 1971) and heat-treated supernatant fractions from cells incubated in insulin or insulin plus triiodothyronine (Fig. 4). This experiment also established that glucagon inhibited the T_3-induced accumulation of enzyme protein.

B. Role of Enzyme Synthesis versus Enzyme Concentration

Changes in the concentration of an enzyme can be caused by changes in the rate of synthesis or rate of degradation of the enzyme protein. Using immunological techniques we established that T_3 stimulated enzyme synthesis (Fig. 5) but had no effect on enzyme degradation (Fig. 6). Malic enzyme synthesis was stimulated 5-fold as early as 3 hours after adding T_3. The rate of enzyme synthesis at early time points after adding T_3 was very low, and background "noise" has prevented us from analyzing changes in enzyme synthesis prior to 3 hours. A new steady-state level of malic enzyme synthesis, about 100 times the control level, was achieved about 30 hours after adding T_3 (Fig. 3). Neither total nor soluble protein synthesis was significantly altered during this experiment (Fig. 3; Goodridge and Adelman, 1976; A. G. Goodridge, unpublished studies).

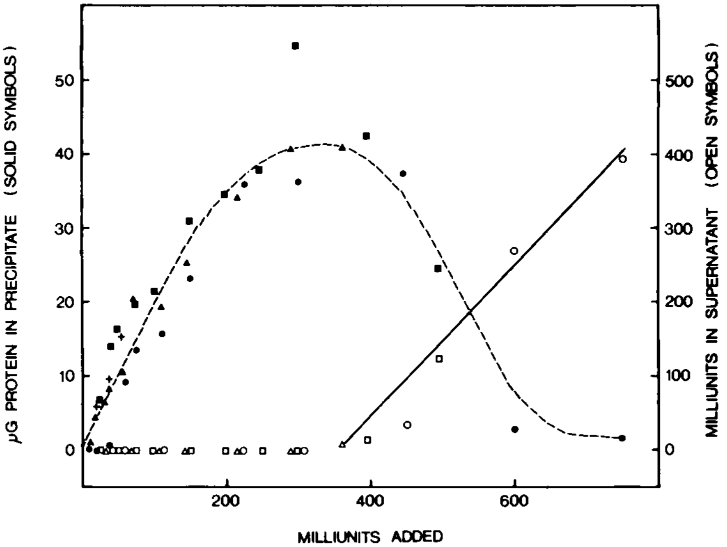


Fig. 4. Quantitative precipitin reactions of malic enzyme in heat-treated extracts of liver cells incubated with different combinations of hormones. Malic enzyme was from liver cells incubated in five different hormone combinations: (a) no triiodothyronine or glucagon for 3 days (+), (activity, 4 mU/mg total cellular protein); (b) triiodothyronine (1 μg/ml) for 3 days (○, ●), (activity, 172 mU/mg total cellular protein); (c) triiodothyronine (50 ng/ml) for 3 days (□, ■), (activity, 70 mU/mg total cellular protein); (d) triiodothyronine (1 μg/ml) plus glucagon (1 μg/ml) for 3 days (+); (activity, 6 mU/mg total cellular protein); (e) triiodothyronine (1 μg/ml) for 2 days and triiodothyronine (1 μg/ml) plus glucagon (1 μg/ml) for a third day (△, ▲), (activity, 63 mU/mg total cellular protein). The upper curve (solid symbols) is protein; the lower curve (open symbols) is activity. Precipitation reaction mixtures contained 25 μl of anti-malic enzyme rabbit serum and various amounts of heat-treated cytosol fraction (Silpananta and Goodridge, 1971) in 1.5 ml of 0.05 *M* Tris-HCl-0.15 *M* NaCl-10 m*M* DL-leucine-1% Triton X-100, pH 7.4. This mixture was incubated at 37°C for 30 minutes and then at 4°C overnight. The precipitates were collected by centrifugation and washed twice with ice cold 0.15 *M* NaCl-10 m*M* DL-leucine-0.5% Triton X-100. A small amount of precipitate was present when serum from nonimmunized rabbits was used. This was subtracted from the total precipitate protein to obtain the values shown above. Reprinted from Goodridge (1975).

C. Involvement of mRNA

The stimulation of malic enzyme synthesis caused by T_3 was blocked by α-amanitin, a specific inhibitor of the enzyme responsible for the transcription of messenger RNA (Lindell *et al.*, 1970; Kedinger and Simard, 1974). The amanitin effect was noted at 6 hours after adding T_3 when total and soluble protein synthesis were only marginally inhibited. Induction of malic enzyme synthesis, therefore, required the continued transcription of cellular mRNA (Goodridge, 1978).

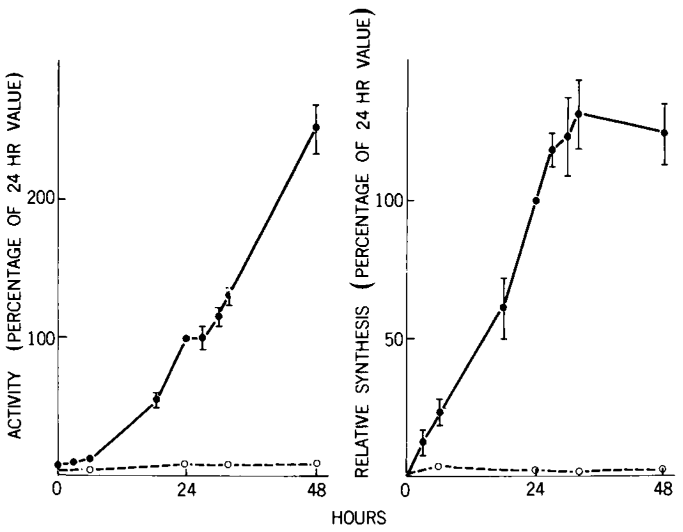


Fig. 5. Time course for the induction of the activity and relative synthesis of malic enzyme in liver cells incubated with triiodothyronine. Cells were isolated from the livers of 18- to 19-day-old chick embryos and incubated for two days in Waymouth medium MD 705/1 containing insulin (0.3 μg/ml). The medium was changed and the cells incubated a further 3 to 48 hours with or without triiodothyronine (1 μg/ml). Activity (left panel) and relative synthesis (right panel) of malic enzyme were measured as previously described (Goodridge and Adelman, 1976). The results are expressed as a percentage of the value in the cells incubated for 24 hours with triiodothyronine. Each point is the average ± SE of 4 to 9 experiments. Activity and synthesis of malic enzyme after 24 hours with triiodothyronine were 158 ± 23 milliunits per milligram soluble protein and 0.97 disintegrations per minute per 100 disintegrations per minute in soluble protein, respectively. From Goodridge (1978), with permission of Elsvier/North Holland.

Fig. 6. Effect of hormones on the degradation of soluble and malic enzyme protein in liver cells in culture. Degradation of soluble protein (A to E) and malic enzyme (F to J) is expressed as a percentage of the radioactivity in the respective proteins at the beginning of the degradation measurement. Cells were incubated for 2 days in the presence of insulin (1 μg/ml) and T_3 (1 μg/ml, solid symbols; or 50 ng/ml, open symbols). After the preincubation period, cellular proteins were labeled with [^{3}H]alanine in medium containing T_3 plus insulin (closed symbols) or insulin alone (open symbols). Initial samples were taken after a 3-hour "chase" period. Cells that had been preincubated with T_3 (1 μg/ml) plus insulin were switched to T_3 plus insulin (A, F, ●); T_3 plus insulin plus glucagon (B, G, ■); or T_3 alone (C, H, ▲). Cells that had been preincubated in T_3 (50 ng/ml) plus insulin were switched to insulin alone (D, I, ○) or insulin plus glucagon (E, J, □). During and after the labeling period, all hormone concentrations were 1 μg/ml. The original data were malic enzyme, 7.1 × 10^3 dpm/2 hours/mg of soluble protein for cells preincubated with both high and low concentrations of T_3; soluble proteins, 1.9 × 10^6 and 2.5 × 10^6 dpm/2 hours/mg of soluble protein for cells preincubated with high and low T_3, respectively. Average results from two or three experiments are shown. Each experiment was performed in duplicate or triplicate. From Goodridge and Adelman (1976) with permission of the *Journal of Biological Chemistry.*

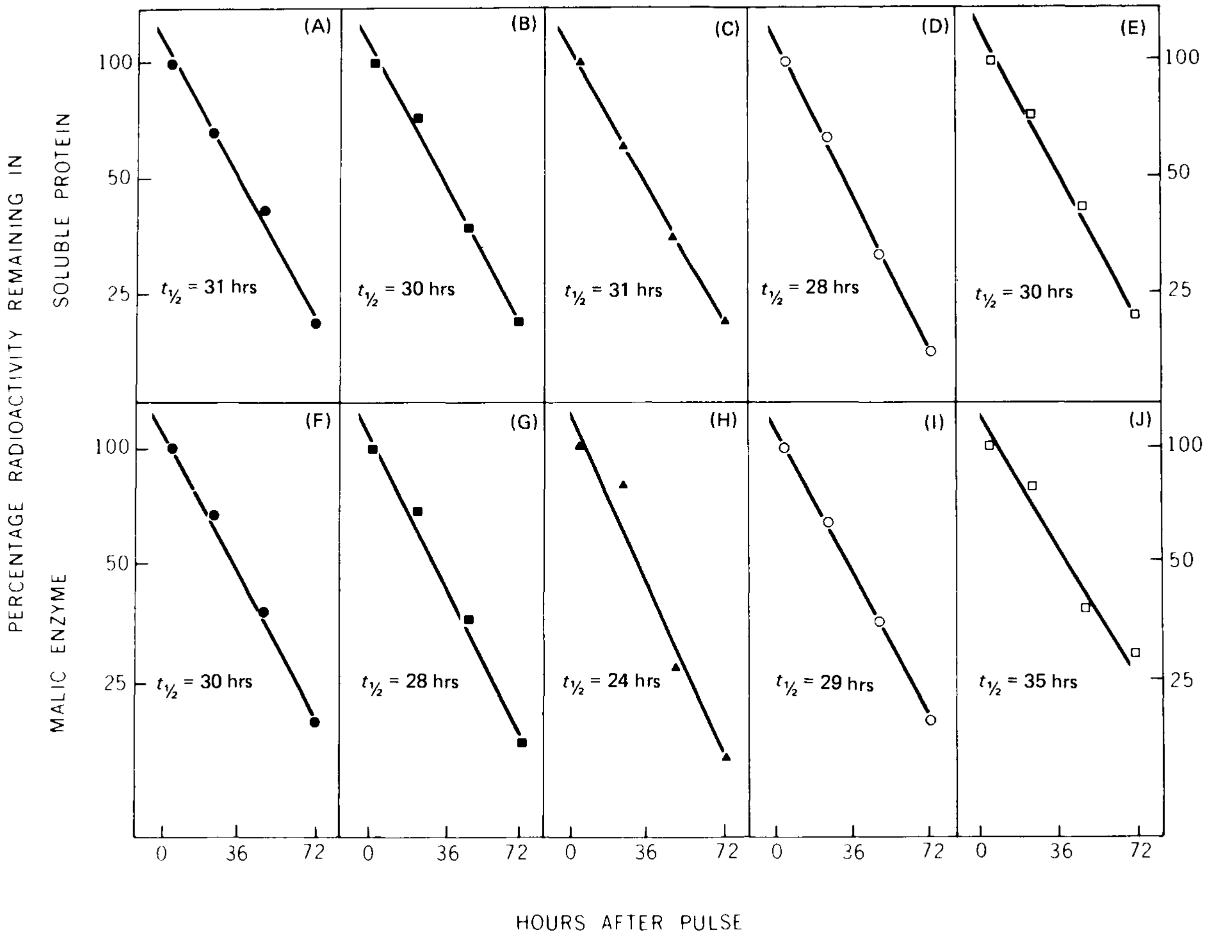

PERCENTAGE RADIOACTIVITY REMAINING IN
SOLUBLE PROTEIN
MALIC ENZYME
(A) $t_{1/2}$ = 31 hrs
(B) $t_{1/2}$ = 30 hrs
(C) $t_{1/2}$ = 31 hrs
(D) $t_{1/2}$ = 28 hrs
(E) $t_{1/2}$ = 30 hrs
(F) $t_{1/2}$ = 30 hrs
(G) $t_{1/2}$ = 28 hrs
(H) $t_{1/2}$ = 24 hrs
(I) $t_{1/2}$ = 29 hrs
(J) $t_{1/2}$ = 35 hrs
100
50
25
0
36
72
HOURS AFTER PULSE

TABLE II

The Effects of Triiodothyronine and Glucagon on the Capacity of Poly(A)$^+$ RNA to Direct the *in Vitro* Synthesis of Total Protein and Malic Enzyme[a]

	Synthesis of total protein	Relative synthesis of malic enzyme
Control	92 ± 42[b]	0.016 ± 0.012
Triiodothyronine	100	0.32 ± 0.04
Triiodothyronine plus glucagon	25 ± 5	0.025 ± 0.011

[a] Freshly isolated hepatocytes were prepared from the livers of 17- to 19-day-old chick embryos. The cells were incubated in Waymouth medium MD 705/1 containing insulin (300 ng/ml) or insulin plus triiodothyronine (1 μg/ml) as indicated in the table. The medium was changed to one of the same composition at 24 hours of incubation except that glucagon (1 μg/ml) was added to one set of petri dishes. The measurements were made at 72 hours of incubation (Siddiqui *et al.*, 1981). The results are the means ± SE of three experiments. Synthesis of total protein in the reticulocyte lysate was originally expressed as percentage of the total [^{35}S]methionine incorporated into acid precipitable protein per microgram added RNA. The results were then normalized by setting the triiodothyronine-treated samples to 100. Relative synthesis of malic enzyme is expressed as counts per minute in malic enzyme per 100 counts per minute in total soluble protein.

[b] Mean ± deviation of 2 experiments.

A more direct test of the effect of thyroid hormone on malic enzyme mRNA level was carried out by measuring the translatable level of malic enzyme mRNA in control and thyroid hormone-treated cells. The techniques for isolating and quantifying malic enzyme mRNA were validated with RNA from the livers of fed ducklings (Siddiqui *et al.*, 1981). The identity of malic enzyme synthesized in the reticulocyte cell-free system was confirmed by virtue of its antigenicity, subunit molecular weight, and proteolytic peptide pattern. The translatable level of malic enzyme mRNA increased 20-fold when hepatocytes were incubated for 3 days with insulin plus triiodothyronine as opposed to insulin alone (Table II). Glucagon blocked the stimulation caused by triiodothyronine.

IV. INHIBITOR AND KINETIC EXPERIMENTS

A. Estimating the Slowest Half-Life from Induction Kinetics

If the increase in enzyme synthesis after addition of T_3 was due to an increased cytoplasmic concentration of malic enzyme mRNA, then the half-life of the cytoplasmic mRNA theoretically can be estimated by determining the time required for enzyme synthesis to achieve 50% of its final steady-state value (Berlin and Schimke, 1965). The results shown in Fig. 5 suggest a half-life of 18–20 hours for cytoplasmic malic enzyme nRNA. Alternatively, this long apparent half-life could be due to another step in T_3 induction which has a half-life longer than that of cytoplasmic malic enzyme mRNA.

B. Estimating the Slowest Half-Life from Decay of Enzyme Synthesis

The rate of decay of enzyme synthesis after removing the inducer should also reflect decay of the event with the slowest turnover. In the experiment shown in Fig. 7, hepatocytes were first incubated with a low concentration of T_3 for 2 days and then switched to a medium without T_3. A low concentration of hormone was used because we wanted to minimize the quantity of active hormone in the culture at the end of the 2 day preincubation period but also to allow enough induction to permit measurement of enzyme synthesis during the subsequent

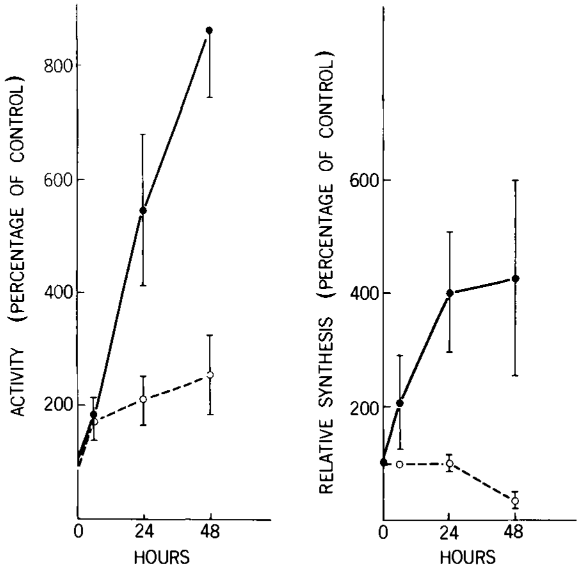
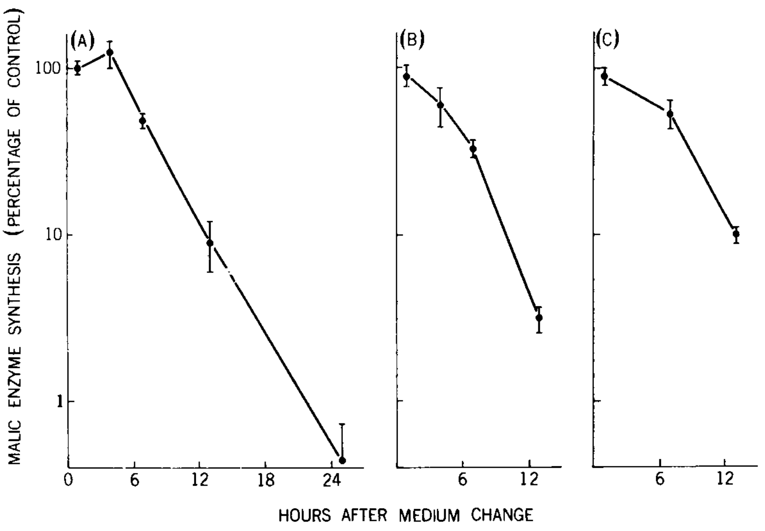


Fig. 7. Activity and relative synthesis of malic enzyme in liver cells incubated with and without triiodothyronine after partial induction by triiodothyronine. Cells were isolated from the livers of 18- to 19-day-old chick embryos and incubated for 2 days in Waymouth medium MD 705/1 containing insulin (0.3 μg/ml) and triiodothyronine (50 ng/ml). The medium was then changed to contain insulin (0.3 μg/ml) with (●) or without (○) triiodothyronine (1 μg/ml). Activity (left panel) and relative synthesis (right panel) of malic enzyme were measured as previously described (Goodridge and Adelman, 1976). The results are expressed as a percentage of the values for cells incubated for 2 days at the low triiodothyronine concentration. Activity and synthesis of malic enzyme at time zero (100%) were 88.2 ± 15.6 milliunits per milligram soluble protein and 0.44 ± 0.13 disintegrations per minute per 100 disintegrations per minute in soluble protein, respectively. Results are the average ± SE of 5 experiments. Three plates were pooled for each determination. Each immunoprecipitation was performed in duplicate. From Goodridge (1978), with permission of Elsevier/North Holland.

deinduction period. The continued sensitivity of the cells to T_3 was demonstrated by adding T_3 to some cells during the second incubation. Both activity and synthesis were markedly stimulated (Fig. 7). By 24 hours after removing T_3, malic enzyme synthesis had begun to decrease in all experiments. Between 24 and 48 hours, enzyme synthesis decayed with an average half-life of 18 ± 2 (n = 5) hours. This estimate for the half-life of the event mediating enzyme induction which has the slowest turnover is remarkably similar to the estimate based on induction kinetics.

C. Estimating Cytoplasmic Malic Enzyme mRNA Half-Life with an Inhibitor of mRNA Transcription

Another method for estimating the half-life of cytoplasmic malic enzyme mRNA is to measure the decay of enzyme synthesis after inhibiting transcription with α-amanitin. In T_3-induced cells, malic enzyme synthesis decayed with first-order kinetics and a half-life of 2.4 hours when α-amanitin was added to the culture medium (Fig. 8). The decay in synthesis of total and soluble protein was much slower, indicating a selective effect on malic enzyme synthesis (Fig. 9). The delay in onset of inhibition of the synthesis of malic enzyme, soluble, and total protein was probably due to the time required for α-amanitin to enter the cell and for preexisting nuclear mRNA to be transported to the cytoplasm. Interestingly, the kinetics of de-induction in the presence of glucagon were very similar to those after adding α-amanitin and very much faster than deinduction after removing T_3 (Figs. 8 and 9).



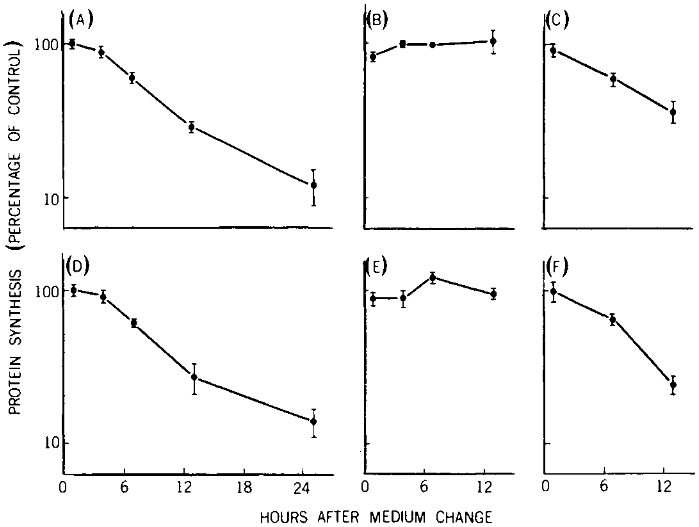


Fig. 9. Decay of total and soluble protein synthesis after adding α-amanitin or glucagon to liver cells previously incubated with triiodothyronine. Details of the incubations and expression of the results are the same as in the legend to Fig. 8. Total protein synthesis is shown in panels A–C; soluble protein synthesis in panels D–F. Additions were α-amanitin (5 μg/ml, panels A and D); glucagon (1 μg/ml, panels B and E); α-amanitin and glucagon (panels C and F). Absolute synthesis of total protein and soluble protein at time zero in the absence of α-amanitin or glucagon were 1.6 ± 0.18 × 10^6 and 2.4 ± 0.4 × 10^6 disintegrations per minute per milligram total or soluble protein per hour, respectively. They did not change significantly during the course of the experiment. From Goodridge (1978), with permission of Elsevier/North Holland.

Fig. 8. Decay of malic enzyme synthesis after adding α-amanitin or glucagon to liver cells previously incubated with triiodothyronine. Cells were isolated from the livers of 18- to 19-day-old chick embryos and incubated for 2 or 3 days in Waymouth medium MD 705/1 containing insulin (1 μg/ml). Triiodothyronine (1 μg/ml) was added during the entire incubation or during only the third day to induce a high rate of malic enzyme synthesis (Goodridge and Adelman, 1976). After the induction period the medium was changed to contain triiodothyronine (1 μg/ml) and insulin (1 μg/ml) plus α-amanitin (5 μg/ml, panel A), glucagon (1 μg/ml, panel B), or α-amanitin and glucagon (panel C). Synthesis of malic enzyme was measured as previously described (Goodridge and Adelman, 1976). The results were originally expressed as disintegrations per minute incorporated into malic enzyme per milligram soluble protein per hour. They were then normalized with respect to the control incubated for the corresponding period of time without α-amanitin or glucagon. Absolute synthesis of malic enzyme at time zero in the absence of α-amanitin or glucagon was 16.4 ± 3.5 × 10^3 disintegrations per minute per milligram soluble protein per hour. It did not change significantly during the course of the experiment. The results are the average ± SE of 3 to 11 experiments. Five to ten plates were pooled for each determination. Each immunoprecipitation was performed in duplicate. From Goodridge (1978), with permission of Elsevier/North Holland.

several other sources (Baxter and Funder, 1979; DeGroot *et al.*, 1978; Oppenheimer *et al.*, 1979), and it will therefore not be discussed in great detail in this chapter. The nuclear T_3BP is a protein with a molecular weight of approximately 50,500 (Latham *et al.*, 1976), with a sedimentation rate constant of 3.4–4.5 S (Bernal and DeGroot, 1977; Latham *et al.*, 1976). On the basis of its nuclear extraction characteristics, it appears to be a nonhistone protein closely associated with the nuclear chromatin (DeGroot *et al., 1974; Surks et al.*, 1973). Recent studies by Eberhardt *et al.* (1979) suggest the hypothesis that the rat liver nuclear receptor is composed of a "core" molecule associated with certain histones. The "holo" receptor binds T_3 with greater affinity than T_4. After dissociation of the histones, the "core" receptor binds T_4 with an affinity comparable to that of the "holo" receptor, but binds T_3 significantly less avidly than T_4. Its equilibrium affinity constant (K_a) for T_3 is in the order of 5×10^9–5×10^{11} M^{-1} by Scatchard analysis (Bernal *et al.*, 1978; Oppenheimer *et al.*, 1974a). The K_a for T_4 is approximately $\frac{1}{20}$–$\frac{1}{40}$ that of T_3 (Oppenheimer *et al.*, 1974a). These affinities are regarded as "high" by virtue of being at least one or two orders of magnitude greater than other nuclear proteins and are comparable to other hormone-binding protein complexes with assumedly physiologically "high" affinities, e.g., estrogen and its receptor (Aten *et al.*, 1978) and insulin and its receptor (Kahn *et al.*, 1974). The maximum T_3 binding capacity of the nuclear T_3BP in rat liver is low, perhaps only 4000–10,000 binding sites per rat liver nucleus (DeGroot and Strausser, 1974; Oppenheimer *et al.*, 1974a), and in the normal rat, these sites are approximately 40% occupied (Oppenheimer *et al.*, 1974b).

Several lines of evidence suggest, but do not prove, that the nuclear T_3BP is a physiologically important receptor or intermediary in the action of thyroid hormone. The T_3BP binds to various thyroid hormone analogs with affinities generally proportionate to the thyromimetic activity of the analogs, when the *in vivo* longevity of the analogs are taken into consideration (Jorgensen, 1978; Oppenheimer *et al.*, 1973; Samuels *et al.*, 1979). The T_3BP content of various tissues are also roughly proportionate to the alleged thyroid hormone responsiveness of the tissues (Oppenheimer *et al.*, 1974b). Variations in nuclear T_3BP content have been observed to occur in several disease states and stages of development, but for the most part tend to suggest potential roles for thyroid hormones in states previously thought to involve minimal, if any, aspects of thyroid deficiency at the tissue level, rather than correlate with obvious thyroid deficiency or excess. Specifically, nuclear T_3BP content in rat liver is diminished in starvation (Burman *et al.*, 1977; DeGroot *et al.*, 1977a; Dillmann *et al.*, 1978c; Schussler and Orlando, 1978), partial hepatectomy(Dillmann *et al.*, 1978c), and after glucagon administration (Dillmann and Oppenheimer, 1979; Dillmann *et al.*, 1978b), but it is not appreciably altered by hypothyroidism, or in some reports by hyperthyroidism (Bernal *et al.*, 1978b; Oppenheimer *et al.*, 1975; Spindler *et al.*, 1975). The physiological import of the nuclear T_3BP is perhaps most strongly implied by its apparent absence in a patient with the syndrome of peripheral resistance to thyroid hormone (Bernal *et al.*, 1976, 1978c). Heart rate and the reciprocals of serum cholesterol, CPK, and Achilles reflex times have also been shown to be linearly related to the estimated T_3BP occupancy in hyper- and hypothyroid patients (Bantle *et al.*, 1980). In other studies it appears that the T_3-induced synthetic rates of fatty acid synthetase, malic enzyme, 6-phosphogluconate dehydrogenase, and α-glycerophosphate dehydrogenase are maximal when the nuclear T_3BP complement is 95–100% saturated (Mariash *et al.*, 1980; Oppenheimer *et al.*, 1977). When T_3 is administered to hypothyroid rats in quantities sufficient to maintain supranormal degrees of T_3BP saturation in hypothyroid rats for 7 days, evidence of thyrotoxicosis, e.g., decreasing increments of weight gain, has been observed (DeGroot and Rue, 1979).

In short, it appears that the existence of a nuclear thyroid hormone binding protein is certain and its role as a physiological receptor or intermediary in the action of thyroid hormone is most probable, based on a variety of experimental and clinical data. The mechanism by which the T_3–T_3BP complexes function is still unknown, but ultimately probably relates to the induction, and perhaps, repression, of multiple gene products.

The mechanisms by which T_3 and T_3-binding proteins enter the nucleus are in many respects unknown. In contrast to the steroid hormones, T_3 and T_4 do not require complexing by a cytoplasmic receptor to enter the nucleus. That T_3 enters isolated nuclei in the absence of cytosol has been demonstrated in many laboratories. DeGroot and co-workers (1976) found that the addition of cytosol or serum proteins to the incubation medium actually reduced the uptake of T_3 by isolated nuclei (Table I). Inclusion of an excess of unlabeled T_3 with these proteins partially reversed this inhibition, presumably by preventing the loss of labeled T_3 to extranuclear protein binding. Tata (1975) and Defer *et al.* (1976) have reported similar findings. T_3–T_3BP complexes formed *in vitro* were virtually the same as those formed *in vivo* when Sephadex G-100 elution profiles, rates of nuclear release, maximal nuclear binding capacities, and salt-extraction characteristics were compared (Surks *et al.*, 1975). These studies indicate that authentic nuclear T_3–T_3BP complexes can be formed in the absence of cytoplasmic factors, but do not rule out the possibility that some of these complexes may have originated outside the nucleus. DeGroot and Rue (1980a), as discussed earlier, have shown that isolated nuclei do, in fact, have an avidity to take up T_3–T_3BP complexes, which is greater than their avidity for a variety of other protein hormones (Fig. 3). Admittedly, the phenomenon observed *in vitro* cannot a priori be assumed to occur *in vivo* in view of the unknown extent of compromise to the integrity of the nuclear membranes suffered during nuclear isolation and washing.

chick embryos and incubated only in serum-free medium. Tarlow *et al.* (1977) have reported that fatty acid synthesis and acetyl-CoA carboxylase activity in hepatocytes prepared from fed 10- to 15-day-old chicks respond to insulin but not T_3. These cells, however, were incubated in a medium containing 5% rooster serum. Since the free concentration of T_3 in diluted serum is the same as that found in whole serum, it is probable that free thyroid hormone levels in the medium were sufficient to stimulate these processes. A clearly stimulatory effect of T_3 was observed by Tarlow *et al.* (1977) when hepatocytes from fasted chicks were incubated in a serum-free medium after a short exposure to medium containing 1% serum. Thus, the response of hepatocytes from embryos is probably the same as that of hepatocytes from growing chicks.

VII. RELATIONSHIP BETWEEN REGULATION OF FATTY ACID BIOSYNTHESIS AND REGULATION OF MALIC ENZYME SYNTHESIS

A. Hypothetical Regulation of Enzyme Synthesis by Pathway Intermediates

Malic enzyme activity and rates of fatty acid biosynthesis are positively correlated under a wide variety of *in vivo* conditions. The activity of fatty acid synthetase is similarly correlated with fatty acid biosynthesis. These strong positive correlations have led to the hypothesis that intermediates of the fatty acid biosynthesis pathway may be involved in the regulation of the synthesis of lipogenic enzymes (Silpananta and Goodridge, 1971; Goodridge, 1973b, 1978; Volpe and Vagelos, 1976). This hypothesis implies that increased fatty acid synthesis will be accompanied or quickly followed by increased synthesis of malic enzyme.

B. Testing the Hypothesis

A direct test of this hypothesis is not possible in an intact animal. When hepatocytes in culture are incubated in the presence of glucose and under varying hormonal states, the same positive correlation between fatty acid synthesis and malic enzyme is observed (Fig. 10). Unlike the situation *in vivo,* however, fatty acid biosynthesis in hepatocytes in culture can be essentially eliminated by incubating the cells in the absence of glucose (Table IV). Addition of glucose without T_3 stimulated fatty acid biosynthesis 7.5-fold but had no effect on malic enzyme. Addition of T_3 in the absence of glucose caused a slight stimulation of fatty acid synthesis but almost a 7-fold increase in malic enzyme activity. In the

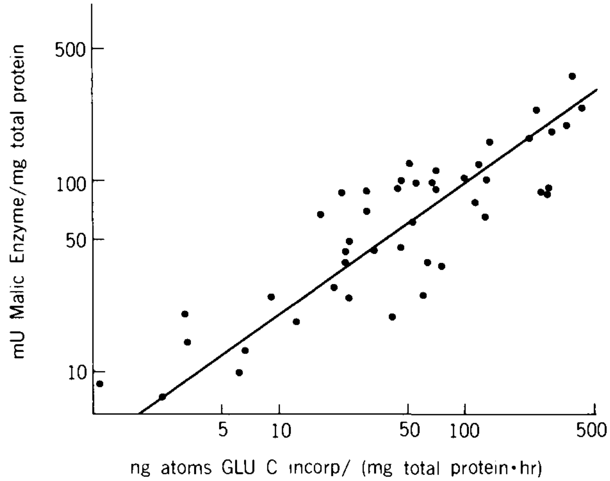


Fig. 10. Correlation between incorporation of glucose into fatty acids and malic enzyme activity in liver cells in culture. Cells were isolated from the livers of 18- to 19-day-old embryos or 1-day-old, unfed chicks (Goodridge, 1973a) and incubated for 3 days in Nutrient Mixture F-10 containing 15% horse serum, 2.5% fetal calf serum, 17 m*M* glucose, and varying combinations of triiodothyronine, insulin, glucagon, cAMP, and free fatty acids. The hormones were added at 1 μg/ml; cAMP and free fatty acids at 0.5 m*M*. In some experiments the sera were dialyzed. Cells were harvested and malic enzyme activity and incorporation of [U-^{14}C]glucose into total fatty acids measured (Goodridge *et al.*, 1974).

TABLE IV

The Effects of Triiodothyronine and Glucose on the Incorporation of ^3H_2O into Total Fatty Acids and on Malic Enzyme Activity[a]

Additions	$^3H_2O \rightarrow$fatty acids	Malic enzyme activity
None	2 ± 0.4	9 ± 0.7
Triiodothyronine	4 ± 0.6	67 ± 5
Glucose	15 ± 2	9 ± 0.8
Triiodothyronine plus glucose	100	100

[a] Cells were isolated from the livers of 18- to 19-day-old chick embryos (Goodridge, 1973a) and incubated for 3 days in Waymouth medium MD 705/1 containing insulin (1 μg/ml) and glucose (28 m*M*) as indicated in the table. At 48 hours of incubation, the medium was changed and triiodothyronine (1 μg/ml) added as indicated in the table. The cells were harvested at 72 hours of incubation and assayed for malic enzyme activity (Goodridge and Adelman, 1976) or incubated for an additional hour in the presence of ^3H_2O. Total fatty acids were isolated and assayed for radioactivity (Goodridge, 1968a). The results are expressed as a percentage of the values for cells incubated with glucose plus triiodothyronine ± SE. There were 4 and 11 experiments for fatty acid synthesis and malic enzyme activity, respectively. Cells from three plates were pooled for each measurement.

presence of glucose, T_3 stimulated both fatty acid biosynthesis and malic enzyme activity.

These results make it unlikely that an intermediate between glucose and fatty acid is the primary intracellular regulator of the synthesis of malic enzyme. Similarly, it is unlikely that the altered ratio of NADP/NADPH caused by increased fatty acid biosynthesis is the intracellular signal for increased malic enzyme synthesis. The involvement of certain other intermediates is still not eliminated. For example, concentrations of long chain fatty acyl-CoA are negatively correlated with fatty acid biosynthesis under a wide variety of conditions. This end product of the pathway could be involved in the regulation of enzyme synthesis independent of actual flow of carbon through the lipogenic pathway. Alternatively, the hormones may regulate fatty acid biosynthesis and lipogenic enzyme synthesis by independent pathways.

VII. PHYSIOLOGICAL AND TELEOLOGICAL SIGNIFICANCE

The results discussed in this review indicate that thyroid hormones have a direct stimulatory effect on the synthesis of the lipogenic enzymes in the liver. The physiological significance of these effects remains to be clarified. Thyroid hormone is well known for its general effects on growth and differentiation. Stimulation of the lipogenic enzymes may be part of the general stimulation of anabolic processes elicited by thyroid hormone. A second possibility is that thyroid hormones play a permissive role, being required for the synthesis of several enzymes of diverse pathways. *In vivo,* the total activities of several enzymes involved in both anabolic and catabolic pathways are increased by thyroid hormones (Colton *et al.*, 1972). Finally, several recent reports have described decreased plasma levels of T_3 in the starved state (Portnay *et al.*, 1974; Vagenakis *et al.*, 1977). Whether this change in T_3 levels is involved in the regulation of lipogenic enzyme concentration is not known.

Although the teleological significance of the regulation of malic enzyme synthesis by thyroid hormone is not clear, our hepatocyte culture system does mimic a phenomenon that occurs *in vivo.* The effects of thyroid hormone on malic enzyme synthesis in hepatocytes in culture are very large, selective, rapid, and elicited at physiological concentrations of hormone. Thus, this system is ideally suited to the analysis of the molecular basis of thyroid hormone action.

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Note added in proof: We have recently isolated a recombinant plasmic containing 970 base pairs of DNA complementary to goose malic enzyme mRNA. This cDNA probe has been used in a "Northern blot" analysis of RNA extracted from chick embryo hepatocytes in culture. Exposure of hepatocytes to T_3 for 3 days caused a more than 100-fold increase in malic enzyme mRNA level.

9

Thyroid Hormone–Carbohydrate Interaction

CARY N. MARIASH AND JACK H. OPPENHEIMER

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I. GENERAL REVIEW

A. Introduction

Recent evidence has emphasized a strong relationship among carbohydrate, lipid, and thyroid hormone metabolism. For example, there is a striking response of hepatic lipogenic enzymes to both thyroid hormone and administration of simple sugars (Fitch and Chaikoff, 1960; Tepperman and Tepperman, 1964; Reed and Tarver, 1975; Mariash *et al.*, 1980a;b; Oppenheimer *et al.*, 1981). At the same time, the peripheral conversion of thyroxine to triiodothyronine appears to be determined in part by the availability of carbohydrate (Spaulding *et al.*, 1976; Glass *et al.*, 1978; Burman *et al.*, 1979; Davidson and Chopra, 1979). Although the precise molecular nature of these interactions and their biological significance remains unknown, substantial progress has been made in defining these interrelationships. In this chapter, we propose first to review selected areas and contributions to the general field of carbohydrate and thyroid hormone interaction, and second, to discuss in greater detail our own studies dealing with the interaction of thyroid hormone and carbohydrate in the induction of lipogenic enzymes and their specific mRNA's.

B. Influence of Thyroid Hormone on Glucose Tolerance

In 1909, Murray first reported that diabetic patients who develop Graves' disease exhibit a marked exacerbation of the diabetic state (Murray and Durh, 1909). Subsequently, several investigations have documented that hyperthyroid patients may have elevated fasting blood sugars and hypothyroid patients may have relatively low fasting blood glucose concentrations (Sanger and Hun, 1922; Rabinowitch. 1931; Althausen, 1944; Houssay, 1946; Doar *et al.*, 1969; McDaniel *et al.*, 1977). Moreover, both in man as well as in other mammals, elevated levels of fasting blood glucose in the hyperthyroid state are associated with elevated basal plasma insulin concentration (Doar *et al.*, 1969; Renauld *et al.*, 1971; Erle *et al.*, 1973; Anderson *et al.*, 1977; Wajchenberg *et al.*, 1978b; Bartels *et al.*, 1979). Conversely, low fasting blood sugars in hypothyroidism are associated with low basal insulin concentrations (Jolin and Montes, 1974; Montes *et al.*, 1977).

A better description of the abnormalities in glucose homeostasis in various thyroidal states can be obtained from examination of the oral glucose tolerance test. Most studies have shown that the hyperthyroid state is associated with glucose intolerance with both peak and total integrated glucose concentrations elevated after oral glcucose administration (Sanger and Hun, 1922; Rabinowitch, 1931; Althausen, 1944; Renauld *et al.*, 1971, 1979; Marecek and Feldman, 1973; Andersen *et al.*, 1977; Wajchenberg *et al.*, 1978b; Houssay, 1948). Curiously, the glucose tolerance more often than not is normal in hypothyroidism (Renaud *et al.*, 1972; Saunders *et al.*, 1980).

Two mechanisms have been proposed to account for the elevated blood sugars in the hyperthyroid state following administration of oral glucose. First, an increased rate of glucose absorption occurs in hyperthyroidism. Althausen (1944) showed in both man and rats that in hyperthyroidism there is increased rate of intestinal absorption of all carbohydrates and that in hypothyroidism this is decreased. The increased rate of transport of glucose across the intestinal mucosa in hyperthyroidism appeared independent of changes in blood flow. The second mechanism that has been proposed to result in elevated blood sugars in hyperthyroidism following oral glucose administration is a decreased rate of blood glucose disappearance. This suggestion was prompted by several reports documenting a decreased rate of glucose disappearance in hyperthyroidism after intravenous administration (Doar *et al.*, 1969; Andersen *et al.*, 1977). Most studies in hyperthyroid patients, however, are in conflict with this conclusion and suggest that the intravenous glucose tolerance test is associated with either an unchanged (Elrick *et al.*, 1961; Renauld *et al.*, 1971) or an accelerated glucose disappearance rate (Amatuzio *et al.*, 1954; Erle *et al.*, 1973; Marecek and Feldman, 1973; Wajchenberg *et al.*, 1978b). These findings lend further support to the concept that the primary determinant of the glucose intolerance is the rapid rate of glucose absorption. The apparent discrepancy between these reports can be understood by examining the role of thyroid hormone in the release of insulin.

C. Thyroid Hormone Regulation of Insulin Secretion

Many of the studies discussed above showed that hyperthyroidism is associated with increased and hypothyroidism with decreased insulin secretion during glucose tolerance testing. Thus, hyperthyroid patients with elevated insulin secretion would be expected to have increased glucose disposal rates, and hypothyroid patients, decreased glucose disposal rates. Nevertheless, if the degree of hyperthyroidism is severe or the course of hyperthyroidism is prolonged, diminished insulin secretion may supervene (Malaisse *et al.*, 1967; Lenzen *et al.*, 1975; Wajchenberg *et al.*, 1978a). Perhaps this may account for the occasional reports of diminished glucose disappearance.

Recent studies using isolated perfused pancreas preparations have also provided additional insight into the determinants of the glucose tolerance in altered thyroid states (Okajima and Ui, 1978). Lenzen (1978) has shown that the immediate release of insulin to a glucose stimulus in the hypothyroid pancreas is less than the normal pancreas. The sluggish response, however, is compensated by a subsequent marked increase in total insulin secretion during the period of perfusion. Since hypothyroidism is associated with a low basal plasma glucose and a delay in glucose absorption, these studies provide a potential explanation for the relatively normal glucose tolerance tests associated with hypothyroidism. An immediate release of insulin is not necessary because of the slow absorption. Of further interest was their finding of diminished total insulin secretion from pancreases taken from severely hyperthyroid rats. Although the immediate insulin response appeared intact, the β cell was incapable of maintaining insulin secretion to the same degree as a normal pancreas (Lenzen *et al.*, 1976; Lenzen, 1978). Such apparent β cell exhaustion, however, could be overcome if the perfusion media was supplemented with the glycolytic intermediate, pyruvate (Lenzen *et al.*, 1976). As indicated above, β cell exhaustion may explain the discrepancy between the accelerated glucose disappearance in mild hyperthyroidism and the diminished glucose disappearance seen in some severely hyperthyroid patients.

D. Control of Hepatic Glucose Production by Thyroid Hormone

In addition to the ability of thyroid hormone to alter glucose absorption and glucose disposal, thyroid hormone also is capable of altering the rate of glucose production. Several studies by Wieland and co-workers (Menahan and Wieland, 1969; Bottger *et al.*, 1970) documented the ability of thyroid hormone to increase hepatic gluconeogenesis. Likewise, hypothyroid livers displayed a diminished capacity for gluconeogenesis. These studies have been extended by Sestoft and co-workers (1977) who showed that the conversion of glycerol to glucose was greater in the hyperthyroid than either the euthyroid or hypothyroid liver. In addition, other investigators (Menahan and Wieland, 1969; Rognstad, 1977; Keyes and Heimberg, 1979; Muller and Seitz, 1980a) have reported that glucose is produced at a greater rate in hyperthyroid than in hypothyroid livers. Although these studies used liver perfusion or isolated hepatocyte techniques, more recent studies have suggested that the hepatic production rate of glucose is also accelerated in the intact hyperthyroid animal (Okajima and Ui, 1979; Muller and Seitz, 1980b). Recent studies by Wahren and co-workers (1981) have suggested that in hyperthyroid man there is a greater rate of conversion of gluconeogenic precursors to glucose.

It is apparent that the increased production rate of glucose in hyperthyroidism, β cell exhaustion, and the increased rate of glucose absorption can all lead to findings of a slightly elevated fasting blood sugar and an abnormal glucose tolerance test in hyperthyroid patients. Likewise, the diminished rate of gluconeogenesis in hypothyroidism can contribute to the relatively low fasting blood sugars in hypothyroid patients.

E. Regulation of Glycogen Content by Thyroid Hormone

Although thyroid hormone clearly modifies both the disappearance and production rate of glucose, the role of thyroid hormone in glycogen metabolism is less clear (Paul and Dhar, 1980). Within 24 hours after its administration, thyroid hormone causes a marked reduction in hepatic glycogen content (Cramer and Krause, 1913; Tata *et al.*, 1963; Lenzen, 1978). The mechanism leading to the fall in hepatic glycogen, however, has not been elucidated. Glycogen depletion does not appear to depend on ongoing protein or RNA synthesis (Arrondo *et al.*, 1977). If glycogen stores were directly related to thyroid hormone status, one would also anticipate that the hypothyroid state would be associated with elevated liver glycogen content. This has generally not been found to be the case (Battarbee, 1974; Takahashi and Suzuki, 1975; Porterfield and Hendrich, 1976; Llobera *et al.*, 1978; Okajima and Ui, 1979). Therefore, the possibility should be considered that the effects of thyroid hormone on glycogen content represent a secondary effect of pharmacological thyroid hormone excess.

F. Thyroid Hormone Regulation of Hepatic Glycolysis

In contrast to the questionable significance of thyroid hormone regulation of glycogen content, it appears well established that thyroid hormone augments the rate of hepatic glycolysis. Hyperthyroidism is associated with enhanced and hypothyroidism with a diminished rate of glycolysis (Glock *et al.*, 1956; Murad and Freeland, 1967; Bottger *et al.*, 1970; Porterfield and Hendrich, 1976; Baquer *et al.*, 1976; Keyes and Heimberg, 1979). However, because the hepatic contribution to net glycolysis *in vivo* is quite small, and because the liver is primary a gluconeogenic and glucose-storing organ, one must rely on isolated hepatocyte, liver slice, or liver perfusion techniques to study the effect of thyroid hormone on hepatic glycolysis. Thus, the relative contribution of hepatic glycolysis to overall *in vivo* glucose homeostasis under various thyroidal states remains uncertain.

The conclusion that thyroid hormone accelerates hepatic glycolysis is based on several lines of evidence. Since a major product of glycolysis is lactate, many authors have studied the influence of thyroidal state on the generation of this product from liver under *in vitro* conditions (Schimassek *et al.*, 1966; Menahan and Wieland, 1969; Hillbom and Lindros, 1971; Goodridge, 1973a,b; Sestoft *et al.*, 1977). In hyperthyroid livers, the production of lactate from glucose is enhanced. The inverse is true in hypothyroid livers.

Another line of evidence derives from studies using radiolabeled glucose. Incubation of liver slices with uniformly labeled [^{14}C]glucose yields enhanced rates of production of $^{14}CO_2$ from hyperthyroid livers (Glock *et al.*, 1956). Conversely, hypothyroid livers yield decreased amounts of radiolabeled CO_2

(Griffin and Miller, 1973; Baquer *et al.*, 1976). Because hyperthyroidism is associated with an increased rate of glucogenesis, the absolute rate of production of various glycolytic intermediates cannot be easily determined from the generation of radiolabeled CO_2 or lactate. Since these intermediates can be reutilized to reform glucose, they are involved in "futile cycles." Examples of such futile cycles include the cycling between glucokinase and glucose-6-phosphatase and the cycling between the phosphofructokinase and fructose-1,6-diphosphatase enzymes. Several authors have now shown that, because of futile cycling, glycolytic flux is substantially greater than estimated by techniques that only measure lactate production or CO_2 generation (Katz and Rognstad, 1976; Cohen *et al.*, 1979; Okajima and Ui, 1979; Huang and Lardy, 1981). Whether futile cycling plays a significant role in the increased thermogenesis associated with hyperthyroidism (Huang and Lardy, 1981) is not clear. Katz and Rognstad (1976) have also proposed that the physiological role of futile cycling is primarily related to the regulation of net flux of substrate through the cycle. When the cycle is operating rapidly, a small change in the activity of one enzyme in the cycle will lead to rather large changes in the net flux across that cycle. Thus, large changes in net glycolytic flux can occur with only minimal changes in the activity of the key regulatory enzymes. Indeed, significant changes in glycolytic substrate concentrations have been measured under various thyroidal conditions with only small changes in the key regulatory enzymes (Aranda *et al.*, 1972; Tobin *et al.*, 1979).

G. Thyroid Regulation of Adipocyte Carbohydrate Utilization

The adipocyte is another cell type in which carbohydrate utilization has clearly been shown to be under the influence of thyroid hormone. Since the primary substrate for fat synthesis in the adipocyte is glucose, the ability of thyroid hormone to regulate glucose uptake and utilization is quite important.

Studies of glucose uptake and utilization in the adipocyte have primarily used collagenase-treated cells. Although this preparation has the advantage of a pure cell type, a major problem is the great variability in results obtained from one preparation to another (Czech, 1976). Despite this disadvantage, several interesting observations have been made with this experimental model and generally have confirmed studies using the fat pad (Bray and Goodman, 1968).

Adipocytes from thyroidectomized rats incubated with physiological concentrations of glucose show increased basal uptake of glucose (Correze *et al.*, 1977; Czech *et al.*, 1980). In contrast to the findings with the liver, the conversion of this glucose to CO_2 is actually enhanced in hypothyroid fat tissue (Bray and Goodman, 1968). Moreover, the conversion of glucose to lipids from hypothyroid adipocytes under basal conditions also has generally been found to be increased (Correze *et al.*, 1977; Czech *et al.*, 1980).

Most authors agree that insulin plays a decisive role as the stimulus for glucose utilization and conversion to lipid in adipose tissue. Adipocytes obtained from euthyroid animals display marked increases in glucose uptake and lipid synthesis when insulin is added to the incubation medium (Czech *et al.*, 1980). It is therefore a major interest to note that most (Seibel *et al.*, 1978; Schoenle *et al.*, 1979; Czech *et al.*, 1980) but not all reports (Verhaegen *et al.*, 1979) indicate that adipocytes obtained from hypothyroid animals are incapable of responding to insulin either by increasing glucose uptake or the rate of lipid formation from glucose. These studies show that in the presence of insulin the euthyroid adipocyte has a greater rate of glucose uptake, CO_2 production, and lipid synthesis compared to the hypothyroid adipocyte *in vitro*. Since insulin is always present *in vivo*, one might anticipate that the hypothyroid fat tissue has a decreased capacity for lipid synthesis.

A possible mechanism that leads to insulin insensitivity of the adipose tissue in hypothyroidism has been suggested from studies with hypophysectomized rats (Schoenle *et al.*, 1979). Thyroid hormone administered to hypophysectomized rats increased both the basal uptake and metabolism of glucose and the rate of lipid formation in isolated adipocytes. However, thyroid hormone was incapable of restoring the insulin induced stimulation of glucose uptake and metabolism. On the other hand, growth hormone administration did restore responsivity of adipocytes to insulin. Since thyroid hormone deprivation is known to lead to growth hormone deficiency in rats (Hervas *et al.*, 1975; Montes *et al.*, 1977), it is possible that the lack of adipocyte sensitivity to insulin in thyroidectomized rats is due to the lack of growth hormone secretion. Studies with the adopocyte effectively demonstrate that not only thyroid hormone and insulin, but other hormones as well, may play a role in the interaction between thyroid hormone and carbohydrates on cellular function.

H. Effect of Carbohydrates on Plasma Thyroid Hormone Levels

Not only does thyroid hormone influence carbohydrate metabolism, but dietary carbohydrate influences the production rate and plasma levels of thyroid hormones. Carbohydrate deprivation, in the form of fasting, significantly lowers plasma triiodothyronine levels of both man and the rat (Portnay *et al.*, 1974; Spaulding *et al.*, 1976; Harris *et al.*, 1978; Pittman *et al.*, 1979). In man, this phenomenon appears to be due to a decreased rate of peripheral conversion of thyroxine to triidothyronine (Spaulding *et al.*, 1976; Burman *et al.*, 1979; Pittman *et al.*, 1979). The decreased conversion of T_4 to T_3 may in part be due to the decreased availability of cytosolic reducing equivalents in the fasting state (Harris *et al.*, 1979). On the other hand, in the rat thyroxine as well as T_3 are proportionately decreased (Harris *et al.*, 1978). Whether decreased peripheral conversion in the fasted rat plays a major role in the decrease in plasma T_3 has not yet been unequivocally documented. Admittedly, diminished conversion of T_4 to T_3 by homogenates of livers from fasted animals has been amply demonstrated (Kaplan and Utiger, 1978).

In man, overfeeding with a diet high in carbohydrate appears capable of the opposite effect, that of increasing plasma T_3 and free T_3 concentrations (Danforth *et al.*, 1979; Davidson and Chopra, 1979). Studies in the rat have been less convincing. Glass *et al.* (1978) have shown a small increase in plasma T_3 with high carbohydrate feeding. The physiological significance of changes in T_3 levels remains unclear. The decreases in plasma T_3 with starvation and the increases with overfeeding have been interpreted to represent an adaptive phenomenon. Thus, speculations have been advanced that during starvation a decreased metabolic requirement may be beneficial to survival. Metabolic studies in fasting volunteers have shown a slight increase in protein catabolic rate when T_3 levels are restored to normal levels (Vignati *et al.*, 1978; Gardner *et al.*, 1979). A similar nitrogen-sparing effect by a reduction in circulating thyroid hormones could not be demonstrated in the starving rat (Schwartz *et al.*, 1980). The possibility has been advanced that a reduction in plasma T_3 in starvation contributes to the physiologically important reduction in lipogenesis (Oppenheimer and Schwartz, 1980). The diminished metabolic rate that accompanies starvation, however, does not appear to be primarily related to a decrease in circulating T_3 (Wimpfheimer *et al.*, 1979).

II. REGULATION OF HEPATIC LIPOGENIC ENZYMES RESPONSIVE TO BOTH T_3 AND CARBOHYDRATE

A. Reactions Involved in Hepatic Fatty Acid Synthesis

An important characteristic of carbohydrates is their ability to induce lipogenic enzymes (Fitch and Chaikoff, 1960; Bruckdorfer *et al.*, 1972; Mack *et al.*, 1975). A diet free of fat and rich in sucrose or glucose will markedly induce liver enzymes responsible for fatty acid synthesis (Gibson *et al.*, 1972). Since thyroid hormone also shares the ability to induce many of the same lipogenic enzymes (Glock and McLean, 1955; Tarentino *et al.*, 1966; Freedland *et al.*, 1968; Diamant *et al.*, 1972; Roncari and Murthy, 1975; Kumar *et al.*, 1977), we undertook a systematic analysis of the relationship between carbohydrates and T_3 in the induction of lipogenic enzymes.

The overall reaction involved in hepatic fatty acid synthesis is:

$$\text{Acetyl-CoA} + 7\ \text{malonyl-CoA} + 14\ \text{NADPH}^+ \rightarrow \text{palmitic acid} + 8\ \text{CoA} + 14\ \text{NADP}^+ + 7\ CO_2 + H_2O$$

Three sets of enzymes regulate the synthesis of the saturated fatty acids: (1) those involved in the generation of the two major substrates, acetyl-CoA and malonyl-CoA; (2) those involved in the generation of the required cofactor NADPH; and (3) the large multienzyme complex, fatty acid synthetase, which catalyzes the condensation of acetyl-CoA and malonyl-CoA to form palmitic acid.

The formation of malonyl-CoA is the first committed step in fatty acid synthesis. This substrate is formed from acetyl-CoA by acetyl-CoA carboxylase. Although the mass of this enzyme decreases with starvation, and increases to values above the fed base line in animals given a high carbohydrate diet (Volpe and Vagelos, 1976), the most important regulation of this enzyme is the minute-to-minute changes occurring in its activity independent of changes in enzyme mass (Guynn *et al.*, 1972; Klain and Weiser, 1973; Beynen *et al.*, 1979). Such regulation is believed to be effected by variation in citrate and fatty acid concentrations, as well as by the phosphorylation state of the enzyme (Witters *et al.*, 1979). Citrate stimulates enzyme activity, whereas free fatty acids and phosphorylation inhibit.

The other enzymes involved in fatty acid synthesis are regulated primarily by changes in enzyme mass. Fatty acid synthetase, a large multienzyme complex, adds successive malonyl-CoA residues to an acetyl-CoA nucleus in the presence of NADPH to form palmityl-CoA. This enzyme has been studied by many groups and has been shown by immunological techniques to have a relatively constant mass to acitivity ratio (Volpe and Vagelos, 1976; Joshi and Wakil, 1978). Similar to acetyl-CoA carboxylase, this enzyme falls during starvation and increases above the fed base line when the animal is given a high carbohydrate fat-free diet. Changes in mass appear to be due to alterations in enzyme synthesis rather than enzyme degradation (Volpe and Vagelos, 1974; Lakshmanan *et al.*, 1975; Volpe and Vagelos, 1976). Moreover, several studies have shown that the synthesis rate of this enzyme is correlated with the amount of specific mRNA coding for this enzyme complex (Flick *et al.*, 1978). These data suggest that changes in the enzyme are regulated at a pretranslational level, either by stimulating the mRNA synthesis or inhibiting the specific mRNA degradation.

Finally, there are three enzymes involved in the formation of NADPH. The two hexose–monophosphate shunt enzymes, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, and malic enzyme. The activities of these enzymes are also directly related to the mass of the enzymes (Gibson *et al.*, 1972; Goodridge, 1975; Procsal *et al.*, 1976; Winberry and Halten, 1977; Goodridge, 1978). Moreover, the activity of these enzymes have also been shown to correlate with the amount of specific mRNA's for the respective enzymes (Hutchison and Holten, 1978; Sun and Holten, 1978; Towle *et al.*, 1980).

B. Relationship between T_3 Nuclear Occupancy and Enzyme Response

As discussed in other chapters, the site of initiation of thyroid hormone action in the liver is the T_3 nuclear receptor. Since previous studies have documented that T_3 can regulate the activity of the lipogenic enzymes, it appeared important first to establish the relationship between T_3 nuclear occupancy and enzyme response.

In order to determine the occupancy–response relationship, several pieces of information are needed. One must be able to determine precisely the amount of nuclear receptor occupied. It has been found, using a variety of techniques, that the euthyroid rat has approximately 50% of its nuclear receptor sites saturated with T_3 (Oppenheimer *et al.*, 1974). In addition, the hypothyroid rats used in our laboratory have virtually no T_3 on their nuclear receptors (Mariash *et al.*, 1980b). Also, one can treat rats with very large doses of T_3 to maintain nuclear occupancy at nearly 100% (Oppenheimer *et al.*, 1977). It is, therefore, relatively easy to obtain rats, at steady state, whose T_3 nuclear occupancy spans the entire possible range.

The final piece of information required to establish the occupancy–response characteristics is the relationship between the rate of formation of enzyme and the steady-state level of enzyme activity. If the thyroidal state does not alter enzyme degradation, then any alteration in enzyme activity simply reflects a change in enzyme formation. Since this has been established for the lipogenic enzymes studied (Mariash *et al.*, 1980b), one can use the enzyme activity at steady state as a measurement of the specific response to thyroid hormone.

A quantitative index of the relationship between nuclear occupancy and response is embodied in the following equation:

$$F = \frac{E_{\text{hyper}} - E_{\text{hypo}}}{E_{\text{eu}} - E_{\text{hypo}}} \qquad (1)$$

We have defined F as the amplification factor (Oppenheimer *et al.*, 1978). E is the enzyme response under the various steady state conditions experimentally easily attained, i.e., hyperthyroidism (hyper), euthyroidism (eu), and hypothyroidism (hypo). If there was a linear relationship between enzyme response and nuclear occupancy, then simple substitution of the known nuclear occupancy into Eq. (1) would yield an amplification factor, F, of 2.0. A value substantially greater than 2 indicates significant amplification of the T_3 receptor signal. In a strongly amplified system the transition from the hypothyroid to the euthyroid animal, leading to occupation of 50% of the T_3-nuclear receptors, should yield

TABLE I

Influence of Thyroidal Status on Lipogenic Enzyme Activity[a]

	Enzyme activity (U/mg)			
	ME	G6PD	6PGD	FAS
Hypo	3.9	9.5	43.3	4.9
Eu	16.0	16.2	67.8	9.2
Hyper	145.6	37.7	154.1	33.7
Amplication factor	11.7	4.2	4.5	6.7

[a] Each value represents the mean of at least 13 animals. Enzyme activity and abbreviations are as in Fig. 1. Hypo, hypothyroid; Eu, euthyroid; Hyper, hyperthyroid rats given 200 μg T_3/100 mg body weight for 4 days. From Mariash *et al.* (1980b).

substantially much less than 50% of the maximal response observed when all the T_3 receptors are occupied. Table I shows that for all four lipogenic enzymes studied, the amplification factor is significantly greater than 2. These data indicate a high degree of amplification of the T_3 nuclear receptor signal (Mariash *et al.*, 1980b). More specific studies with malic enzymes using nonsteady analytic state techniques have confirmed the high degree of amplification predicted by the steady-state methods (Oppenheimer *et al.*, 1978). If T_3 induction of these enzymes is due to increased enzyme synthesis, and increased enzyme synthesis is secondary to increased specific mRNA formation, then one must conclude that some postreceptor mechanism is responsible for the amplification of the T_3 nuclear signal.

Of further interest is the finding that not all responses yield the same degree of amplification. Moreover, since the amplification factor is different for the enzymes examined, there must be different postreceptor events modifying the T_3 nuclear receptor signal for each of the enzymes. If only a single factor interacted with the T_3 nuclear signal to induce the various enzymes, one would anticipate an identical amplification factor. This is clearly not the case.

C. Relationship of T_3 and Carbohydrate Feeding in Induction of Lipogenic Enzymes

Experiments designed to elucidate the nature of the interaction between thyroid hormone and a high carbohydrate diet are illustrated in Fig. 1 (Mariash *et al.*, 1980b). Carbohydrate feeding led to a smaller enzyme induction in hypothyroid animals than in euthyroid animals. These findings could not be accounted for by a diminished dietary intake. Although hypothyroid rats indeed eat less than euthyroid rats, the decreased ability of carbohydrate feeding to induce

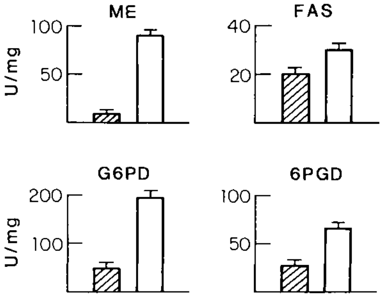

Fig. 1. Carbohydrate induced changes in lipogenic enzymes. Each bar depicts the mean ± SEM of the carbohydrate induced increment in enzyme activity above the regular diet baseline. The hatched bars represent hypothyroid rats, open bars represent euthyroid rats. The difference between the two thyroidal states is significant at $p < .001$ for each enzyme except FAS ($p < .05$). ME, malic enzyme; FAS, fatty acid synthetase; G6PD, glucose-6-phosphate dehydrogenase; 6 PGD, 6-phosphogluconate dehydrogenase. Enzyme activity is expressed as nanomoles of NADPH oxidized or reduced per minute per milligram protein (U/mg). From Mariash *et al.* (1980a).

enzymes in the hypothyroid rat is far greater than the decrease in calories consumed by these animals. Thus, thyroid hormone in some way interacts at the tissue level with carbohydrate feeding in the induction of lipogenic enzymes.

Figure 2 shows the response of the lipogenic enzymes to T_3 in hypothyroid rats fed either a regular or high carbohydrate diet. The data cover the full range of thyroidal status, from extreme hypothyroidism to marked hyperthyroidism. At all levels of thyroid hormone replacement, carbohydrate is capable of inducing each of the four lipogenic enzymes. Furthermore, the ability of the carbohydrate diet to induce the lipogenic enzymes was markedly enhanced with only very small doses of thyroid hormone. As a consequence, thyroid hormone-induced increases in enzyme activity were readily demonstrated in animals maintained on a high carbohydrate diet with doses of T_3, which did not cause a perceptible increase in enzyme activity in animals maintained on a regular chow diet.

The dose–response relationship to thyroid hormone can be quantitated from computer generated dose–response curves (Mariash *et al.*, 1980a). Several important parameters can be obtained from these curves including ED_{50}, the dose required to achieve 50% of the maximum response, and the instantaneous slope at any given dose.

Table II presents the ED_{50} for the four lipogenic enzymes on both a regular chow diet and the high carbohydrate diet. In addition, this table lists the maximum T_3 induced increment in activity obtained on both diets as well as the slope of the dose–response curve at the lowest dose of T_3 administered, 30 ng/100 g body weight. It is apparent that administration of a high carbohydrate diet lowers the ED_{50} for T_3 by 3- to 8-fold for each of the enzymes examined. Note also that carbohydrate feeding markedly increases, up to 40-fold in the case of G6PD, the

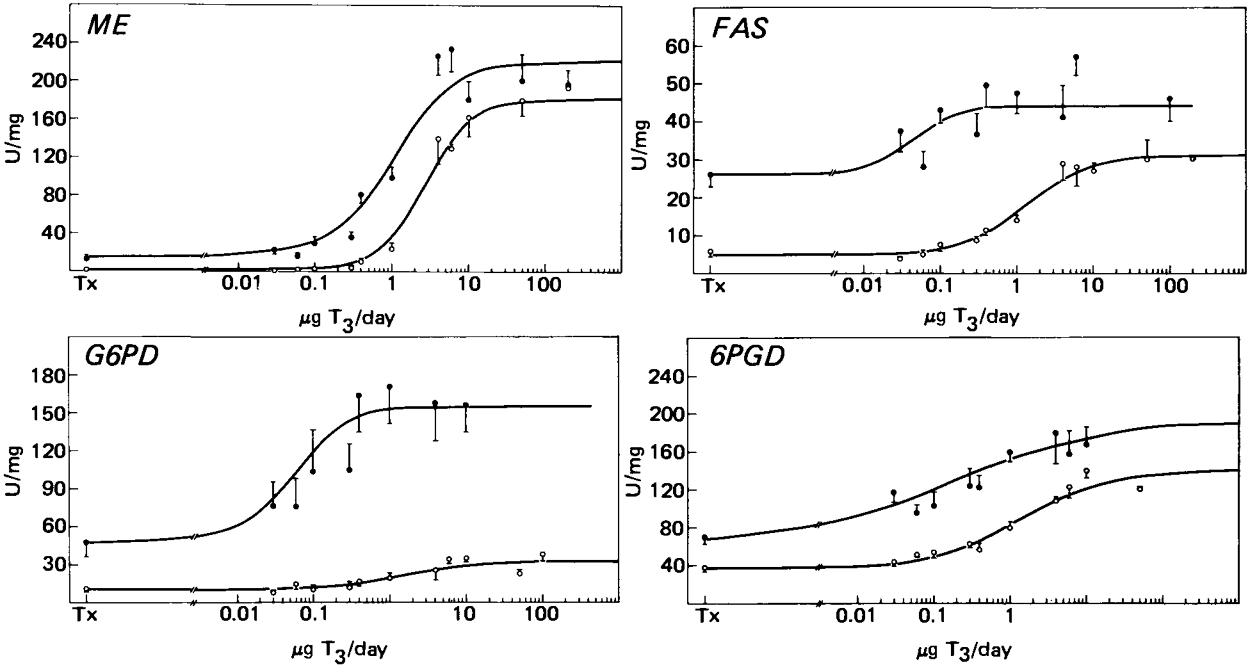

Fig. 2. Complete dose–response relationship of the lipogenic enzymes to T_3 on regular and high carbohydrate diets. Thyroidectomized rats were given graded doses of T_3 for 7 days and either given a high carbohydrate diet (●) or left on a regular diet (○) for the last 4 days. Each point represents the mean ± SEM of at least four animals. Enzyme abbreviations are as in Fig. 1. From Mariash *et al.* (1980a).

instantaneous slope of the dose–response curve at 30 ng T_3. These two findings can be interpreted to demonstrate a synergistic interaction between carbohydrate feeding and thyroid hormone administration on the induction of hepatic lipogenic enzymes. If there were no synergism between T_3 and carbohydrate feeding, then one would expect no shift in the dose–response curve and identical instantaneous slopes at low doses of thyroid hormone.

We explored several possible mechanisms underlying the synergism (Mariash *et al.*, 1980a) between carbohydrate feeding and thyroid hormone administration. We first excluded the possibility that carbohydrate feeding, by decreasing the metabolic clearance rate for T_3, would increase the effective plasma and nuclear T_3 content for a given dose of T_3. As can be seen in Table III, the metabolic clearance rate for T_3 in euthyroid rats given a high carbohydrate diet is not significantly different from the corresponding values in animals on a regular chow diet. Another possibility was also excluded, namely, that the high carbohydrate diet increases either the number or relative affinity of the nuclear receptors. The binding of tracer T_3 to the nuclear receptor *in vivo*, as determined from the nuclear/plasma ratio, was similar in animals on the high carbohydrate diet and regular chow diets. Since plasma T_3 levels were unchanged, the concentrations of T_3 at the nucleus necessarily were also similar. This conclusion was reinforced by the finding that the maximal nuclear binding capacity and the equilibrium association constant of T_3 determined in *in vitro* studies were the same in animals maintained on both diets. Therefore, we concluded that the synergism between carbohydrate feeding and thyroid hormone in the induction of lipogenic enzyme

TABLE II

T_3 Dose–Response Parameters on Regular (Reg) and High Carbohydrate (Carbohydrate) Diets[a]

Enzyme	Diet	ED_{50} (μg/100g)	Max (U/mg)	Slope (U/μg T_3)
ME	Reg	2.8	180	11.2
	Carbohydrate	1.1	209	151.4
FAS	Reg	1.4	26.5	17.2
	Carbohydrate	0.043	17.8	173.9
G6PD	Reg	1.4	22	22.2
	Carbohydrate	0.057	109	881.1
6PGD	Reg	1.4	102	122.1
	Carbohydrate	0.18	123	464.3

[a] The values are derived from computer generated best fit of the data in Fig. 2. ED_{50} is the dose of T_3 that produces 50% of the maximum response (Max). The slope is the first derivative of the dose–response curve at 30 ng T_3/100 g body weight. Enzyme abbreviations are as in Fig. 1. From Mariash *et al.* (1980a).

TABLE III

Fate of T_3 and the T_3 Nuclear Receptor on a Regular (Reg) and High Carbohydrate (Carbohydrate) Diet[a]

	Reg	Carbohydrate
MCR (ml/hour/100 g)	17	15
Nuclear/plasma ratio	0.361	0.387
Max binding (ng T_3/mg DNA)	0.31	0.32
K_a (M^{-1})	7.7×10^8	6.2×10^8

[a] MCR represents the metabolic clearance rate of $[^{125}I]T_3$ given intravenously. The nuclear/plasma ratio was obtained 30 minutes after injection of $[^{125}I]T_3$. The maximum binding of T_3 to its nuclear receptor (Max binding) and the affinity for T_3 (K_a) were determined *in vitro* by whole nuclear Scatchard analysis. From Mariash *et al.* (1980a).

cannot be attributed to any changes in T_3 metabolism or T_3 binding to the nuclear receptor as a consequence of the dietary manipulation.

D. Role of Insulin in T_3–Carbohydrate Interaction

In order to gain further insight into the mechanism of interaction between T_3 and carbohydrate feeding, we investigated the potential role of insulin. It seemed quite possible that the synergistic action of the high carbohydrate diet was mediated by a carbohydrate-induced increase in insulin secretion. Therefore, we studied the interaction of T_3 and carbohydrate feeding in rats rendered diabetic with streptozotocin (Kaiser *et al.*, 1980). After the diabetic state was verified, rats were placed on various diets including a regular chow and a 60% fructose diet. Since fructose, unlike glucose, can be metabolized by the liver of diabetic animals (Volpe and Vagelos, 1976), it represents a potential source of intracellular carbohydrate in the absence of insulin. Twenty-four hours prior to killing, half the rats in each group received a maximal dose of T_3 (200 μg/100 g body weight).

The results of these studies, summarized in Table IV, confirm the results of others and show that the diabetic state markedly inhibits the response to thyroid hormone (Ruegamer *et al.*, 1965). Fructose feeding, however, restored the ability of T_3 to induce malic enzyme. The diabetic rats that received both T_3 and fructose exhibited an increase in malic enzyme of 30 U/mg protein above the chow fed diabetic controls, a value not significantly different from that produced in nondiabetic rats given both T_3 and fructose (54 U/mg).

Plasma insulin levels were measured in rats both before being placed on the test diet and at the time of their killing. In most cases the insulin concentration was below 5 μU/ml in the diabetic rats at all times and there was no increase in insulin concentration as a result of any treatment.

TABLE IV

Effect of Diabetes on Malic Enzyme (ME) Response to T_3 and Fructose[a]

Treatment	ME (U/mg) Diabetic	ME (U/mg) Normal
Control	7.1 ± 1.8	19.3 ± 3.2
T_3	15.6 ± 2.0	56.6 ± 9.3
Fructose	8.2 ± .6	58.7 ± 8.6
T_3 + fructose	37.1 ± 7.9	74.2 ± 7.7

[a] The fructose diet was given for 48 hours prior to killing. Each value represents the mean ± SEM of at least 5 animals. Enzyme activity is expressed as in Fig. 1. From Kaiser *et al.* (1980).

These studies indicate that insulin is not the proximate agent responsible for the synergistic interaction between thyroid hormone and carbohydrate feeding. Since fructose enters the liver and is metabolized independent of insulin, fructose appears capable of generating the necessary intracellular substrate required to interact with thyroid hormone in the induction of malic enzyme. These data also strongly suggest that a product of glucose metabolism is the primary factor that interacts with the T_3 receptor signal to induce malic enzyme. It appears probable that in the intact animal insulin plays a "permissive" role by increasing glucose phosphorylation and subsequent metabolism and thus generating the product that interacts with the T_3-receptor signal (Sassoon *et al.*, 1968).

E. Effect of Carbohydrate Deprivation on Malic Enzyme Induction by T_3

We have also used starvation and aging to study the interaction between T_3 and carbohydrate in malic enzyme induction. Both these models are associated with decreased carbohydrate utilization. In starvation there is enhanced gluconeogenesis (Pilkis *et al.*, 1978), and in the aging rat there is evidence for glucose intolerance (Reaven *et al.*, 1979).

It had been suggested by Tarentino *et al.* (1966), and subsequently confirmed by us (Oppenheimer and Schwartz, 1980), that the total malic enzyme response to large doses of thyroid hormone was depressed in starved animals. Since hepatic protein turnover is increased (Schwartz *et al.*, 1980) and the content of T_3 nuclear receptors is decreased (DeGroot *et al.*, 1977; Burman *et al.*, 1977; Schussler and Orlando, 1978; Dillmann *et al.*, 1978), it seemed possible that the inhibited malic enzyme response to T_3 in starvation might be due to these two factors. Nevertheless, when the rate of malic enzyme appearance after T_3 administration was expressed per nanogram T_3 nuclear complex, we found that the values in starvation were only 12% of the corresponding estimates in fed controls

(Oppenheimer and Schwartz, 1980). Because the calculations were corrected for the content of T_3 at the nuclear receptor site, we have concluded there must also be starvation related postreceptor factors, perhaps related to intracellular carbohydrate metabolism, which lead to the decreased malic enzyme response. It is possible that starvation leads to a diminution of the carbohydrate-induced signal associated with the synergistic interaction between T_3 and carbohydrate feeding.

Another physiological model of the carbohydrate–T_3 interaction is the aging rat. In the aging rat (Brasho-Romero and Reaven, 1977), the level of insulin is high in relationship to the fasting blood sugar, a finding that suggests a form of insulin resistance (Reaven *et al.*, 1979).

In previous studies (Schwartz *et al.*, 1979) we had noted that there was an age-related decline both in the basal level of hepatic malic enzyme as well as the increment in enzyme activity which could be induced by T_3 administration. In a subsequent analysis, Forciea *et al.* (1981) observed that the response to carbohydrate administration also declined with age. Furthermore, the T_3-induced increase in animals maintained on a high carbohydrate diet declined in a parallel fashion (Table V).

Of particular interest was the finding that T_3 led to a constant fold increase over the base line value at all ages. Thus, on a regular diet, T_3 produced approximately a 10-fold increase in malic enzyme; on a high carbohydrate diet, there was a 1½- to 2-fold increase in malic enzyme. These data suggest that T_3 acts as a multiplier of a carbohydrate generated signal. In aging there appears to

TABLE V

Effect of Age, Diet, and T_3 on Malic Enzyme[a]

Age (months)	T_3	Regular diet ME	Regular diet D	Regular diet R	High carbohydrate diet ME	High carbohydrate diet D	High carbohydrate diet R
1.0	−	669			6232		
	+	6284	5615	9.4	9051	2819	1.5
1.5	−	482			4432		
	+	3866	3384	8.0	7059	2627	1.6
6.0	−	215			2427		
	+	3179	2964	14.8	3811	1384	1.6
12.0	−	262			782		
	+	3375	3095	12.9	2947	2165	3.8
18.0	−	251			1053		
	+	2577	2326	10.3	2695	1642	2.6

[a] T_3 (15 μg/100 g body weight) was administered for 7 days. The high carbohydrate diet was given for 4 days prior to killing. Each value represents the mean of at least 4 animals. D, the increment in activity induced by T_3. R, the ratio of ME in T_3-treated to controls. Enzyme activity is expressed as units per milligram DNA. From Forciea *et al.* (1981).

be a diminution in the ability of carbohydrate to generate this signal. The diminution in this signal with aging, or starvation, leads to a postreceptor inhibition of the T_3 response (Forciea *et al.*, 1981).

F. Molecular Basis of the Interaction between T_3 and Carbohydrates

In order to localize the site of interaction between carbohydrate feeding and thyroid hormone administration, we examined the relative rate of malic enzyme synthesis following the administration of either T_3 or a high carbohydrate diet (Mariash *et al.*, 1980a). The relative rate of hepatic synthesis was determined by immunoprecipitation of cytosolic [^{3}H]leucine labeled malic enzyme and comparing the amount of radioactivity in malic enzyme to the amount of radioactivity in total cytosolic TCA precipitable protein. In euthyroid rats on a regular chow diet, the amount of malic enzyme synthesized is approximately .05% of total cytosolic protein. Figure 3 illustrates the time course of the relative rate of synthesis of malic enzyme following both administration of maximal doses of T_3 intravenously and a high carbohydrate diet delivered by direct intragastric installation. Extrapolation of the two curves to the base line level indicates that both stimuli increase the rate of synthesis of malic enzyme within 2 hours following their administration. It is apparent that both stimuli act extremely rapidly to induce the synthesis of malic enzyme and both appear to induce a simultaneous

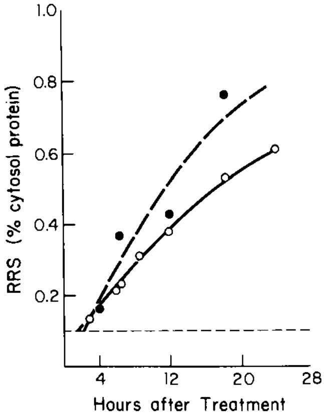

Fig. 3. Effect of T_3 and carbohydrate on malic enzyme synthesis. Rats were given T_3 (200 μg/100 g body weight) or gavaged with a high carbohydrate diet at t=0 and given 0.5 mCi [^{3}H]leucine i.p. 45 minutes prior to killing. The relative rate of malic enzyme synthesis (RRS) was determined by comparing the cpm in immunopercipitable malic enzyme to the cpm in total cytosolic protein. (○) T_3-induced rats; (●) carbohydrate-induced rats. The dashed line represents the untreated control RRS. From Mariash *et al.* (1980a).

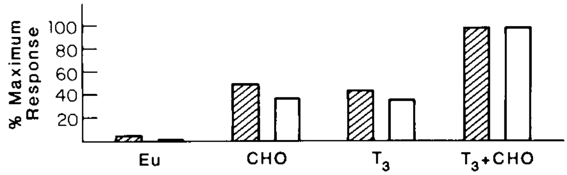

Fig. 4. Effect of T_3 and carbohydrate feeding on malic enzyme activity and mRNA. Each bar represents the mean of four rats. Hatched bars represent malic enzyme activity; open bars represent malic enzyme mRNA. Eu, euthyroid controls; CHO, high carbohydrate diet for 7 days; T_3, 15 μg/100 g body weight for 7 days; T_3 + CHO, combined treatment for 7 days. From Towle *et al.* (1980).

increase in the rate of malic enzyme synthesis. Furthermore, the slope of the curve described by the T_3-injected animals suggests that a maximal rate of induction is attained immediately.

These experiments show that the interaction between thyroid hormone and carbohydrate feeding occurs immediately and therefore suggest that neither stimulus is effective through an activation of the other stimulus. Thus, if T_3 had acted simply by increasing carbohydrate metabolism, then one would have anticipated a significantly longer lag time in the increase in malic enzyme synthesis after T_3 than after carbohydrate feeding. In addition, one would also expect to find a gradually increasing rate of enzyme synthesis after T_3 administration, instead of what appears to be a maximal rate of increase in enzyme synthesis after the administration of T_3.

The experiments clearly confirm the finding of others that T_3 and dietary carbohydrate increase the level of malic enzyme by augmenting the synthesis rate. In order to determine whether this increased rate of synthesis was due to changes in translation efficiency or due to changes in quantities of messenger RNA for malic enzyme, we determined the content of malic enzyme mRNA from rats given T_3, carbohydrate diet, or both. These determinations were made with a mRNA-dependent rabbit reticulocyte assay system (Towle *et al.*, 1980). The results of these studies are shown in Fig. 4. The hepatic content of mRNA for malic enzyme under all four conditions studied (base line, T_3 alone, carbohydrate diet alone, and the combined stimulus of T_3 and a high carbohydrate diet) correlated well with the activity of the enzyme. Since these studies were performed under steady-state conditions, and T_3 does not alter the rate of malic enzyme mRNA degradation (Towle *et al.*, 1981), both thyroid hormone and carbohydrate feeding must stimulate the synthesis or processing of the specific mRNA for malic enzyme at the nuclear level. The site of interaction between carbohydrate feeding and thyroid hormone administration thus appears to be the hepatic nucleus itself.

In very recent experiments, we have used two-dimensional gel electrophoresis to examine the population of mRNA's influenced by either thyroid hormone or carbohydrate feeding (Seelig *et al.*, 1981a,b). Following translation of total poly(A^+)-RNA and separation of the radioactively labeled products by two-dimensional gel electrophoresis, we have found that approximately 21 out of 250 identifiable products are either augmented or suppressed by thyroid hormone. Of further interest was the finding that 8 of these 21 are also changed by carbohydrate feeding. Moreover, the direction of change induced by carbohydrate feeding was identical to that induced by T_3.

These studies point to the very broad overlap between the genes regulated by thyroid hormone and those regulated by carbohydrate. Approximately 40% (8/21) of the T_3 responsive genome is also responsive to carbohydrate feeding. Thus, the interaction between T_3 and carbohydrate feeding probably is considerably more extensive than the interplay leading to the formation of the lipogenic enzymes discussed above.

G. Efforts to Identify the Carbohydrate Associated Factor Responsible for Malic Enzyme Induction

Since multiple hormones and metabolites change following either carbohydrate or T_3 administration *in vivo*, we have developed an isolated adult rat hepatocyte culture model in order to identify more precisely the carbohydrate associated factor responsible for malic enzyme induction (Mariash *et al.*, 1981). This model permitted us to manipulate the concentration of various hormones and metabolites which could be potentially responsible for triggering malic enzyme induction.

Because our previous experiments with intact animals suggested that carbohydrate itself is the most likely candidate responsible for the increase in malic enzyme, we examined the ability of glucose *in vitro* to induce malic enzyme. The

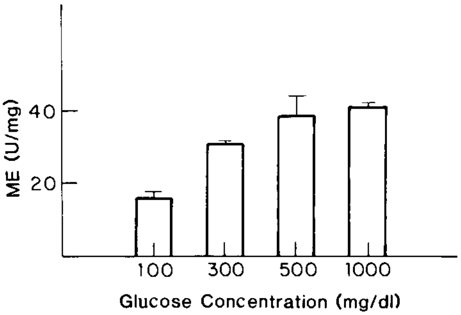

Fig. 5. Induction of malic enzyme by glucose. Hepatocytes were maintained in culture for 5 days in 10% T_3-free serum at the indicated initial glucose concentration. Each bar represents the mean and range of duplicate cultures. Malic enzyme activity is expressed as in Fig. 1. From Mariash *et al.* (1981).

TABLE VI

Effect of Insulin, Glucose, and Dexamethasone on Malic Enzyme[a]

	ME activity in 6 day hepatocytes, serum-free media		
Insulin	Glucose (100 mg/dl)	Glucose (500 mg/dl)	Dexamethasone
+	12.9	70.0	+
–	8.8	14.6	+
+	15.5	50.2	–
–	8.2	19.8	–

[a] Hepatocytes were cultured for 6 days in serum-free medium. Dexamethasone was present at a concentration of 10^{-6} *M*. Insulin was present at a concentration of 100 mU/ml. Enzyme activity is expressed as in Fig. 1. From Mariash *et al.* (1981).

results summarized in Fig. 5 demonstrate a direct relationship between the concentration of glucose in the media and the level of malic enzyme achieved in hepatocytes cultured for 5 days. In this experiment, the hepatocytes were cultured in 10% calf serum that had been rendered T_3 free. Insulin and dexamethasone were present in all cultures. Since the only variable constituent was the glucose concentration, these studies conclusively show that the glucose effect is not mediated by an extrahepatic hormone or factor. Moreover, this experiment documents that *in vitro* T_3 is not a necessary cofactor for the carbohydrate induction of malic enzyme. Also, major glucose-induced increases in malic enzyme occur with physiological concentrations of glucose. Although there is a small increase in enzyme activity above 500 mg% glucose, the largest increase in enzyme activity occurs at glucose concentrations under 500 mg%, levels that approximate the postprandial levels of portal vein glucose.

In our model, the hepatocytes remain viable for the duration of the experiment, even if cultured in the absence of any added serum in the media (Sirica *et al.*, 1979). This fact has allowed us to examine the potential role of glucocorticoids and insulin on glucose induction of malic enzyme. When dexamethasone was eliminated from the media, there appeared to be no decrease in the ability of carbohydrate to induce malic enzyme.

On the other hand, insulin plays an important permissive role in the carbohydrate induction of malic enzyme. In an experiment summarized in Table VI, hepatocytes were cultured in the absence or presence of a large amount of insulin (100 mU/ml) both with glucose concentrations of 100 and 500 mg/dl. When insulin was eliminated from the culture media, glucose was incapable of inducing malic enzyme. However, note also that with a glucose concentration of 100 mg/dl, insulin appeared to exert little or no effect on malic enzyme activity. This finding confirms our *in vivo* experiments in diabetic animals in which we sug-

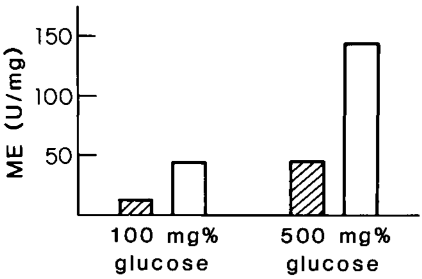

Fig. 6. Interaction between glucose and T_3 on malic enzyme induction. Hepatocytes were cultured for 6 days in serum-free media at the indicated initial glucose concentration. Each bar represents the mean of duplicate plates. The hatched bar represents cultures maintained in the absence of T_3; the open bar represents cultures maintained in the presence of 10^{-7} *M* T_3. Malic enzyme activity (ME) is expressed as in Fig. 1.

gested that insulin was not the primary carbohydrate associated factor responsible for malic enzyme induction. Insulin does appear to play an important role in as much as it facilitates the metabolism of glucose in the hepatocytes in order to produce the glycolytic factor responsible for malic enzyme induction.

The isolated hepatocyte model also allows us to examine the interaction between carbohydrate and T_3 in the induction of malic enzyme. Figure 6 illustrates the level of malic enzyme in cultures grown either in the absence of T_3 or in the presence of maximal concentrations of T_3 at low and high glucose concentrations. In the face of rapid T_3 metabolism, relatively high initial concentrations of T_3 (10^{-7} *M*) were required to maintain T_3 nuclear occupancy at nearly 100% for the duration of the experiment. T_3 increases the activity of malic enzyme at both glucose concentrations utilized. However, the increment in enzyme activity induced by T_3 is greater in cultures grown at 500 mg% glucose than in cultures grown at 100 mg% glucose. Further inspection of the data reveals that T_3 acts as a constant multiplier of the carbohydrate associated signal, precisely as under *in vivo* circumstances. Thus, T_3 increases the level of malic enzyme 3.5-fold at both 100 and 500 mg% glucose. A similar relationship characterized the T_3–glucose interaction in the induction of malic enzyme at other glucose concentrations. We conclude that the synergism between T_3 and carbohydrate is related to the ability of T_3 to act as a constant multiplier of the carbohydrate generated signal. Moreover, since T_3 is not essential for glucose induction of malic enzyme, it is the carbohydrate signal that acts as the primary inducer of malic enzyme.

In additional studies, we showed that T_3 did not increase net glucose metabolism, and that glucose did not alter the rate of T_3 metabolism. Moreover, by measuring the relative rate of malic enzyme synthesis as previously described, we demonstrated that the changes in malic enzyme activity resulting from manipulations in the glucose and T_3 concentrations were due to changes in enzyme synthesis (Mariash *et al.*, 1981).

We have not succeeded so far either in identifying the glycolytic product responsible for the induction of malic enzyme or in elucidating the structural basis of the synergistic interaction between T_3 and carbohydrate. Since the cultured hepatocyte system seems to simulate most aspects of the phenomena observed in the intact animal, further studies with this model should be helpful in clarifying these issues.

III. CONCLUDING REMARKS

The data reviewed in this chapter provide ample evidence of the close nexus among carbohydrate metabolism, lipogenesis, and thyroid hormone action. Alterations in carbohydrate availability profoundly influence the level of circulating thyroid hormones, especially T_3. The older literature fully documents the shifts in glucose tolerance which accompany various thyroidal states. More recent studies at the molecular level emphasize that the expression of hormonal action is strongly influenced by dietary carbohydrate. An interesting parallelism exists between hypothyroidism and starvation, on the one hand, and hyperthyroidism and carbohydrate excess, on the other. A detailed analysis of the relationship between carbohydrate administration and thyroid hormones in the generation of lipogenic enzymes indicates that thyroid hormones achieves its effect by multiplying a nuclear signal originating from an as yet unidentified glycolytic product.

Despite the growing body of relevant data, the formation available to us is still too fragmentary to allow us to provide a comprehensive synthesis. Thus, the full implications of the reduction in circulating level of thyroid hormone in starvation have not as yet been defined. The significance of thyroid hormone-related changes in glucose tolerance cannot be fully evaluated since we do not understand precisely how such changes influence glucose fluxes and glucose metabolism. Moreover, we comprehend only partially the physiological impact of the induction of lipogenic enzymes by T_3. Whereas T_3 increases the rate of synthesis of fatty acids, T_3 is also known to accelerate lipolysis and lipid metabolism. As in many aspects of thyroid hormone action, T_3 appears to be stimulating a futile cycle, simultaneously augmenting the synthesis and degradation of biological products.

In these studies, we have focused considerable attention on the interaction between carbohydrate and T_3 in the generation of malic enzyme. It is premature to suggest that this interrelationship can serve as a general model for other thyroid hormone actions. Nevertheless, the interrelationship between carbohydrate and T_3 are very similar to the multiplicative multihormonal interaction with which T_3, dihydrotestosterone, growth hormone, and cortisol engage in the formation of the specific mRNA coding for α_{2u}-globulin (Kurtz *et al.*, 1976; Roy and Dowbenko, 1977) and the synergistic interaction between T_3 and cortisol in the formation of the mRNA coding for growth hormone (Martial *et al.*, 1977; Shapiro *et al.*, 1978).

Regardless of these considerations, it appears worthwhile to reemphasize that thyroid hormone action in the intact organism can only be understood in the context of the entire pathophysiological setting and that the state of nutrition constitutes an important component of that setting. Much of the confusion abounding in the interpretation of thyroid function tests, especially in patients with nonthyroidal disease and nutritional disturbances, may be related to this issue.

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10

Regulation of Thermogenesis by Thyroid Hormones

D. L. GUERNSEY AND I. S. EDELMAN

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I. CELLULAR METABOLIC HEAT PRODUCTION

The major site of heat production in eukaryotes is the mitochondrion, wherein oxidation of substrates (such as $NADH_2$ and succinate) provide most of the chemical energy needed to maintain the various physiological functions of the living cell. The energy liberated from the oxidation of nutrients is not used directly, but is stored in the form of phosphate ester bonds, i.e., adenosine

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triphosphate (ATP). The energy released by combustion of substrates is assumed to be the same whether *in vitro* or *in vivo* (Lusk, 1931; Lehninger, 1971). The total available energy that is conserved in ATP *in vivo*, however, is not known with certainty. The estimated enthalpy of ATP formation (Prusiner and Poe, 1968, 1970) indicates that only about 25% of the energy of substrate oxidation (e.g., glucose to CO_2 and H_2O) is conserved in ATP, and that 75% is liberated as heat. Hydrolysis of ATP coupled to cyclical processes (e.g., muscle contraction, ion transport) liberates the remainder as heat. Utilization of ATP for biosynthetic processes would conserve some of this energy in the synthesized products. In animals in stationary states (constant body weight and composition) heat production is determined almost completely by mitochondrial oxidative metabolism. Under normal circumstances oxygen and substrates are present in abundance and the rate of oxidation is regulated by the phosphorylation state ratio: $[ATP]/[ADP][P_i]$ (Owen and Wilson, 1974). In turn, the phosphorylation state ratio is directly modulated by all processes that utilize ATP, thus forming ADP and P_i. The findings of Wilson *et al.* (1974) and Erecinska and Wilson (1978) support the idea of a near-equilibrium model of mitochondrial respiration implying that thermogenesis is primarily regulated by hydrolysis of ATP in a variety of cellular processes.

The cellular processes that hydrolyze ATP include (1) ion transport, (2) synthesis of proteins, DNA, RNA, complex carbohydrates, and lipids, (3) muscle contraction, (4) secretory and absorptive processes, and (5) a variety of metabolic pathways. These processes will regulate heat production uniquely when oxidation is tightly coupled to phosphorylation. Alternative mechanisms for significant heat production include mitochondrial oxidation–reduction reactions (i.e., electron transport) in the uncoupled state and futile metabolic cycles.

A. Basal Metabolism

Basal metabolism of an intact mammal is the rate of oxygen consumption under the specified conditions of (1) rest, (2) postabsorptive state, and (3) at thermoneutral temperatures. The basal metabolic rate (BMR) is directly related to the basal rate of energy turnover at the cell level. Oxygen consumption is used as the measure of overall metabolism since the amount of heat produced for an amount of oxygen reduced to water is almost constant regardless of whether fat, carbohydrate, or protein is oxidized: 5.0 kcal per liter O_2 for glucose, 4.5 kcal for protein, and 4.7 kcal for neutral fat (Schmidt-Nielsen, 1975).

Swan (1972) and Girardier (1977) termed the basal metabolic rate as the "minimal heat production," which can be subdivided into "essential energesis" and "obligate heat." "Essential energesis" is the total of three energy expenditures: (1) heat liberated from catabolic processes in the cellular balance of anabolism and catabolism, (2) work maintaining physiological ion gradients, and (3) the energy needed to supply food to cells. The heat production from essential energy expenditure is not enough to maintain a body temperature above ambient and might be considered equivalent to the metabolic rate of a poikilotherm (Girardier, 1977). Templeton (1970) pointed out, for example, that the rate of heat production of a rat is five times greater than that of a reptile of the same body weight corrected for the same temperature. These and other findings imply that acquisition of mechanisms generating additional metabolic heat was associated with the development of homeothermy. Girardier (1977) emphasized that since normal heat production, estimated from the BMR of a homeotherm *in vivo,* is approximately equal to the sum of the QO_2's of each of the body tissues measured *in vitro,* the control mechanisms operating *in vivo* appear to be preserved *in vitro.* This would also suggest that enhancement of the BMR at least under some conditions may have a long time constant.

B. Thyroid Hormone and Basal Metabolic Rate

In 1895, Magnus-Levy noted that patients with myxedema exhibited an abnormally low oxygen consumption when compared to normal individuals and that unusually high amounts of oxygen were consumed by hyperthyroid patients. It was subsequently found that tissues isolated from hypothyroid and hyperthyroid animals demonstrated decreased and increased oxygen consumption, respectively (Foster, 1927; Barker, 1951; Barker and Klitgaard, 1952). The finding that the metabolic effect of thyroid hormone was preserved *in vitro* from animals treated *in vivo* implied that the cellular and biochemical mechanisms responsible could be investigated at the biochemical level.

The thermogenic effect of thyroid hormones (T_4 and T_3) is characterized by a latent period of 12–24 hours. The length of this lag phase depends on the size, species, and environment of the animal. The direct addition of T_4 to tissues *in vitro* does not elicit the increased metabolism in a short time (i.e., hours) (Wiswell *et al.,* 1954). In adult homeotherms, the thermogenic response is evident in all tissues of the body, with the notable exceptions of brain, spleen, and gonads (Barker and Klitgaard, 1952; Ismail-Beigi and Edelman, 1971). On a molar basis, triiodothyronine is 3–5 times more potent in activity than thyroxine (Gross and Pitt-Rivers, 1954).

C. Mechanism of Thyroid Thermogenesis

The first biochemical theory of thyroid thermogenesis proposed that thyroid hormone directly uncouples mitochondrial oxidative phosphorylation, analogous to the action of dinitrophenol (DNP). This theory predicted thyroid-dependent increases in oxygen consumption without concomitant increases in ATP production (decreased P/O ratio). In accord with this proposal, Lardy and Feldott

(1951), Martius and Hess, (1951), and Hoch and Lipmann (1954) presented evidence that mitochondria prepared from T_4 treated rats exhibited lower P/O ratios than that from euthyroid controls. In the early 1960's, the validity of the uncoupling hypothesis, however, was challenged for several reasons: (1) inactive iodinated compounds were as active as thyroxine in producing uncoupling of oxidative phosphorylation (Tapley and Cooper, 1956); (2) isolated mitochondria from animals injected with high doses of thyroxine did not show increased rates of respiration, despite the low P/O ratios (Lardy and Feldott, 1951; Hoch and Lipmann, 1954); (3) after single injections the peak thermogenic action of T_4 occurs long after the hormone has been metabolized and excreted (Brigg *et al.*, 1953; Gross and Leblond, 1947); (4) the biological actions of thyroid hormones were not mimicked by DNP, *in vivo* (Tapley and Cooper, 1956); (5) the uncoupling observed could have represented pharmacological rather than physiological effects since the doses used were 100 to 10,000 times higher than the daily amount of thyroxine released by the thyroid gland in the rat. With less toxic burdens of T_4 or T_3, significant thermogenesis is elicited with preservation of normal P/O ratios (Tata *el al.*, 1962; Fletcher *et al.*, 1962).

Fletcher and co-workers (1962; Fletcher and Myant, 1962) noted that hyperthyroid rats had decreased liver ATP content and normal mitochondrial P/O ratios concomitant with increased tissue respiration. Tata *et al.* (1962) proposed that thyroid hormone induced the biosynthesis of mitochondrial respiratory units in that mitochondria isolated from hyperthyroid rats exhibited increased capacity for oxidative phosphorylation and higher rates of amino acid incorporation into mitochondrial proteins than in comparable preparations from euthyroid rats. Histological studies supported this concept in that thyroid hormone increases the number and size of cristae of skeletal muscle mitochondria (Gustafsson *et al.*, 1965). Several studies further implicated activation of RNA and protein synthesis in the thermogenic action of thyroid hormones (Tata, 1963; Tata and Widnell, 1966). The thermogenic action of thyroid hormone, however, is not easily explained by an increase in the number and respiratory capacity of mitochondria, since the local concentration of ADP determines cellular respiration when sufficient substrate and oxygen are available. Further, the amount of energy used in RNA and protein synthesis indicated that this pathway can only account for a small fraction of the increase in QO_2 (Ismail-Beigi *et al.*, 1976). The induction of RNA and protein synthesis, however, may be involved in the thermogenic action of thyroid hormones by synthesizing proteins that necessitate the utlization of ATP.

As previously stated, under normal conditions with adequate substrate and normal O_2 tensions, the local concentration of ADP or the [ADP] $[P_i]$/[ATP] ratio determines the rate of mitochondrial respiration (Owen and Wilson, 1974). In accord with this inference, the cell content of energy rich nucleotides (in

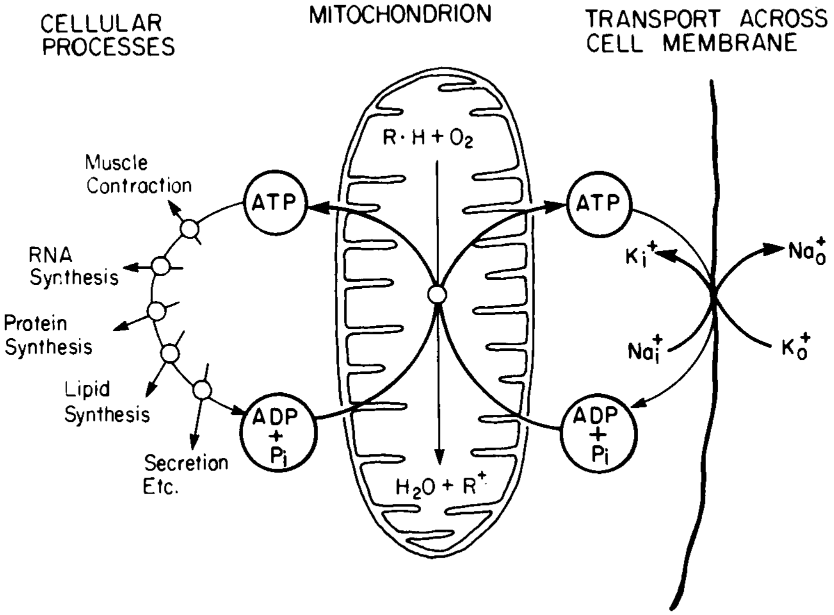

Fig. 1. Energy cycle for oxidative metabolism. ATP generated by mitochondrial oxidative phosphorylation is hydrolyzed to ADP + P_i to provide the energy for a variety of cellulalar processes (e.g., muscle contraction, macromolecular synthesis) and for active Na^+ transport (or linked $Na^+:K^+$ transport) across cell membranes. In the coupled state with NADH, succinate, and oxygen in abundance, QO_2 will be paced by rate of formation of ADP + P_i as a result of ATP hydrolysis. Na_o = extracellular Na; Na_i = intracellular Na. Reprinted from Edelman (1974).

warm-blooded animals) is very small when compared to the rate of energy consumption either by independent measurements of QO_2 and ATP contents or as indicated by the turnover time of ATP → ADP + P_i → ATP, which is of the order of a minute or less (Edelman, 1974). Cellular processes that increase ATP consumption, therefore, should dominate mitochondrial oxidative activity.

A unitary theory of the mechanism of thyroid thermogenesis should include evidence that the pathway uses enough ATP to account for the thermogenic responses and be present in all target cells. Figure 1 illustrates a variety of cellular biological energy cycles coupled to oxidative metabolism. Of the many ATP hydrolyzing processes, in resting cells, active Na^+ transport is unique in that some estimates assign a large portion of the total energy turnover (15–85% of QO_2) to this pathway, and it is present in all cells. Accordingly, Ismail-Beigi and Edelman (1970) initiated studies to test the possibility that thyroid hormone increases active transmembrane Na^+ transport and that the ADP and P_i so generated would pace mitochondrial oxidative activity and thereby account for a significant part of the thermogenic action.

II. THYROID THERMOGENESIS AND THE Na^+ PUMP

A. Active Na^+ Transport

Several reviews have extensively discussed the biochemistry, pharmacology, and physiology of the Na^+ pump and/or its enzymatic equivalent the (Na^+ + K^+ + Mg^{2+})-dependent adenosine triphosphatase (Na^+,K^+-ATPase) (Skou, 1973; Askari, 1974; Schwartz *et al.*, 1975; Wallick *et al.*, 1979; Sweadner and Goldin, 1980). The Na^+ pump serves two general functions: (1) net transepithelial salt transport (e.g., toad bladder, frog skin, intestine, renal tubule), and (2) extrusion of Na^+ and accumulation of K^+ in all cells. In the latter case, no net transport occurs because of the finite permeability of the membrane to Na^+ and K^+ via passive pathways. To maintain the steady-state transmembrane electrochemical difference of Na^+ and K^+, there must be a constant expenditure of cellular energy.

The energy expended in active Na^+ transport in intact cells and tissues has been estimated from the difference in oxygen consumption (QO_2) in optimal media from that in the presence of ouabain or in Na^+-free media (QO_2'), that is,

$$QO_2(t) = QO_2 - QO_2' \tag{1}$$

where $QO_2(t)$ is the Na^+ transport-dependent respiration. The assumptions underlying this strategy is that complete inhibition of the Na^+ pump by the digitalis glycoside, ouabain, does not impair any other process, and that cessation of Na^+ pump activity does not alter significantly other energy-utilizing pathways.

The assumption that ouabain is a highly specific inhibitor of the Na^+ pump has been justified by a series of findings: (1) No other enzyme system is known to be inhibited at concentrations < 3 m*M*. Enzyme systems that are ouabain-resistant include mitochondrial ATPase, sarcoplasmic Ca-ATPase, myosin ATPase, membrane associated Mg-ATPase, 5′-nucleotidase, and adenylate cyclase (Ismail-Beigi and Edelman, 1971). (2) The estimated $QO_2(t)$ was the same using high concentrations of ouabain or Na^+-free media [substituted with isomolar choline chloride or K^+ sucrose (Asano *et al.*, 1976)]. That Na^+-free media stops the Na^+ pump by exhausting intracellular Na^+ was documented by Asano *et al.* (1976).

Van Rossum (1970) and Himms-Hagen (1976) suggested that the ouabain-dependent fall in QO_2 results from changes in intracellular composition (Na_i^+ and K_i^+). This criticism was effectively negated by the findings of Asano *et al.* (1976). Incubation of rat diaphragm in ouabain (K^+-free) media reduced K_i^+ to 15 mEq/liter and increased Na_i^+ to 155 mEq/liter. Incubation in Na^+-free sucrose Ringer's supplemented with 40 m*M* K^+ maintained K_i^+ at 125 mEq/liter while intracellular Na^+ was virtually nil. It was found that $QO_2(t)$ of diaphragm measured by substituting Na^+-free, K^+-supplemented media yielded the same results as the addition of ouabain ($-K^+$ medium) (Asano *et al.*, 1976). These results indicate that the respiratory indices [and subsequent estimation of $QO_2(t)$] were independent of distortions in intracellular Na^+ and K^+ concentrations of the diaphragm during the time required to make the measurement.

B. Na^+ Transport-Dependent Respiration and Thyroid Thermogenesis

As shown in Table I, the fractional contribution of $QO_2(t)$ to QO_2 in isolated tissues obtained from euthyroid rats implies that active Na^+ transport consumes from 15 to 40% of total energy turnover in mammalian cells (Ismail-Beigi and Edelman, 1970; also unpublished results). These estimates are in accord with the earlier findings of Whittam (1964) and Blond and Whittam (1964). The magnitude of the contribution of increased Na^+-dependent energy utilization to thyroid thermogenesis has been evaluated using similar techniques in rat liver, kidney, skeletal muscle, and jejunal mucosa (Ismail-Beigi and Edelman, 1970; Asano *et al.*, 1976; Liberman *et al.*, 1979). In the rat diaphragm, increased $QO_2(t)$ accounted for 45% of the increase in QO_2 in the transition from the hypothyroid to the euthyroid state, and for 85% of the increase in the transition from the euthyroid to the hyperthyroid state, estimated either with ouabain or with Na^+-free, K^+-supplemented media (Ismail-Beigi and Edelman, 1970; Asano *et al.*, 1976). Similarly, increased $QO_2(t)$ accounted for 90–100% of the increased QO_2 in the transition from hypothyroid to euthyroid or to hyperthyroid levels in rat liver. The comparable contributions of $QO_2(t)$ to QO_2 in the transition from the hypothyroid to the euthyroid or hyperthyroid states were 29 and 46% in kidney

TABLE I

Comparison of Respiratory Indices of Various Tissues of the Rat[a]

Tissue	QO_2	QO_2'	$QO_2(t)$	$QO_2(t)/QO_2$
Brain cortex (slices)	10.3	6.2	4.1	0.40
Kidney cortex (slices)	26.2	16.9	9.3	0.35
Liver (slices)	8.2	5.1	3.1	0.38
Diaphragm	7.7	6.5	1.2	0.16
Jejunal mucosa	16.5	10.9	5.6	0.34

[a] Respiration is given in units of μl O_2/mg dry weight of tissue/hour. QO_2 denotes respiration in optimal media; QO_2' denotes ouabain-insensitive respiration; and $QO_2(t)$ denotes ouabain-sensitive or Na^+ transport-dependent respiration (Ismail-Beigi and Edelman, 1971; Liberman *et al.*, 1979).

slices, and 57 and 29% in jejunal mucosa. It is important to note that in the adult rat brain, a thermogenically nonresponsive tissue, *in vivo* thyroid hormone administration had no effect on either QO_2 or $QO_2(t)$ of slices assayed *in vitro* (Ismail-Beigi and Edelman, 1971). These results indicate that the energy demands for active Na^+ transport are a major portion of the metabolic response to thyroid hormone. This is presumably a result of ATP hydrolysis providing energy for active Na^+ extrusion and uptake of K^+.

It should be noted that some investigators have questioned the accuracy of the estimates of the contributions of $QO_2(t)$ to QO_2 in thyroid thermogenesis. Folke and Sestoft (1977) questioned the results obtained with liver slices on the grounds of possible injury to hepatocytes subjacent to the cut surface with resultant abnormally high rates of passive Na^+ entry and spuriously high estimates of $QO_2(t)$. To circumvent this possibility, they studied the effects of T_3 on the isolated perfused rat liver and found that pretreatment with T_3 raised hepatic QO_2 by 30%, but addition of ouabain to the circulating medium had little effect on the measured rate of respiration. This maneuver, however, was attended by progressive rises in perfusion pressure within 20 minutes, too brief a period to ensure effective inhibition of hepatocyte Na^+,K^+-ATPase. Moreover, in primary monolayer cultures of rat hepatocytes (no cut surfaces), the increase in $QO_2(t)$ accounted for 90% of the increase in QO_2 (Ismail-Beigi *et al.*, 1979), and in stripped intestinal mucosa (mucosa separated at a connective tissue boundary) without any cut surfaces, the increases in $QO_2(t)$ accounted for 30–60% of the thyroid induced increases in QO_2.

Reservations concerning the possibility of injury artifacts have also been expressed by Chinet *et al.* (1977) in the use of the diaphragm to evaluate $QO_2(t)$. To avoid possible effects of cutting across muscle fibers they used intact soleus cut at the ligamentous ends and measured heat production by direct calorimetry. At 30°C and in the presence of 10 m*M* Mg^{2+}, ouabain-sensitive heat production in rat and mouse soleus was only 5–8% of the total. Chinet *et al.* (1977) and Biron *et al.* (1979) concluded, therefore, that changes in $QO_2(t)$ could not account for a significant fraction of thyroid thermogenesis. These results, however are unreliable for two reasons: (1) Optimum Na^+,K^+-ATPase activity is obtained at a Mg:ATP ratio close to unity (i.e., 1–3 m*M*). As demonstrated by Gregg and Milligan (1980) in soleus, an external Mg^{2+} concentration of 10 m*M* inhibits estimated $QO_2(t)$ considerably. (2) Optimum active Na^+ transport is obtained at 37°C, and both $QO_2(t)$ and Na^+,K^+-ATPase are characterized by high energies of activation ($E_a \sim 16$ kcal) (Rahimifar and Ismail-Beigi, 1977). Accordingly, the use of an incubation temperature of 30°C should result in a differential inhibition of the Na^+ pump (Smith and Edelman, 1979). Indeed, estimates of ouabain-sensitive respiration in intact soleus muscle incubated in 1 m*M* Mg^{2+} and at 37°C assign 19.7% of the QO_2 to $QO_2(t)$ (Gregg and Milligan, 1980).

1. *Na^+,K^+-ATPase Enzyme Activity*

Evidence of thyroid hormone induced increases in Na^+,K^+-ATPase activity (the enzymatic expression of the Na^+ pump) has been adduced in many tissues. Liver homogenates from T_3-treated, thyroidectomized rats exhibited a 54% greater Na^+,K^+-ATPase activity than paired controls, and a 81% higher activity from T_3-treated euthyroid rats (Ismail-Beigi and Edelman, 1971). In kidney cortex homogenates, T_3 administered to thyroidectomized rats gave a 69% enhancement of enzyme activity, and a 21% increase when T_3 was given to euthyroid rats. It is significant that the cerebral cortex. which showed no thyroid induced changes in QO_2 or $QO_2(t)$, also exhibited no modulation of Na^+,K^+-ATPase activity with thyroid state. The specificity of the enhanced Na^+,K^+-ATPase activity is indicated by the fact that T_3 had insignificant effects on Mg-ATPase and 5′-nucleotidase activities (Ismail-Beigi and Edelman, 1971); and in liver homogenates prepared from hypo-, eu-, and hyperthyroid rats adenylate cyclase activities (basal, epinephrine-stimulated, and Fl^--stimulated) were unchanged (Jones *et al.*, 1972). Thyroid status also modulates Na^+,K^+-ATPase activity in rat skeletal muscle (Asano *et al.*, 1976), jejunal mucosa (Liberman *et al.*, 1979), and cardiac muscle (Philipson and Edelman, 1977a; Hegyvary, 1977). These results indicate that one locus of thyroidal regulation is the Na^+,K^+-ATPase system.

2. *Quantitative Relationships among QO_2, $QO_2(t)$, and Na^+,K^+-ATPase Activity*

Several investigations have clearly shown temporal coordination in enhanced QO_2, $QO_2(t)$, and Na^+,K^+-ATPase activity by thyroid hormone. After injection of T_3 into either thyroidectomized or euthyroid rats, liver QO_2, $QO_2(t)$, and Na^+,K^+-ATPase activity showed a characteristic 12 hour lag period, then reached peak increases at 48 hours. Control levels were reestablished after 4 days. Repeated injections of T_3 (every 48 hours) produced parallel increases in QO_2, $QO_2(t)$, and Na^+,K^+-ATPase activity; the steady-state levels were attained at 48 hours (Ismail-Beigi and Edelman, 1974).

The quantitative correlation in the T_3-dose dependent changes in $QO_2(t)$ and Na^+,K^+-ATPase activity of skeletal muscle is shown in Fig. 2. In thyroidectomized rats injected with single doses of T_3 (10, 50, or 250 μg/100 g body weight) $QO_2(t)$ increased linearly with Na^+,K^+-ATPase activity when measured 48 hours postinjection. QO_2 and $QO_2(t)$ demonstrated similar dose dependence as Na^+,K^+-ATPase; the mean change in absolute $QO_2(t)$ was about one-half that in QO_2 at all doses (Asano *et al.*, 1976). Further studies with rat jejunal mucosa demonstrated constant ratios of $QO_2/Na^+,K^+$-ATPase and $QO_2(t)/Na^+,K^+$-ATPase in various thyroid states, implying proportionate increases in these respiratory and enzymatic indices of Na^+ transport (Liberman *et al.*, 1979). Recent data show parallel dose-dependent increases in hepatic and renal

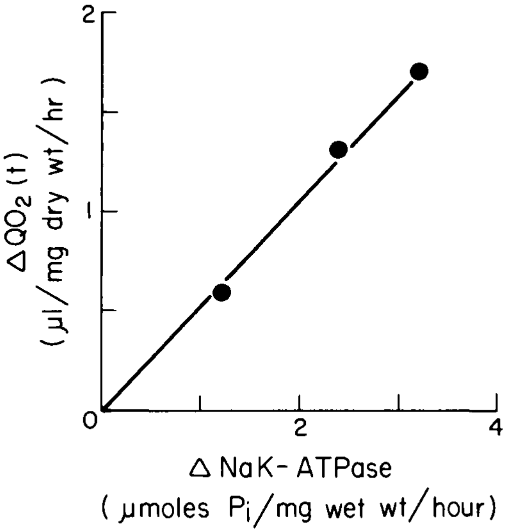

Fig. 2. Relationship between the change in QO_2(t) and the change in Na^+,K^+-ATPase activity. Rats were injected with a single dose of T_3 1 week after surgical thyroidectomy and assayed 48 hours later. Respiratory analyses were done on diaphragm and enzyme activity on gastrocnemius. Reprinted from Asano *et al.* (1976).

cortical QO_2, QO_2(t), Na^+,K^+-ATPase, and α-GPDH activities on administration of single doses of T_3 to hypothyroid rats (Figs. 3 and 4) (Somjen *et al.*, 1981). The half-maximal increases in all of the response parameters in both kidney and liver were obtained at dosages of 6–32 μg T_3/100 g body weight. These correlations are consistent with the inference that increased Na^+ transport contributes significantly to thyroid thermogenesis.

3. *Direct Action of Thyroid Hormone in Vitro*

To determine that T_3 acts directly on target tissues rather than indirectly via other circulating hormones or intermediates requires that the physiological response be demonstrated in isolated cell systems. This was accomplished in adult rat hepatocytes in primary monolayer culture prepared from hypothyroid rats and subjected to the direct addition of T_3 to the culture medium (Ismail-Beigi *et al.*, 1979). Addition of T_3 to the thyroid-depleted culture medium increased QO_2 detectably by 24 hours and maximally by 72–96 hours, relative to control cultures. The thyroid responsive enzyme activities, Na^+,K^+-ATPase and α-GPDH, increased in parallel with QO_2, but Mg-ATPase remained unchanged. The increase in QO_2(t) (based on ouabain) accounted for 90% of the increase in QO_2. Moreover, the fractional increases in Na^+,K^+-ATPase, α-GPDH, and

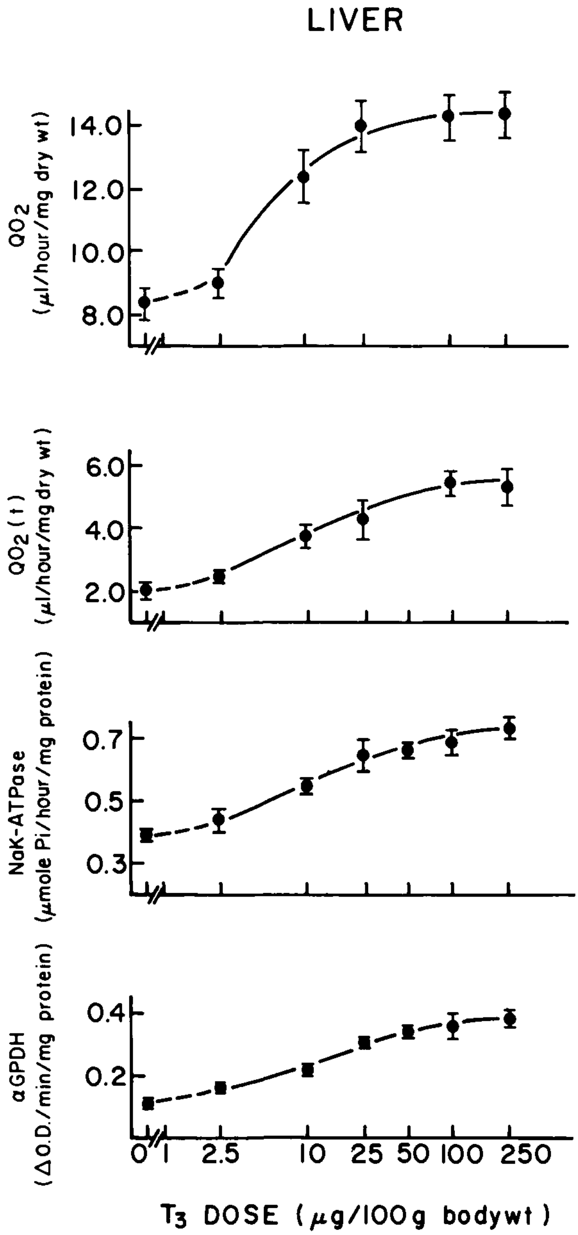

Fig. 3. Dependence of hepatic QO_2, $QO_2(t)$, Na^+,K^+-ATPase, and α-GPDH on the total dose of T_3. The tissue was sampled 48 hours after administration of T_3 by constant intravenous infusion (1 hour). The vertical lines indicate + SEM, $n = 8$ or more for each point. The basal values were determined in liver from the diluent-treated control hypothyroid rats sampled 48 hours after infusion with the diluent. Reprinted from Somjen *et al.* (1981).

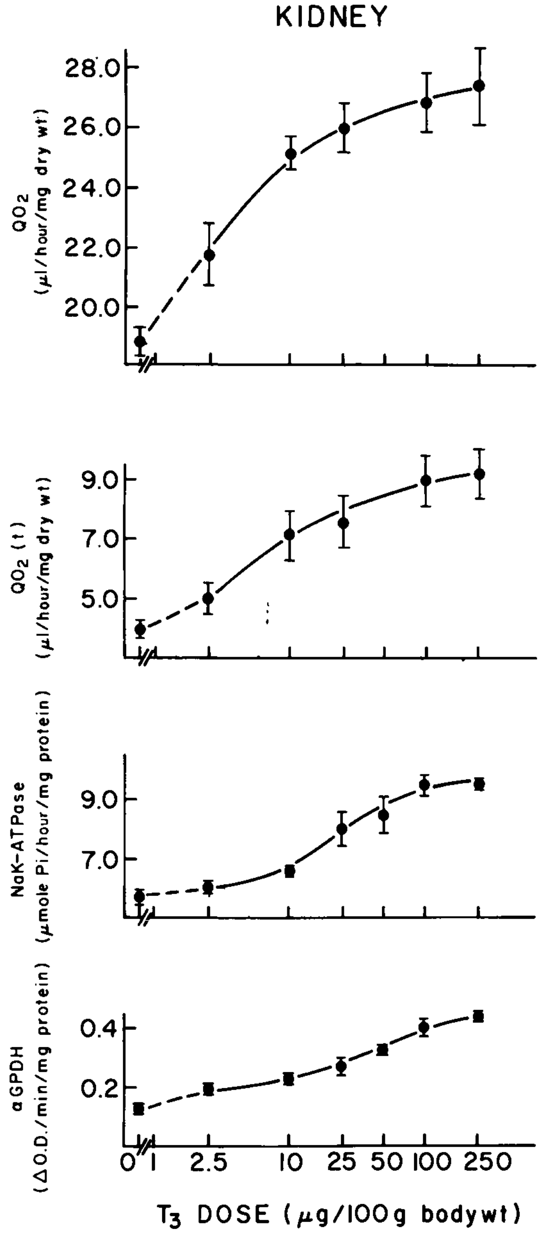

Fig. 4. Dependence of renal cortical QO_2, QO_2(t), Na^+,K^+-ATPase, and α-GPDH on the total dose of T_3. The tissue was sampled 48 hours after administration of T_3 by constant intravenous infusion (1 hour). The vertical lines indicate ± SEM, $n = 8$ or more for each point. The basal values were determined in kidney from the diluent-treated control. Reprinted from Somjen *et al.* (1981).

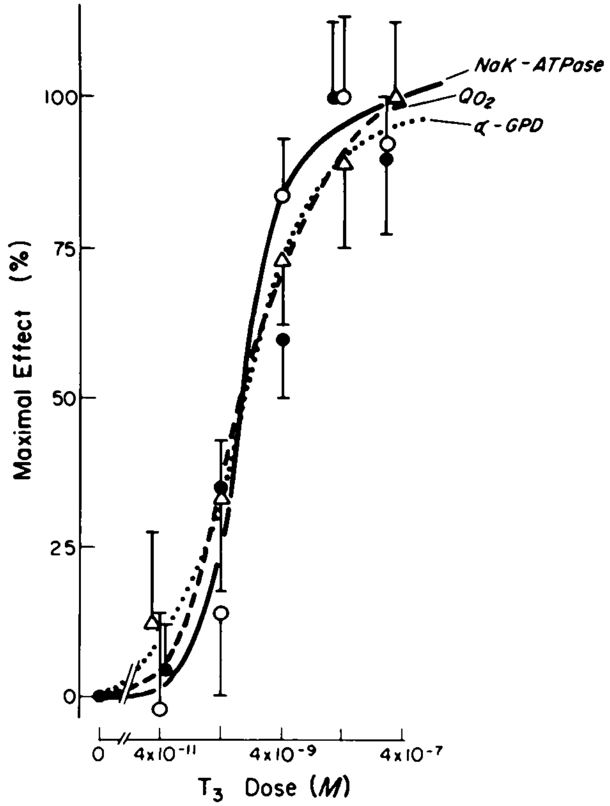

Fig. 5. Relationship between concentration and effect of T_3 on QO_2 (●), Na^+,K^+-ATPase (○), and mitochondrial α-GPD (△) activity of primary hepatocyte monolayer cultures. Cells were exposed to various concentrations of T_3 4–6 hours after plating and daily thereafter. Measurements were carried out 3 days after initial exposure to T_3. The maximum response in each parameter (calculated per microgram DNA) was taken as 100%, and the data are plotted as a percentage of the maximal effect. Mean ± SE, $n = 4$, all determinations in duplicate. Reprinted from Ismail-Beigi *et al.* (1979).

QO_2 were indistinguishable at equivalent concentrations of T_3 (Fig. 5). The half-maximal responses were attained at 8×10^{-10} *M* T_3. The enzymatic and respiratory responses to direct addition of T_3 also were obtained in a defined culture medium; and the increase in QO_2 was independent of the presence or absence of serum, corticosterone, or insulin in the medium. These data indicate that thyroid thermogenesis can be demonstrated in a single population of cells by direct addition of T_3. The enhanced respiration, mitochondrial α-GPDH, and Na^+,K^+-ATPase activities were coordinate with respect to dose and cotemporal (Ismail-Beigi *et al.*, 1979).

C. Mechanism of Thyroid Hormone Stimulation of Active Na^+ Transport

An increase in the cellular energy expenditure for Na^+ transport could be a result of several events as shown in Fig. 6. If thyroid hormone increases the permeability of the cell membrane to Na^+ and K^+ (site 1 in Fig. 6) the resultant increases in internal Na^+ (Na_i^+) and in the local concentration of external K^+ (K_o^+) could stimulate the activity of the transport enzyme directly. Second, thyroid hormone may alter the efficiency of the Na^+ pump such that more ATP would be hydrolyzed in order to transport the same quantity of Na^+ (site 2, Fig. 6). A third possibility is an action on the mitochondria to enhance synthesis of ATP thereby providing more ATP or increasing the ATP/ADP + P_i ratio, which would increase the metabolic driving force for the Na^+ pump (site 3, Fig. 6). Finally, Na^+ transport activity could be increased by activation of or an increase in the total number of functional pumps (site 4, Fig. 6).

If the predominant effect of thyroid hormone was via pathways labeled 1 and 2 in Fig. 6, then an increased Na_i^+/K_i^+ ratio would be predicted. In investigating this possibility it was found that administration of T_3 to hypothyroid and euthyroid rats decreased Na_i^+ and increased K_i^+ of rat liver, skeletal, and cardiac muscle, which resulted in 20–34% declines in the Na_i^+/K_i^+ ratios (Ismail-Beigi and Edelman, 1973). Moreover, consideration was given to the possibility of an early increase in Na_i^+ (within the first several hours of treatment), an increase in the number of Na^+ pumps at 24 hours, secondary to the rise in Na_i^+ and a tertiary fall (overshoot) in the Na_i^+/K_i^+ ratio at 48 hours. This speculation arose from the report of Boardman *et al.* (1972) who found that partial inhibition of the Na^+ pump in cell culture raised the Na_i^+/K_i^+ ratio, which was followed by an increase in Na^+,K^+-ATPase activity. Accordingly, the time courses of the ionic and enzymatic responses to a single injection of T_3 in thyroid-oblated (^{131}I-treated) rats were measured by Philipson and Edelman (1977b). At 6 and 16 hours after injection, T_3 had to significant effect on cardiac Na_i^+, K_i^+, or microsomal Na^+,K^+-ATPase activity. At 24 and 48 hours, proportionate increases in K_i and Na^+,K^+-ATPase activity were exhibited. The observed changes support the implication of a primary increase in Na^+ pump activity and secondary changes in the ion gradients. These results indicate that either pathway 3 or 4 (Fig. 6) dominate the response to thyroid hormone. If activation or induction of the Na^+ pump is the dominant response to T_3, there should be an inverse relationship between changes in the ATP/ADP ratio and in QO_2. If, on the other hand, thyroid hormone predominantly stimulates ATP production, then there should be a rise in ATP/ADP ratios after administration on the hormone (site 3, Fig. 6). Measurement of cellular ATP/ADP ratios failed to provide support for a T_3-mediated augmentation of the ATP/ADP ratio. Using a rapid freeze *in situ* technique, Ismail-Beigi *et al.* (1973) found that administration of

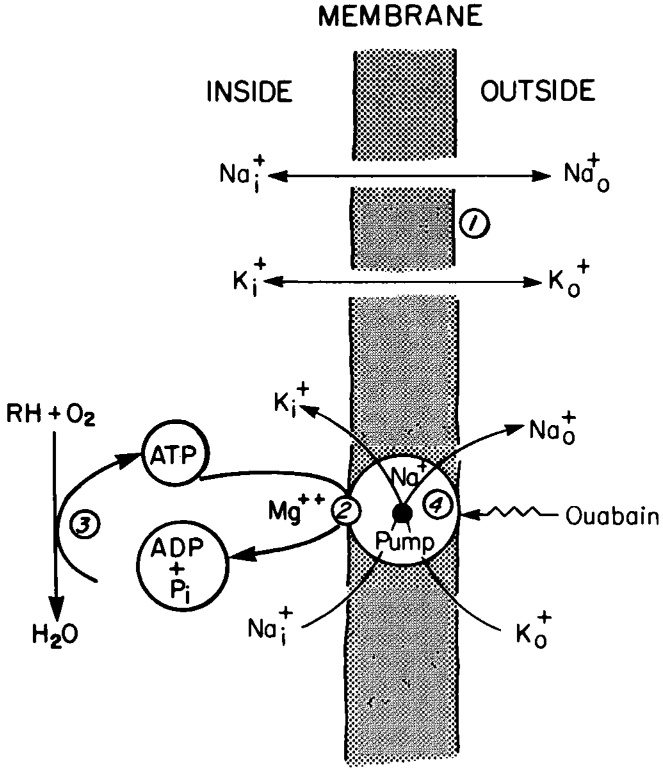

Fig. 6. Sites of regulation of Na^+ transport across cell membrane. Site 1 indicates passive Na^+ and K^+ permeability channels. Site 2 is the coupling of ATP hydrolysis to active Na^+ transport (or Na^+,K^+-linked transport). Site 3 designates the regeneration of ATP coupled to oxidation of reduced substrates (e.g., NADH, succinate). Site 4 indicates actions on the Na^+ pump either by stimulation of a set of preexisting pumps or by increasing the number of pumps. Reprinted from Edelman (1974).

T_3 to thyroidectomized or euthyroid rats increased hepatic QO_2 by 119 and 69%, respectively, and lowered the ATP–ADP ratio in both groups. The observed fall in the hepatic ATP/ADP ratio in T_3-treated thyroidectomized rats, however, was statistically insignificant and implies simultaneous augmentation of mitochondrial oxidative phosphorylation and Na^+ pump activity. Indeed T_3-dependent augmentation of this capacity has been inferred by Tata *et al.* (1963) and is implied by the reported increases in a variety of mitochondrial enzymes and transport pathways (Oppenheimer, 1979; Babior *et al.*, 1973).

The available evidence indicates that T_3 augments Na^+ and K^+ transport, via activation of the Na^+ pump. As noted above, administration of T_3 results in steady-state decreases in the Na_i^+/K_i^+ ratio (with no change in plasma Na^+ or

K^+ concentrations) in liver, skeletal, and cardiac muscles (Ismail-Beigi and Edelman, 1973). And in the heart, the increase in K_i^+ (no significant effect on Na_i^+) correlated temporally with the increase in Na^+,K^+-ATPase activity (Philipson and Edelman, 1977b). In addition, the Na^+ efflux rate constant in rat liver increased by 105% in response to administration of T_3 to hypothyroid rats and by 30% in the response to T_3 of euthyroid rats (Ismail-Beigi and Edelman, 1973). Lo and Lo (1979) have demonstrated that the early increase in kidney cortex Na^+,K^+-ATPase activity (24 hours) after T_3 administration to rats precedes an increase in glomerular filtration rate and Na^+ load. These results suggest that T_3 directly regulates Na^+,K^+-ATPase activity in rat kidney cortex, rather than a secondary response to a primary increase in filtered Na^+.

The evidence cited above supports the conclusion that activation of a preexisting set of Na^+ pumps or augmentation of the number of Na^+ pumps (per cell) mediates the observed increases in $QO_2(t)$, Na^+ efflux, and Na^+,K^+-ATPase activity (measured at V_{max}) elicited by thyroid hormone. This inference based on the results obtained in a variety of target tissues, including liver, kidney, jejunal mucosa, and skeletal and cardiac muscle on injection of T_3 to intact rats and assays of the isolated tissues. Thyroidal enhancement of Na^+,K^+-ATPase activity was not a simple consequence of a generalized increase in plasma membrane protein content in that other enzymes associated with the membrane-rich fraction, notably Mg-ATPase and 5′-nucleotidase, were unaffected by thyroid status.

The increased activity of the Na^+,K^+-ATPase system elicited by thyroid hormone could result from activation of a fixed number of enzyme units or from an increase in the total number of functional transport enzymes. Activation of a fixed number of enzyme units could result in decreases in the K_m for ATP and the $K_{1/2}$ for Na^+ and K^+. On the other hand, synthesis of new transport units or activation of a latent population to the same state as the normal enzyme (unmasking) should be expressed as an increase in V_{max}. Several studies have addressed this question: Gastrocnemius muscle was assayed in an ATP regenerating system 48 hours after a single injection of T_3 (250 μg/100 g body weight) into hypothyroid rats (Asano *et al.*, 1976). There was no change in K_m and a significant increase in V_{max} of the Na^+,K^+-ATPase for ATP. Similarly in the renal cortex of the rat, after three doses of T_3 given on alternate days, the V_{max}'s for ATP, Na^+, and K^+ were increased by 134, 79, and 46%, respectively, and there were no significant changes in the K_m for ATP or the $K_{1/2}$ values for Na^+ and K^+ (Lo *et al.*, 1976). Kinetic analysis of the enzyme in rat heart yielded equivalent results. Administration of T_3 had no effect on the K_m for ATP or the $K_{1/2}$'s for Na^+ and K^+ but significantly increased the V_{max}'s for ATP, Na^+, and K^+ (Philipson and Edelman, 1977b). All these results suggest that thyroid hormone augments the complement of Na^+ transport units in target cells.

1. *Thyroid Hormone Induction of* Na^+,K^+*-ATPase*

The inference that thyroid hormone increases the number of Na^+ pump sites was tested using two methods to quantify the number of enzyme units: (1) Na^+- and Mg^{2+}-dependent incorporation of ^{32}P into the phosphorylated intermediate at the catalytic site of the Na^+,K^+-ATPase, using $AT^{32}P$, and (2) Na^+-, Mg^{2+}-, and ATP-dependent binding of [^{3}H]ouabain to the Na^+,K^+-ATPase. In rat kidney cortex there was a linear correlation between the specific activity of Na^+,K^+-ATPase activity (determined at V_{max}) and the amount of phosphorylated intermediate formed from thyroidectomized rats injected with T_3 (Lo *et al.*, 1976). Regression analysis of the dependence of Na^+, K^+-ATPase activity on the amount of ^{32}P bound revealed a 1:1 correspondence, implying that the entire effect is consequent to a corresponding increase in the number of pump sites. In these experiments there was also a linear correlation between the activity of Na^+,K^+-ATPase (V_{max}) and the amount of [^{3}H]ouabain specifically bound (Na^+-, Mg^{2+}-, ATP-dependent) to Na^+,K^+-ATPase. Similarly, in rat jejunal musosa, the maximum number of [^{3}H]ouabain-binding sites and Na^+,K^+-ATPase activity increased to similar extents after injection of T_3, with no change in the apparent [^{3}H]ouabain dissociation constant (K_d) (Liberman *et al.*, 1979). In this study, the amount of phosphorylated intermediate formed also correlated linearly with the Na^+,K^+-ATPase activity. Moreover, Lin and Akera (1978) confirmed that accumulation of Na^+,K^+-ATPase units accounts quantitatively for the T_3-induced increase in activity of the enzyme. Administration of T_3 to rats increased maximal specific binding of [^{3}H]ouabain, with no change in the K_d in a variety of tissues.

Three mechanisms could account for an increase in the number of functional Na^+ pump sites: (1) unmasking of latent Na^+ pump sites, (2) increased synthesis of the Na^+,K^+-ATPase enzyme, or (3) decreased rate of degradation of the enzyme. To distinguish between these possibilities estimates were made of the effects of T_3 on the synthesis and degradation of the subunits of Na^+,K^+-ATPase.

Analysis of the turnover of Na^+,K^+-ATPase was made possible by successful purification and identification of the subunits by SDS-polyacrylamide gel electrophoresis (Kyte, 1972; Hokin, 1974; Jorgensen, 1974). The enzyme consists of two subunits: one containing the (Na^+-Mg^{2+})-dependent, K^+-sensitive phosphorylation site (molecular weight ~95,000) and a smaller glycoprotein subunit (molecular weight ~50,000). The effect of T_3 on the synthesis and degradation of the major component (α subunit) of the Na^+,K^+-ATPase enzyme from renal cortex was first investigated by Lo and Edelman (1976), using T_3-treated thyroidectomized rats. Twenty hours after injection of T_3 (50 μg/100 g body weight) or diluent, the rats received ^{3}H- or ^{35}S-labeled methionine

TABLE II

Effect of T$_3$ and Reverse T$_3$ on Incorporation of [^{3}H]- or [^{35}S]Methionine into the Polypeptides of Partially Purified Renal Cortical Na$^+$,K$^+$-ATPase[a]

No. of experiments	Isotope combinations	Infusion/postinfusion times (hours)	Isotope band ratios		
			Band III / Band II	Band IV / Band II	Band IV / Band III
7	^{35}S/^{3}H	1/8	1.00 ± 0.04	1.03 ± 0.03	0.99 ± 0.02
	^{35}S (T$_3$)/^{3}H (T$_3$)				
7	^{35}S (T$_3$)/^{3}H		1.35 ± 0.05	1.36 ± 0.05	1.00 ± 0.01
	^{3}H (T$_3$)/^{35}S		$p < .005$	$p < .005$	N.S.[b]
8	^{35}S/^{3}H	1/22	0.99 ± 0.03	0.95 ± 0.03	0.95 ± 0.02
	^{35}S (T$_3$)/^{3}H (T$_3$)				
8	^{35}S (T$_3$)/^{3}H		1.48 ± 0.05	1.46 ± 0.06	0.97 ± 0.02
	^{3}H (T$_3$)/^{35}S		$p < .001$	$p < .001$	N.S.[b]
8	^{35}S/^{3}H	1/22	1.04 ± 0.03	1.04 ± 0.04	1.01 ± 0.04
	^{35}S (RT$_3$)/^{3}H (RT$_3$)				
7	^{35}S (RT$_3$)/^{3}H		1.03 ± 0.02	0.97 ± 0.02	0.97 ± 0.03
	^{3}H (RT$_3$)/^{34}S		N.S.[b]	N.S.[b]	N.S.[b]

[a] Surgically thyroidectomized rats were injected with one dose of T$_3$ or reverse T$_3$ (50 μg/100g body weight) or the diluent. Twenty hours after injection, [^{3}H]- or [^{35}S]methionine was infused at a constant rate for 1 hour. At various postinfusion times, the rats were killed and the kidneys pooled. The partially purified Na$^+$,K$^+$-ATPase was resolved by DodSO$_4$$^+$-PAG electrophoresis (9% total acrylamide). All values are mean ± SE. Reprinted from Lo and Lo (1980).

[b] p values are not significant.

administered as a constant infusion into the tail vein for 1 hour (in repeat series the isotopes were reversed). The kidneys were excised either 8 or 20 hours after T_3 infusion and pooled. T_3 augmented labeled methionine incorporation into the α-subunit by 44%, 8 hours after infusion of the labeled amino acid, and by 61%, 20 hours after infusion. Incorporation of methionine into two adjacent polypeptides in the SDS gels was not modulated by thyroid status. The magnitude of the T_3-dependent augmentation of methionine incorporation (+70%) into band III (identified as the α-subunit of Na^+,K^+-ATPase) was the same as the increase in renal cortical Na^+,K^+-ATPase activity measured in aliquots of the same preparations. Moreover, thyroid status had no effect on the rate constant of α-subunit degradation. Lo and Lo (1980) used a similar experimental design and confirmed the equivalence in T_3-dependent increases in renal cortical synthesis of the α-subunit and Na^+,K^+-ATPase activity. They also extended these studies to an analysis of the synthesis of the β-subunit (band IV) and found parallel effects (Table II). No changes were detected in the degradation rate constants, confirming the uniqueness of the action of T_3 on the biosynthesis of the Na^+,K^+-ATPase.

The specificity of the induction of Na^+,K^+-ATPase by T_3 was indicated by the failure of reverse T_3 to enhance methionine incorporation into either of the subunits (Table II). Moreover, neither T_3 nor reverse-T_3 altered the incorporation of labeled methionine into other membrane proteins.

2. *Possible Pathways in T_3 Induction of Na^+,K^+-ATPase*

Much evidence has accumulated that the tissue effects exerted by T_3 are initiated by binding to high affinity nuclear receptors (Oppenheimer *et al.*, 1972, 1974, 1966; Samuels and Tsai, 1974). This is consistent with the evidence that thyroid hormone is a potent regulator of RNA and protein synthesis (Tata, 1963; Tata and Widnell, 1966; Dillmann *et al.*, 1978). Recently, T_3 has been shown to induce specific mRNA's (Kurtz *et al.*, 1976; Martial *et al.*, 1977). These and related findings imply that the action of T_3 is initiated by binding to nuclear receptors and mediated by induction of specific mRNA's. This mechanism may also apply to thyroidal regulation of Na^+,K^+-ATPase and the associated effects on $QO_2(t)$. If the induction mechanism also mediates the thermogenic response to thyroid hormone, then similar degrees of nuclear receptor occupancy should elicit similar changes in various indices of response. This prediction was tested by infusing T_3 or $[^{125}I]T_3$ into hypothyroid rats and determining the relationships between binding of $[^{125}I]T_3$ to nuclear receptors and the increments in hepatic and renal cortical QO_2, $QO_2(t)$, Na^+,K^+-ATPase, and α-GPDH activity (Somjen *et al.*, 1981). Striking correlations were obtained between the respiratory and enzymatic responses and nuclear binding to T_3 (Fig. 7). These findings are consistent with the hypothesis of thyroidal induction of the Na^+ pump at the nuclear level.

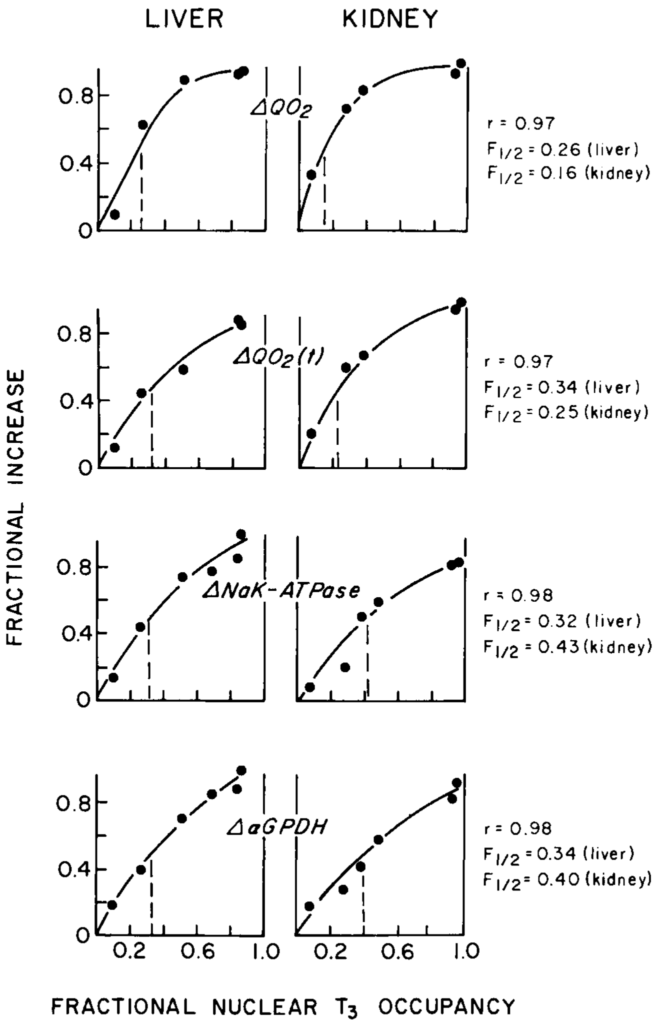

Fig. 7. Relationships between binding of $[^{125}I]T_3$ to the high affinity nuclear binding sites (N*) and the increments in hepatic and renal cortical QO_2, $QO_2(t)$, Na^+,K^+-ATPase, and α-GPDH. These curves are expressed in fractional responses [V_{max} = 1.0 and fractional occupancies (N_m = 1.0)]. r denotes the correlation coefficient for linear regression of the response in liver vs. kidney; $F_{1/2}$ is the fractional nuclear T_3 occupancy for a fractional response of 0.5. Reprinted from Somjen *et al.* (1981).

D. Mitochondrial Involvement in Thyroid Thermogenesis

The available evidence indicates that thyroid hormone increases energy utilization by the Na^+ pump, mediated by enhanced synthesis of Na^+,K^+-ATPase. Ouabain or Na^+-free media has been used to demonstrate the pacemaker activity of Na^+ transport activity with respect to mitochondrial aerobic respiration. Recently, Balaban *et al.* (1980), using fluorometry, provided data supporting the role of Na^+ transport as a determinant of mitochondrial respiratory rates via changes in cellular ATP/ADP ratios [as originally proposed by Whittam (1961)]. If mitochondrial ATP synthesis remains coupled to respiration, in the steady state, a sustained thermogenic response would not be primarily attributable to an increase in mitochondrial oxidative capacity, since the ADP P_i/ATP ratio will determine QO_2. Thyroidal enhancement of ATP utilization by active Na^+ transport, however, would be augmented by an increase in mitochondrial oxidative phosphorylative capacity, as implied by previously reported increases in the number, size, and cristae of skeletal muscle mitochondria in response to thyroid hormone (Tata *et al.*, 1963; Gustafsson *et al.*, 1965; Winder *et al.*, 1975; Winder and Holloszy, 1977; Nishiki *et al.*, 1978; Barsano *et al.*, *1977; Herd et al.*, 1974). The mitochondrial hyperplasia is accompanied by increases in a variety of mitochondrial enzymes, and components including cytochrome *c*, cytochrome oxidase, α-GPDH, malate dehydrogenase, and the adenine nucleotide transport system (Booth and Holloszy, 1975; Oppenheimer, 1979; Babior *et al.*, 1973).

Since mitochondria contain DNA and have the capacity for both RNA and protein synthesis, the possibility of thyroidal induction of both intrinsic mitochondrial proteins and the complement of mitochondrial enzymes that are synthesized extramitochondrially deserves serious consideration. The detection of high affinity binding sites for T_3 in mitochondria (Sterling and Milch, 1975; Greif and Sloane, 1978) raises the possibility of direct modulation of mitochondrial gene expression by T_3.

The majority of mitochondrial proteins are encoded by nuclear genes, synthesized on cytoplasmic ribosomes, and subsequently transported into the mitochondria (e.g., α-GPDH). To date, only 10–12 proteins are known to result from mitochondrial protein synthesis and these comprise hydrophobic polypeptides of the inner mitochondrial membranes (for reviews, see Tzagoloff 1977; Schatz and Mason 1974; Tzagoloff *et al.*, 1973; Tzagoloff and Macino, 1979). Of those polypeptides identified, most are subunits of mitochondrial enzymes, and their synthesis by the mitochondria shows a dependence on the presence of cytoplasmically synthesized subunits. Mitochondrial synthesis is responsible for 3 of the 6 polypeptide subunits of cytochrome *c* oxidase, the cytochrome *b* subunit of coenzyme QH_2–cytochrome reductase, and 2–4 of the 10 polypeptides of oligomycin-sensitive ATPase. The rate and extent of mitochondrial protein synthesis are influenced by cytoplasmic translation products. Most cytoplasmic products, however, including those that are destined to be integrated into enzymes containing mitochondrially derived subunits, are synthesized at near normal levels when mitochondrial synthesis is inhibited (Tzagoloff *et al.*, 1973; Tzagoloff and Macino, 1979).

Recently, Nelson *et al.* (1980) prepared isolated rat hepatocytes from hypothyroid rats 24 hours after injection of T_3 or diluent, and assayed the incorporation of radiomethionine into mitochondrially encoded peptides. T_3 induced ~2.5-fold increases in the labeling of four of the mitochondrially translated proteins, implying a generalized increase in translation/transcription of endogeneous mitochondrial proteins.

In many target tissues, T_3 exerts hyperplastic and hypertrophic influences on mitochondrial populations. These effects are probably mediated by coordinate regulation of nuclear and mitochondrial gene expression. Further studies are needed on the molecular mechanisms operative in both pathways and on the role of induction of mitochondrial proteins in the thermogenic response.

III. THYROID INVOLVEMENT IN COLD ACCLIMATION

Thyroidectomy markedly reduces the ability to tolerate sudden decreases in ambient temperature. For example, the time for body temperature to drop to 30°C when guinea pigs are exposed to −1.5°C is 122 minutes in intact animals, but 49 minutes in thyroidectomized animals (Pichoyka *et al.*, 1953, cited by Laites and Weiss, 1959). Moreover, thyroidectomized rats maintained at low ambient temperatures will die within hours unless exogenous T_4 or T_3 is administered. Carlson (1960) found that thyroidectomy causes a gradual reduction of cold induced nonshivering thermogenesis (NST) (i.e., the increase in metabolic rate as a result of exposure to cold that does not involve shivering). Conversely, LeBlanc and Villemaire (1970) reported that injecting rats daily with 10 μg of T_4 for 35 days improved cold tolerance, as measured at −25°C. However, Cottle (1960) noted that 24 μg of T_4 injected daily for 3 weeks did not increase survival of rats that were maintained at 5°C.

Another approach has been to study the effects of cold acclimation on metabolism of thyroid hormone and physiology of the thyroid gland. Thus, Woods and Carlson (1956) found that the amount of exogenous T_4 needed to prevent thyroid hypertrophy in partially thyroidectomized rats at 5°C was at least twice as much as that required at room temperature, implying an environmental drive to release of TSH. Similarly, Jobin *et al.* (reported in LeBlanc, 1971) noted that plasma TSH is higher in rats kept at 5°C for 32 days than in controls. They further noted that plasma thyroxine is lower in cold-adapted animals; but, the secretion rate of T_4 is comparable in both groups, this being due to a greater metabolic clearance rate after cold acclimation. Oppenheimer and his co-workers (1970; Schwartz *et al.*, 1971) found that, in the rat, approximately 20% of the total body T_3 is derived from deiodination of T_4. Since T_3 has been estimated to have 3–5 times greater biological activity than T_4, this conversion could be important in thyroid hormone activity. Accordingly, Bernal and Escobar del Rey (1975) investigated the effect of cold exposure on extrathyroidal conversion of T_4 to T_3 and on intramitochondrial α-GPDH activity in thyroidectomized rats injected with iodine labeled thyroxine. The cold exposed rats exhibited a decreased percentage of incorporation of total radioiodine into T_4 in the carcass, various organs, and the plasma, and a significant increase in labeling of T_4-derived T_3. The T_3/T_4 ratios increased more than twofold in the cold-exposed rats. The absolute and relative increase in tissue T_3 content was accompanied by parallel increases in α-GPDH activity. Similarly, Balsam and Leppo (1974) exposed rats to cold for 3–5 months and noted that the tissue-plasma concentration gradients of radioactive T_3 in liver, kidney, and muscle were all significantly increased in cold-exposed animals. Balsam and Sexton (1975) also reported enhanced conversion of T_4 to T_3 in rats exposed to cold for only 2 weeks. These findings imply a significant role for thyroid hormone in the NST component of the adaptation to cold.

Cellular Mechanisms Mediating Nonshivering Thermogenesis

No single mechanism appears to account for NST at the tissue or cellular level. Moreover, the nature of this regulatory response could vary from tissue to tissue (for a review of NST, see Jansky, 1973). Earlier studies related NST of cold-acclimated animals to increased metabolism that was demonstrable in excised tissues measured *in vitro* (You and Sellers, 1951; Weiss, 1954, 1957; Davis *et al.*, 1960). Recent reports documented enhanced Na^+ transport-dependent respiration in tissues from cold acclimated mice, rats, and hamsters (Stevens and Kido, 1974; Videla *et al.*, 1975; Guernsey and Stevens, 1977; Horwitz and Eaton, 1977; Guernsey and Whittow, 1981). The estimated increases in QO_2 and $QO_2(t)$ of total skeletal muscle, kidney, and liver in rats exposed to cold for up to 50 days are shown in Fig. 8 (Guernsey and Stevens, 1977). The shaded area represents that portion of the increase in tissue QO_2 that is attributable to enhanced Na^+ transport-dependent respiration, $QO_2(t)$. In intact rats, NST is not activated until day 5 or 6 of cold exposure and reaches maximum levels by day 9 or 10 (shivering thermogenesis is the major mechanism of heat production during this time) (Depocas, 1959; Bartunkova *et al.*, 1971; Davis *et al.*, 1960). This pattern corresponds closely to the tissue responses; QO_2 and $QO_2(t)$ (Fig. 8). Enhanced active Na^+ transport, therefore, qualifies as a major mechanism in the thermoregulatory response to cold. Support for this suggestion was derived from

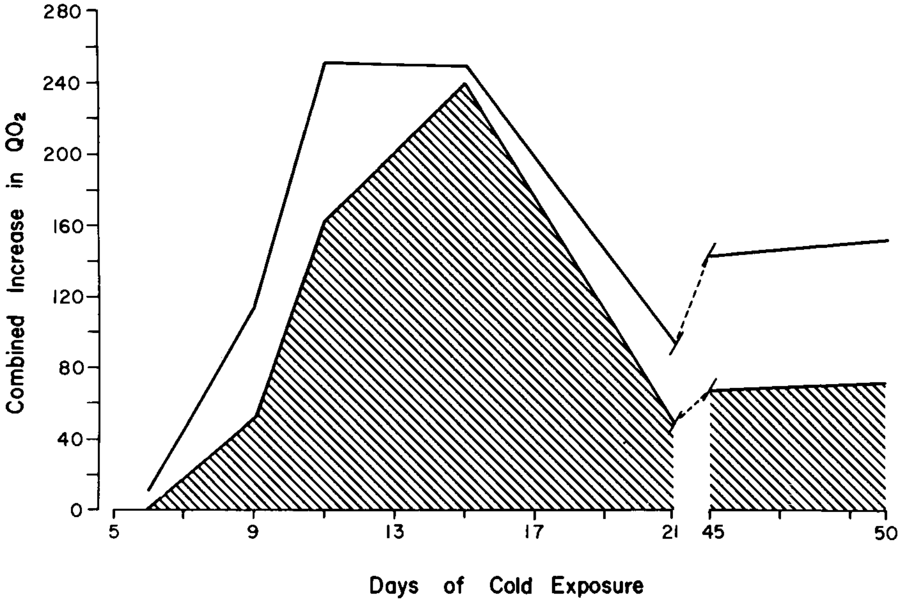

Fig. 8. The combined increase of total oxygen consumption (microliters of O_2 per hour per body weight$^{3/4}$) due to cold exposure calculated for the entire skeletal muscle mass, liver, and kidneys of animals. The shaded portion is that fraction of the increased oxygen consumption that is attributible to increased $QO_2(t)$. These data were determined by (1) calculating the change in QO_2 due to cold exposure for each tissue at each period on the basis of wet weight of tissue, (2) determining the percentage weight contribution of each tissue mass to the mean body weight of the group, and (3) calculating the metabolic increase for each of the three tissue masses and summing these values for each period of exposure. Reprinted from Guernsey and Stevens (1977).

studies on genetically obese (*ob/ob*) mice. The *ob/ob* mouse exhibits an impaired thermogenic response on acute exposure to cold (Davis and Mayer, 1954; Trayhurn and James, 1978), and both $QO_2(t)$ and the abundance of Na^+,K^+-ATPase units are reduced in skeletal muscle and liver (Guernsey and Morishige, 1979; Ramsos, 1981).

Convincing evidence that thyroid hormone is responsible for the increased QO_2 and $QO_2(t)$ of tissues from cold-acclimated mammals has not yet appeared. Cold-acclimated rats, however, are characterized by an increase in basal metabolic rate that is similar in magnitude and time course to the increases in tissue QO_2 (LeBlanc and Cote, 1967; Stromme and Hammel, 1967; Jansky *et al.*, 1967; LeBlanc and Villemaire, 1970; Guernsey and Whittow, 1981). Thus, the possibility of an increase in the effective intracellular T_3 concentration on exposure to cold deserves further consideration. Recently, Himms-Hagen (1976) has suggested that cold-acclimated rats do not exhibit a higher basal metabolic rate compared to controls. This suggestion was based on a study by Hsieh

(1963), which reported that cold-acclimated rats given sufficient time to reach a steady state after transfer to 25°–28°C for measurement demonstrated no difference in basal oxygen consumption compared to warm controls. However, a great many studies (including those listed above) report a significantly higher basal metabolic rate of cold acclimated rats when given time to reach steady state and when the measurements were determined between 25° and 28°C (instead of 30°C). Therefore, the preponderance of available data suggests that acclimation of rats to cold temperatures results in an increased basal metabolic rate.

Some data in the literature implicate coordinate actions of thyroid hormone and norepinephrine in the thermogenic response during cold acclimation (Cassuto and Amit, 1968; Tanche and Therminarias, 1969; Swanson, 1957; Harrison, 1964). For example, rats pretreated daily with T_4 (10 μg) for 35 days exhibited improved tolerance to acute exposure to cold (−25°C) (LeBlanc, 1971). This improvement was greater than pretreatment with norepinephrine (30 μg/100 g/day). Simultaneous administration of T_4 and norephinephrine, however, resulted in even greater tolerance to acute cold exposure and the combined effect was nearly equal to the cold tolerance of cold-acclimated rats. Fregly *et al.* (1976, 1979) also found that thyroid hormone augmented the thermogenic response to catecholamines in warm-adapted rats and that cold-acclimated rats demonstrated an increased responsiveness to exogenously administered β-adrenergic catecholamines without pretreatment with thyroid hormone.

The synergistic thermogenic effects of thyroid hormone and norepinephrine may be mediated to a significant extent by the Na^+,K^+-ATPase system. Horwitz *et al.* (1969) and Horowitz *et al.* (1971) noted that in brown fat, *in vitro,* norepinephrine increases cell membrane permeability to Na^+. The resultant increase in intracellular Na^+ concentration apparently stimulates Na^+ pump activity. Similarly, norepinephrine appears to increase Na^+ permeability of skeletal muscle plasma membrane, as judged by a fairly rapid depolarization response (Horowitz *et al.*, 1976; Dockry *et al.*, 1966; Teskey *et al.*, 1975; Turkanis, 1969). Increased activity of ouabain-sensitive Na^+,K^+-ATPase has been observed following the addition of norepinephrine to membrane fractions prepared from brown adipocytes (Herd *et al.*, 1970). Additionally, the calorigenic effect of norepinephrine is inhibited 60% in hamster brown fat (Horwitz, 1973) and rat brown fat (Herd *et al.*, 1973) in the presence of ouabain. Moreover, several studies documented direct stimulation, by norepinephrine of Na^+,K^+-ATPase activity and Na^+/K^+ transport in rat skeletal muscles (Rogus *et al.*, 1977; Cheng *et al.*, 1977; Clausen and Hansen, 1977; Clausen and Flatman, 1977). Rogus *et al.* (1977) postulated that β-adrenergic stimulation of coupled Na^+-K^+ transport requires the intracellular synthesis of a mediating factor induced by cyclic AMP.

Further studies may reveal a dual participation of thyroid hormone and norepinephrine, at the cell membrane level, as mediators of enhanced metabolic heat production during cold adaptation. Particular issues deserving of further analysis include the possibility of enhanced synthesis of active Na^+ transport units in cold acclimation and whether norepinephrine modulates these units by a direct or indirect mechanism.

IV. THE GENETICALLY OBESE (*ob/ob*) MOUSE

Genetically obese (*ob/ob*) mice are a well-studied model of obesity, similar in many respects to hyperplastic human obesity (Bray and York, 1971). It has been postulated that obesity results from a greater-than-normal efficiency in storing dietary energy associated with a subnormal metabolic rate rather than an elevated food intake (Miller, 1974; James and Trayhurn, 1976; Djazayery *et al.*, 1979; Ramsos, 1981). The *ob/ob* mouse is hypothermic and hypometabolic at normal ambient temperatures and succumbs within hours after acute exposure to cold (i.e., 5°C), as a result of hypothermia (Mayer *et al.*, 1952; Davis and Mayer, 1954; Mayer and Barrnett, 1953). The combination of decreased capacity for NST and the supernormal efficiency in conservation of dietary energy may be a consequence of a generalized decrease in energy utilization for thermoregulatory thermogenesis (Trayhurn *et al.*, 1977; Trayhurn and James, 1978). It is of interest that Jung *et al.* (1979) recorded a reduced NST capacity in human subjects with familial obesity.

Since active sodium transport has been implicated as an important effector in both thyroid thermogenesis and in NST, there has been an interest in cellular Na^+ pump activity in obese mice (Ismail-Beigi and Edelman, 1970, 1971; Guernsey and Stevens, 1977). Na^+,K^+-ATPase activity as well as the number of Na^+,K^+-ATPase units are abnormally low in the tissues of *ob/ob* mice (York *et al.*, 1978; Guernsey and Morishige, 1979; Lin *et al.*, 1978, 1979a). These results correlate with reduced tissue QO_2 and a significantly decreased $QO_2(t)$ in liver and skeletal muscle (Conway and Kaplan, 1977; Guernsey and Morishige, 1979). Thus, the greater-than-normal metabolic efficiency of the *ob/ob* variant may be a result, at least in part, of reduced active Na^+ transport and a consequently decreased thermogenic capacity (York *et al.*, 1978; Lin *et al.*, 1978; Guernsey and Morishige, 1979).

There has been conflicting data regarding thyroid hormone induction of Na^+,K^+-ATPase in the *ob/ob* mouse. York *et al.* (1978) reported that administration of T_3 did not increase hepatic Na^+,K^+-ATPase activity of the *ob/ob* mouse (12 weeks old) but did so in the lean littermate controls. In contrast, Lin *et al.* (1979b) found that administration of T_4 increased the number of Na^+,K^+-ATPase enzyme units in liver, skeletal muscle, and kidney of 6-week-old *ob/ob* mice.

Several features of these experiments may account for the differences in the recorded results, including: the ages of the subjects, the use of T_3 vs. T_4, dosages, and duration of treatment. For example, acute exposure to cold (3°–5°C) is rapidly lethal in the adult but not in the young (5 weeks old) *ob/ob* mouse (Lin *et al.*, 1980). In addition, exposure to 14°C for 3 weeks evoked significant increases in the abundance of Na^+,K^+-ATPase units of various tissue in 6-week-old *ob/ob* mice (Lin *et al.*, 1980). It is possible, therefore, that in the immature stages, the *ob/ob* mice are normally responsive to T_3 or T_4 and that during maturation, this response (i.e. induction of Na/K-ATPase) is lost in some tissues. Alternatively, the *ob/ob* variant may be paradoxically more sensitive to T_4 than to T_3, or may require larger doses for longer periods to elicit the same response as in their lean counterparts. The answers to some of these unresolved questions may be derivable from studies on the nuclear events in the induction of Na^+,K^+-ATPase by thyroid hormone and cold exposure in young and old *ob/ob* mice. It is noteworthy that adult *ob/ob* mice maintained at 25°C have significantly reduced T_3 nuclear binding capacities, concomitant with subnormal QO_2(t) and Na^+,K^+-ATPase activity in liver and skeletal muscle (Guernsey and Morishige, 1979).

V. POSSIBLE ROLE OF Na^+ TRANSPORT AND THYROID HORMONE IN THE EVOLUTIONARY TRANSITION FROM POIKILOTHERMY TO HOMEOTHERMY

The implication of Na^+,K^+-ATPase in thyroid thermogenesis and thermoregulatory NST prompted exploration of the possibility that thyroid hormone modulation of Na^+ transport could have played a major role in the evolutionary transition from poikilothermy to homeothermy (Stevens, 1973; Edelman, 1976; Rossier *et al.*, 1979).

Stevens (1973) suggests that thermoregulatory responses to cold and to thyroxine and the Na^+ pump are related functionally and phylogentically. Fish regulate their body temperature by variations in physical exertion. This increased activity demands increased oxygen consumption with subsequent transfer of ions across the gill membranes, resulting in obligatory stimulation of the Na^+ pump. According to Stevens (1973) the evolution of NST (endothermy) in fish and other poikilotherms was accomplished by direct stimulation of the Na^+ pump to produce heat; and thyroxine is the most likely controlling mechanism. The thermogenic response to thyroid hormone, however, was recruited at the level of poikilothermic vertebrate evolution and may be utilized in an enhanced functional capacity in homeotherms simply because of higher body temperatures (Edelman, 1976). This suggestion is based on (1) the thermogenic response to thyroid hormone is present in several poikilothermic vertebrates (Maher and Levedahl, 1959; Maher, 1965; Packard *et al.*, 1974; Packard and Packard, 1973;

Lagerspetz *et al.*, 1974), and (2) the temperature dependence of the thermogenic action of thyroid hormone is very steep. For example, in rat liver, the increase in $QO_2(t)$ accounts for only 58% of the T_3-induced increase in QO_2 when assayed at 31°C; but when assayed at 37°C, $QO_2(t)$ accounts for 90% of the enhanced QO_2. Thus, the enhanced thermogenesis derived from Na^+ transport in homeotherms may be a consequence of the positive temperature dependence of active Na^+ transport and the acquisition of hypothalamic mechanisms for controlling heat loss.

Rossier *et al.* (1979) reported that in the liver and urinary bladder of the toad the thyroxine-dependent increases in QO_2 are insensitive to ouabain, suggesting that Na^+ transport is not the mediator of enhanced QO_2. They suggest that one of the steps in the transition from poikilothermy to homeothermy involves the establishment of thyroid hormone control of Na^+,K^+-ATPase gene expression, mediating an increased complement of Na^+ pumps in the homeotherm cell. Clearly, further studies are needed on the mediating events in thyroid thermogenesis of poikilothrems in order to elucidate the probable pathways in vertebrate evolution.

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11

The Effects of Thyroid Hormone on Adrenergic Receptors

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I. INTRODUCTION

Long before our current concepts of adrenergic receptors emerged, the striking similarity between many of the clinical manifestations of thyroid hormone excess and the effects of catecholamine administration had suggested an important relationship between thyroid hormones and the sympathetic nervous system (Kraus and Friedenthal, 1908). Early observations that sympathetic blockade by spinal anesthesia (Knight, 1945) or by depletion of intraneuronal catecholamine stores by reserpine (Brewster *et al.*, 1956) could ameliorate the tachycardia, tremor, and hyperactivity noted in patients with hyperthyroidism lent further support to such a relationship. However, despite decades of investigation and the

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development of powerful new techniques for the analysis of hormone action, the precise nature of the interactions between thyroid hormones and catecholamines remains incompletely understood.

Several different lines of research have been pursued in attempts to elucidate the role of the adrenergic nervous system in mediating the biological effects of thyroid hormone. Most studies have addressed one of the following questions:

1. Are adrenergic mechanisms necessary for the physiological effects of thyroid hormone to be expressed, or do adrenergic mechanisms alter thyroid hormone metabolism?
2. Do thyroid hormones alter plasma or tissue levels of catecholamines, or kinetic aspects of catecholamine metabolism?
3. Do thyroid hormones alter physiological responsiveness to adrenergic agonists or antagonists?
4. Do thyroid hormones alter biochemical responses to adrenergic stimulation, such as activation of adenylate cyclase?
5. Do thyroid hormones affect adrenergic receptors as assessed directly by radioligand binding techniques?

In regard to this last question, the ability to assess the properties of hormone receptors by radioligand binding methods has led to important observations regarding the biochemical mechanisms of physiological phenomena in several areas. Certain disease states, such as familial hypercholesterolemia (Ho *et al.*, 1977) and one form of insulin resistant diabetes mellitus (Flier *et al.*, 1979), represent genetic deficiencies of the cell surface receptors for low density lipoproteins and insulin, respectively. Another form of receptor mediated pathophysiology occurs in myasthenia gravis, where antibodies directed against the acetylcholine receptors of skeletal muscle produce the clinical disorder, probably by accelerating rates of receptor degradation (Drachman *et al.*, 1978). Yet another mechanism whereby altered receptor function may provide the molecular basis for physiological phenomena is the now well-described receptor regulation by homologous hormones. The insulin resistance of obesity appears to be, at least in part, a function of a reduction in the number (down regulation) of insulin receptors induced by high levels of circulating insulin (Harrison *et al.*, 1976). This phenomenon of a hormone exerting a regulatory influence on the properties of its own cell surface receptors occurs for β-adrenergic receptors as well. "Desensitization" of β-adrenergic receptors by adrenergic agonists has been conclusively demonstrated in the frog erythrocyte system (Lefkowitz and Williams, 1978), but also appears to occur in man (Galant *et al.*, 1978).

A fourth mechanism, and the one directly relevant to the discussion of thyroid–catecholamine interactions, is the regulation of hormone receptors by heterologous hormones, that is, changes in one class of hormone receptors induced by hormones or pharmacological agents that do not bind directly to the receptors being altered (Lefkowitz, 1979; Snyder, 1979).

Both theoretical considerations and experimental evidence support the conclusion that changes in the number of membrane receptors can mediate changes in tissue sensitivity to a drug or hormone. A change in receptor number can be reflected either as a change in the maximum physiological response to hormone stimulation, or as a shift in the dose–response curve (the concentration of hormone or drug necessary to produce a given level of response), depending on the manner in which the membrane receptors are coupled to the subsequent effector mechanisms producing the physiological response. As illustrated in Fig. 1A, in a tightly coupled system lacking "spare receptors," an increase in the number of receptors would lead to an increased maximal response, whereas in the system with "spare receptors" shown in Fig. 1B, an increase in the number of receptors would produce an increased sensitivity of the biological response, indicated by a lower dose of agonist necessary to produce a half-maximal response.

Since changes in the properties of adrenergic receptors represent at least one potential molecular mechanism for the apparent state of β-adrenergic excess associated with hyperthyroidism (and the apparently diminished β-adrenergic tone associated with hypothyroidism), considerable interest has been focused on this issue, and this literature will be reviewed in detail. However, to put the role of the effects of thyroid hormones on adrenergic receptors into proper biological perspective, it should be recognized that alterations in adrenergic receptors, per se, represent only one of many possible explanations for the thyroid–catecholamine interaction.

Thyroid hormones could change the apparent state of adrenergic activity observed in an animal or man by inducing alterations at any of several points in the complex chain of biochemical events occurring between central stimulation of sympathetic preganglionic neurons and the eventual biological sequelae noted in responsive tissues. For example, using heart rate as an example of a physiological response in which excessive levels of thyroid hormone induce an apparent state of β-adrenergic excess, this effect of thyroid hormone could be mediated by a change in any one of the following:

1. The rate of nerve traffic in efferent sympathetic fibers leaving the rostral brainstem, synapsing with the postganglionic fibers in the sympathetic ganglia, and proceeding via the cardioaccelerator nerve to the specialized pacemaker tissue of the sinus node.
2. The rate of nerve traffic inducing catecholamine release from the adrenal medulla.
3. The amount of catecholamine released from the cardiac postganglionic neurons or from the adrenal medulla by any given rate of nerve firing.

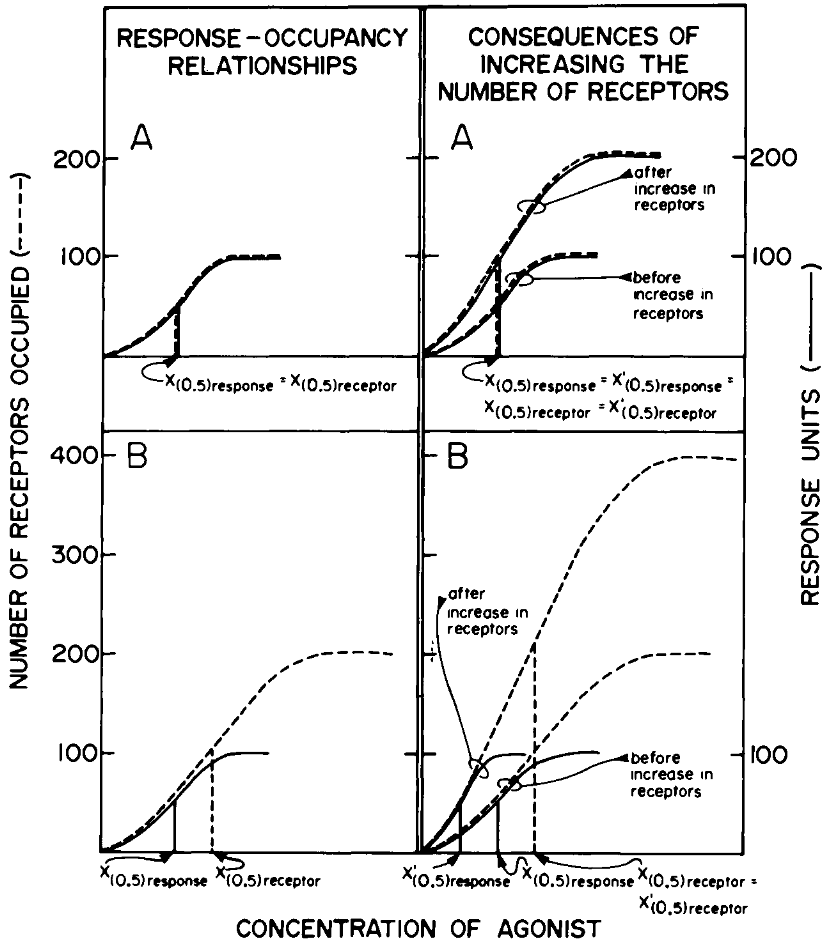

Fig. 1. Effects of response–occupancy relationships on the consequences of increasing the number of receptors. (Left) The biological response is plotted in arbitrary response units as a solid line and the number of receptors occupied (in arbitrary units) in dashed lines. A depicts the situation in the absence and B in the presence of "spare receptors." (Right) These curves are replotted (designated as "before increase in receptors"), and the occupancy and response that would be predicted from a twofold increase in receptor number are also plotted (designated as "after increase in receptors"). Measured on the abscissa is the concentration of agonist. The concentration of agonist giving a half-maximal response is designated as $^{x}(.5)$ response, and the concentration giving 50% receptor occupancy is designated as $^{x}(.5)$ receptor. Primed numbers (for example, $^{x'}(.5)$ response) refer to the corresponding concentrations after an increase in receptor number has occurred. With permission from L. T. Williams and Lefkowitz (1978). "Receptor Binding Studies in Adrenergic Pharmacology," Raven Press, New York.

4. The processes regulating the amount of norepinephrine released by sympathetic nerve stimulation which is available for binding to the postsynaptic adrenergic receptors, such as catechol-*O*-methyltransferase activity or norepinephrine reuptake channels in the synaptic cleft.
5. The number of β-adrenergic receptors located on the cardiac cells, or the affinity of those receptors for adrenergic agonists.
6. The coupling of receptor occupancy of β-adrenergic receptors to the immediate biochemical sequelae of receptor stimulation, such as activation of adenylate cyclase.
7. Other biochemical processes downstream in the cascade of intracellular events occurring between agonist binding to cell surface receptors and the eventual physiological response of increased heart rate, such as activation of protein kinases by cAMP, or phosphorylation of enzymes mediating ion flux.
8. Mechanisms distinct from the β-adrenergic receptor which modulate heart rate, changes in which could be interpreted as alterations in adrenergic responsiveness. For cardiac pacemaker cells the level of cholinergic tone, the activity of cAMP phosphodiesterases, the activity of processes mediated by α-adrenergic receptors, or the activity of ionic channels affecting membrane potentials or intracellular cation concentrations could serve as examples.

In the sections to follow, we shall review several of the areas outlined in the preceding discussion where experimental data exist regarding the effects of thyroid hormone on adrenergic receptors.

II. EFFECTS OF THYROID HORMONE ON ADRENERGIC RECEPTORS IN THE HEART

A. General Considerations

The changes in cardiac function observed with altered thyroid states are among the most clinically prominent aspects that occur. The sinus tachycardia, susceptibility to arrhythmias, and augmented contractility occurring with hyperthyroidism are directly analogous to the effects of cardiac sympathetic nerve stimulation or infused epinephrine. In addition, the chronotropic alterations of hyperthyroidism, if not the inotropic, are significantly ameliorated by β-adrenergic blockade (Grossman *et al.*, 1971a; Lewis *et al.*, 1979; Levey, 1975; Amidi *et al.*, 1968). Conversely in hypothyroidism, bradycardia and reduced inotropic state are noted, suggesting a diminished β-adrenergic influence over cardiac function (Harrison, 1964). These observations are uniformly accepted and represent the major physiological phenomena that led to the hypothesis that altered adrenergic receptors may mediate, at least in part, the effects of thyroid hormone on cardiac function.

There is general agreement in the literature on three other important points regarding the thyroid–catecholamine interaction. First, the changes in plasma catecholamines occurring with hyperthyroidism and hypothyroidism are in the opposite direction from those expected if thyroid hormone augmented neuronal release of catecholamines from sympathetic nerve terminals or from the adrenal medulla. Plasma norepinephrine levels are either low or normal in subjects with hyperthyroidism while norepinephrine levels are almost uniformly elevated in hypothyroidism, and plasma epinephrine levels are largely unaltered by thyroid state (Christensen, 1972, 1973; Gione *et al.*, 1974; Stoffer *et al.*, 1973; Columbe *et al.*, 1977). Urinary excretion of epinephrine, norepinephrine, metanephrines, and VMA are similarly normal or low in hyperthyroidism, and increased in hypothyroidism (Bayliss and Edwards, 1971; Kuschke *et al.*, 1960; Wiswell *et al.*, 1963). Furthermore, dopamine β-hydroxylase activity in plasma has been found to be reduced in hyperthyroidism and augmented in hypothyroidism (Nishizawa *et al.*, 1974; Noth and Spaulding, 1974). These data fail to support either the hypothesis that thyroid hormones produce accelerated rates of sympathetic nerve traffic, or that thyroid hormones accentuate the release of catecholamines from sympathetic neurons at any given neuronal firing rate. A second major point of agreement is that adrenergic blockade in hyperthyroidism does not produce its physiological effects by alterations in thyroid hormone biosynthesis, release, or peripheral metabolism. Adrenergic innervation of intrinsic thyroid microvasculature and of thyroid follicles does exist (Melander *et al.*, 1974), and adrenergic control of thyroid hormone production and release (by both α- and β-adrenergic mechanisms) has been described (Maayan *et al.*, 1971; Melander *et al.*, 1972), but adrenergically mediated release of thyroid hormones is not a mechanism of hyperthyroidism. Although β-adrenergic antagonists have been reported to impair the peripheral monodeiodination of thyroxine (Verhoeven *et al.*, 1977), this effect exerts only minor changes in the overall levels of T_3 and T_4 encountered in hyperthyroidism (Harroner *et al.*, 1977; Saunders *et al.*, 1978; Verhoeven *et al.*, 1977). In addition, patients treated with sympatholytic drug therapy for hypertension do not develop hypothyroidism.

The third major point of agreement is that thyroid hormones do not bind directly to β-adrenergic receptors. T_4 does not displace radiolabeled adrenergic compounds from β-adrenergic receptors in cardiac membrane preparations (Williams *et al.*, 1977). Although thyroid hormones have been reported to stimulate myocardial adenylate cyclase (Levey and Epstein, 1969), this effect is not blocked by propranolol, or diminished by reserpine or guanethidine (Klein *et al.*, 1971). In addition, the biological significance of this direct stimulation of myocardial adenylate cyclase is uncertain, since plasma or tissue levels of cAMP are not elevated in hyperthyroidism (Guttler *et al.*, 1977; McNeill *et al.*, 1969; Frazer *et al.*, 1969), and neither T_4 nor T_3 acutely alters contractility (Skelton *et al.*, 1973) or intrinsic sinus rate (Wahlberg *et al.*, 1977) in isolated heart preparations.

There is, thus, general agreement on these three points: (1) the apparent state of β-adrenergic excess that exists in hyperthyroidism occurs in the context of diminished levels of circulating catecholamines (and the apparently diminished level of β-adrenergic activity seen in hypothyroidism occurs in the context of elevated plasma catecholamines); (2) the ameliorative effects of β-adrenergic blockade occur without major changes in the elevated levels of T_4 and T_3 encountered in hyperthyroidism; and (3) thyroid hormones do not bind directly to β-adrenergic receptors. These findings have fostered the notion that changes in adrenergic receptors might be an important mechanism of the cardiac manifestations of altered thyroid states. What then is the evidence that such changes occur? This issue has been addressed on three levels: (1) changes in physiological responsiveness to adrenergic stimulation, (2) changes in biochemical responsiveness to adrenergic stimulation, and (3) changes in adrenergic receptors assessed directly by radioligand binding.

B. Physiological Responses

The literature addressing physiological responses is extensive and clouded by numerous conflicting reports, as well as by general problems regarding the definition of subsensitivity and supersensitivity of physiological and biochemical responses to catecholamines. For any given biological variable in which the base line measurements in altered thyroid states are different from the control state (such as heart rate or contractility), different interpretations of the same data can be obtained depending on whether the response to adrenergic stimulation is expressed in absolute terms, or as a percentage of the base line level or of the maximal response. This ''law of initial values'' clouds much of the literature regarding the thyroid–catecholamine interaction, but no entirely satisfactory analytical approach has been devised to deal with the problem.

Most recent reviewers have concluded that cardiac sensitivity to catecholamines is unaffected by alterations in thyroid state (Levey, 1971; Spaulding and Noth, 1975; Skelton and Sonnenblick, 1978; Lansberg, 1978). However, reports prior to 1965 largely observed that heart rate and blood pressure responses to catecholamines were enhanced in hyperthyroidism (Goetsch, 1918; Rosenblum *et al.*, 1933; Sawyer and Brown, 1935; McDonald *et al.*, 1935; Hoffman *et al.*, 1947; Schneckloth *et al.*, 1953; Brewster *et al.*, 1956; Murray and Kelley, 1959; Harrison, 1964; Waldstein, 1966) and decreased in hypothyroidism (Eppinger and Levine, 1934; Sawyer and Brown, 1935; Hoffman *et al.*, 1947; Catz and Russell, 1961; Harrison, 1964; Waldstein, 1966), and an augmented cardiac response to epinephrine was even suggested as a diagnostic test for hyperthyroidism (Murray and Kelley, 1959). These early studies have been criticized on methodological grounds for their lack of rigorous controls, failure to study dose–response relationships, and inadequate statistical analyses.

There are numerous studies that fail to demonstrate altered catecholamine sensitivity of cardiac rate or contractility with altered thyroid state (Van der Shoot and Moran, 1965; Cravey and Gravenstein, 1965; Wilson *et al.*, 1966; Levey *et al.*, 1969; Cariroli and Crout, 1967; Goodkind, 1969; McDevitt *et al.*, 1978; Leak and Lew, 1963; Benforado and Wiggins, 1965; Hagino and Shigei, 1976). Frequently quoted studies include those of Buccino *et al.* (1967), Margolius and Gaffney (1965), Young and McNeil (1974), and Aoki *et al.* (1967, 1972). Buccino reported no change in the response of developed tension or rate of tension development to norepinephrine in isolated papillary muscles from hyperthyroid cats. Margolius observed no altered responsiveness of heart rate in anesthesized hyperthyroid dogs to either infused norepinephrine or to direct cardioaccelerator nerve stimulation. Young reported unaltered contractile responses to norepinephrine in isolated perfused hearts from hyperthyroid rats, and Aoki reported unaltered heart rate or blood pressure responses to catecholamines in normal volunteers treated with T_3 (Aoki *et al.*, 1967), or in patients with spontaneous hyperthyroidism (Aoki *et al.*, 1972).

On the other hand, several groups continue to report shifts in the dose–response curves for catecholamine stimulation of cardiac function with changes in thyroid state. Wildenthal (1972) observed an increased sensitivity of the chronotropic response to β-adrenergic stimulation in isolated fetal mouse hearts exposed to T_3 in organ culture. Examining the positive inotropic effects of isoproterenol on isolated guinea pig atria and in rabbit papillary muscles, Hashimoto and Nakishima (1978) reported a significantly increased sensitivity in preparations from T_4-treated animals. Nakishima *et al.* (1971) also observed the converse effect, namely, a rightward shift in the dose–response curve for isoproterenol on contractile force, in isolated atria from hypothyroid rats.

A point of further interest here regards the growing body of literature supporting the existence of functional cardiac adrenergic receptors with pharmacological properties meeting accepted criteria for α-adrenergic receptors (R. S. Williams and Lefkowitz, 1978).* Some investigators have contended that the effects of thyroid hormones on physiological responsiveness to β-adrenergic stimulation in the heart are paralleled by reciprocal changes in the physiological responsiveness to α-adrenergic stimulation. Kunos and others, in several reports (Kunos, 1977; Kunos *et al.*, 1974; Nickerson and Kunos, 1977), have analyzed dose–response curves for force development in response to norepinephrine, isoproterenol, and phenylephrine in isolated atria from control, hypothyroid, and hyperthyroid rats. They report an augmented contractile responsiveness to isoproterenol and a decreased responsiveness to phenylephrine in hyperthyroid atria, and the converse, namely, augmented phenylephrine responsiveness and decreased isoproterenol responsiveness in hypothyroid atria. In addition, blockade of the response to phenylephrine by the α-adrenergic antagonists phentolamine or phenoxybenzamine was markedly enhanced in the hypothyroid state. These observations led these authors to postulate that α- and β-adrenergic receptors may represent interconvertible forms of the same macromolecule and that the relative numbers of α- and β-adrenergic receptors are under control of several stimuli, notably thyroid hormones. Further data regarding this point have been obtained by directing binding studies of adrenergic receptors, and are discussed in Section II,D.

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*In the years since the initial delineation by Ahlquist (1948) of physiological responses to catecholamines into two major groups, termed α and β, several generally accepted criteria for making this distinction have been defined. β-Adrenergic responses are initiated by adrenergic agonists in decreasing order of potency from isoproterenol > epinephrine > norepinephrine, and they are blocked by propranolol and its congeners, whereas α-adrenergic responses are elicited by agonists in the potency series epinephrine ≥ norepinephrine > isoproterenol, and are blocked by phentolamine, phenoxybenzamine, or certain ergot alkaloids. Phenylephrine stimulates both classes of receptors, but has a greater affinity for α-adrenergic receptors. Its physiological effects when tested at low concentrations in heart are unassociated with elevations of cAMP and are blocked by phentolamine, whereas its effects at higher concentrations are associated with elevations in cAMP and are blocked by propranolol (Hashimoto and Nakashima, 1978; Wagner and Brodde, 1978; Osnes and Oye, 1975; Verma and McNeill, 1976).

C. Biochemical Responses

As we observed for adrenergically mediated physiological responses, the effects of thyroid hormones on biochemical responsiveness to catecholamines in the heart are still under debate. At least four groups have reported changes in the sensitivity of adenylate cyclase activity in response to β-adrenergic stimulation with alterations in thyroid state. Wollenberger and Will-Shahab (1976) studied adenylate cyclase activity in cardiac membranes from rats and noted a diminished sensitivity to epinephrine stimulation in hypothyroid animals, as well as a leftward shift of the dose–response curve in hyperthyroid animals. Tsai and Chen (1978) demonstrated a dose-dependent increase in the cAMP production stimulated by 1 μM epinephrine when cultured heart cells from newborn rats were incubated in the presence of T_3 for 24 hours, and Watanabe *et al.* (1978) observed a slight, but statistically significant increase in the cAMP accumulated in isolated perfused hearts from thyrotoxic rats in response to isoproterenol. The interpretation of Wollenberger's results is clouded by the aforementioned problem of interpreting responses that begin at different initial values. In this study the basal level of cAMP was markedly higher in the hypothyroid hearts compared to euthyroid hearts, and thus the physiological significance of a diminished sensitivity to catecholamines expressed as a percentage of basal levels is difficult to interpret.

In contrast to these reports, numerous investigators studying a variety of preparations have not observed significant effects of thyroid hormone on cardiac adenylate cyclase activation by adrenergic agonists (Levey and Epstein, 1969; Sobel *et al.*, 1969; Young and McNeill, 1974; McNeill *et al.*, 1969; Levey *et al.*, 1969; Frazer *et al.*, 1969; Felt *et al.*, 1975). Likewise, no effects of thyroid alteration on myocardial phosphodiesterase (Sobel *et al.*, 1969) or protein kinase activation by norepinephrine or dibutyryl-cAMP (Katz *et al.*, 1977) have been noted.

Somewhat surprisingly, in view of the preceeding discussion, the activation of myocardial phosphorylase by β-adrenergic stimulation has uniformily been noted to be augmented in heart preparations from hyperthyroid animals (Young and McNeill, 1974; Felt *et al.*, 1975; McNeill *et al.*, 1969; Frazer *et al.*, 1969; McNeill and Brody, 1968; McNeill, 1969). McNeill and co-workers have published several reports that pretreatment of rats with T_3 for 3 days enhances the activation of cardiac phosphorylase by norepinephrine, epinephrine, or isoproterenol in intact animals or in the isolated perfused heart. These authors hypothesized that thyroid hormone could increase the stimulation of adenylate cyclase by β-adrenergic agonists *in vivo*, but that the ability to measure this effect is artifactually lost in the preparation of the enzyme for *in vitro* assay (McNeill *et al.*, 1969). Reports of unaltered myocardial cAMP levels in hyperthyroid hearts would not necessary negate this hypothesis, since increased production of cAMP by β-adrenergic receptor coupled adenylate cyclase could occur and immediately exert its effects on protein kinases or other effector proteins located nearby without appreciably altering the levels of cAMP observed in homogenates of whole heart.

D. Radioligand Binding Studies

The effects of thyroid hormones on cardiac β-adrenergic receptors have been studied directly by the binding of the tritiated form of the potent β-adrenergic antagonist dihydroalprenolol (DHA) to membrane fractions derived from the hearts of rats with experimental hyperthyroidism and hypothyroidism in several laboratories.* Though variations in the absolute values for number of binding sites (B_{max}) and for dissociation constants (K_d) for DHA have differed between investigators, and though criticism can be levied against the experimental and analytical methods employed in certain studies, several consistent patterns in the experimental results have appeared.

All investigators to date have observed an increase in the number of β-adrenergic receptors in cardiac membranes derived from hyperthyroid animals, and a

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*For a discussion of the theoretical basis and experimental validation of the use of radioligand binding for the direct assessment of adrenergic receptors at the molecular level, the reader is referred to one of several recent reviews (L. T. Williams and Lefkowitz, 1978; Snyder, 1979; Lefkowitz, 1978, 1979). Since the binding sites identified in early studies using tritiated norepinephrine of low specific activity probably do not reflect binding to physiological β-adrenergic receptors, reports of effects of altered thyroid state on [^{3}H]norepinephrine binding (Wollenberger and Will-Shahab, 1976; Felt *et al.*, 1975) have not been considered in this discussion.

decrease in the number of β-adrenergic receptors in hypothyroid hearts. No significant changes in binding affinity for dihydroalprenolol have been observed by any group. The original report of thyroid hormone regulation of β-adrenergic receptor number by Williams *et al.* (1977) remains representative. Scatchard analysis of the specific binding of dihydroalprenolol to cardiac membranes from euthyroid and hyperthyroid rats is shown in Fig. 2A. By this analysis, the intercepts of the regression lines with the abscissa reflect the B_{max}, demonstrating a roughly twofold increase in the number of β receptors in hyperthyroid membranes. The dissociation constants (K_d) can also be estimated from the negative reciprocals of the slopes of the lines, and the similarity in slope of the two lines demonstrates that binding affinity for DHA was unaltered by experimental hyperthyroidism. The binding affinity for β-adrenergic agonists was also unaltered by hyperthyroidism, as reflected by identical EC_{50} values for the competition of unlabeled isoproterenol for DHA binding sites in euthyroid and hyperthyroid cardiac membranes (Fig. 2B). The relative uniformity of the changes in dihydroalprenolol binding sites from hyperthyroid and hypothyroid cardiac membranes, expressed as percentages of the binding observed in euthyroid membranes, is shown in Table I.

Two other studies analyzing effects of thyroid hormone on cardiac β-adrenergic receptor *in vitro* are of interest. Tsai and Chen (1978) observed a roughly twofold increase in specific DHA binding in cultured heart cells from newborn rats when the cells were incubated with triiodothyronine for 24 hours. In addition, Kempson *et al.* (1978) addressed the processes by which thyroid hormones produce an augmentation in cardiac β-adrenergic receptor number utilizing an *in vitro* model. In their studies, the incubation of rat ventricular slices in the presence of triiodothyronine produced a time-dependent increase in the specific binding of DHA to membrane preparations made from these slices. They observed no effect on DHA binding when cardiac homogenates, rather than the intact cells present in the ventricular slices, were incubated in T_3. The effect of T_3 on DHA binding in ventricular slices was stereospecific, and appeared to have two components: an early (1–3 hour) increase in dihydroalprenolol binding, which was unaffected by inhibitors of protein synthesis, and a late (15 hour) increase, which could be prevented by cycloheximide or puromycin. They postulated two separate mechanisms for the T_3-induced augmentation of membrane β-adrenergic receptor number: the early phase reflecting a posttranslational recruitment of previously synthesized β-adrenergic receptors to the membrane surface; and the late phase representing augmented synthesis of new receptor protein. Because both of these *in vitro* studies assessed DHA binding at only one concentration of radioligand rather than assessing binding isotherms, these conclusions regarding increases in receptor number with T_3 exposure, though provocative, remain somewhat preliminary.

The effects of experimental hyperthyroidism and hypothyroidism on cardiac

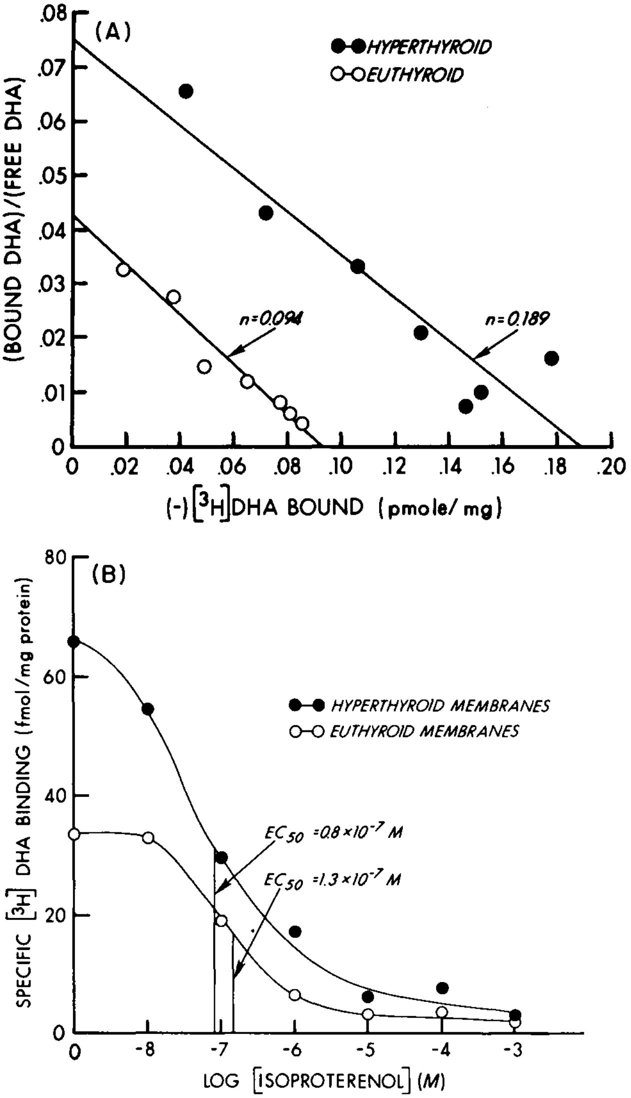

Fig. 2. (A) Scatchard plot of (−)-[^{3}H]dihydroalprenolol (DHA) binding to cardiac membranes from control (euthyroid) and hyperthyroid rats. Hyperthyroidism was induced by injection of triiodothyronine. Cardiac membranes were incubated with a series of concentrations of (−)-[^{3}H]DHA and specific binding was determined. The lines of euthyroid ($r = 0.97$) and hyperthyroid ($r = 0.93$) membranes were determined by linear regression analysis. The intercept with the abscissa indicates the number of binding sites (*n*). Each point represents the mean of duplicate determinations. This experiment is representative of six such experiments. (B) Competition for (−)-[^{3}H]DHA binding sites by isoproterenol in euthyroid and hyperthyroid cardiac membranes. Hyperthyroidism was induced by injection of triiodothyronine. Cardiac membranes (4 mg/ml) were incubated with (−)-[^{3}H]DHA (8 n*M*) in the presence of increasing concentrations of isoproterenol and specific (−)-[^{3}H]DHA binding was measured. The concentration of isoproterenol causing half-maximal inhibition of (−)-[^{3}H]DHA binding (EC$_{50}$) was determined. Each point represents the mean of triplicate determinations. With permission from Williams *et al.* (1977).

TABLE I

DHA Binding to β-Adrenergic Receptors of Rat Cardiac Membranes

Study	Hyperthyroid $B_{\max}$ (% euthyroid)	Hypothyroid $B_{\max}$ (% euthyroid)	Hyperthyroid $B_{\max}$/ hypothyroid $B_{\max}$
Williams *et al.* (1977)	220 (T_4)	—	—
	180 (T_3)	—	—
Ciaraldi and Marinetti (1977)	328	71	4.6
Ciaraldi and Marinetti (1978)	162	66	2.5
Banerjee and Kung (1977)	129	68	1.9
R. S. Williams, and R. J. Lefkowitz (unpublished data, 1979)	165	71	2.3
Kempson *et al.* (1978)	325	—	—
McConnaughey *et al.* (1979)	143	84	1.7

α-adrenergic receptors, as assessed by the binding of the potent α-adrenergic antagonist dihydroergocryptine (DHE), have also been extensively studied, though without the consensus in results between laboratories which has been noted for the effects of thyroid hormones on α-adrenergic receptors.*

The earliest paper addressing this issue reported a decreased $B_{\max}$ for dihydroergocryptine in both hyperthyroid and hypothyroid membranes as compared to control euthyroid hearts (Ciaraldi and Marinetti, 1977). These same investigators later reported different findings when they prepared their membranes from hearts obtained immediately after sacrificing the animals, as compared to those prepared from frozen hearts. In such fresh membranes they noted an increased $B_{\max}$ in hyperthyroidism and a diminished $B_{\max}$ in hypothyroidism (Ciaraldi and Marinetti, 1978). In both experiments they also noted an increased binding affinity for DHE in hypothyroid membranes and a diminished affinity for DHE in hyperthyroid membranes. These initial reports are difficult to interpret because of several methodological shortcomings as delineated in later papers (R. S. Williams and Lefkowitz, 1979), and the findings have not been confirmed by subsequent investigators. For example, Sharma and Banerjee (1978) noted a statistically significant decrease in $B_{\max}$ without change in K_d in two different cardiac membrane preparations derived from hyperthyroid as compared with

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*The binding sites in cardiac tissue identified by tritiated phenoxybenzamine have not been characterized as representative of cardiac α-adrenergic receptors. The nonequilibrium kinetics displayed by phenoxybenzamine binding to cardiac membrane components (L. T. Williams and Lefkowitz, 1977) and the low specific activity of the tritiated ligand make it unlikely that such sites represent physiological α-adrenergic receptors. Therefore, reports of effects of altered thyroid state on [^{3}H]phenoxybenzamine binding in rat heart (Kunos *et al.*, 1974; Kunos, 1977) have not been considered in this discussion.

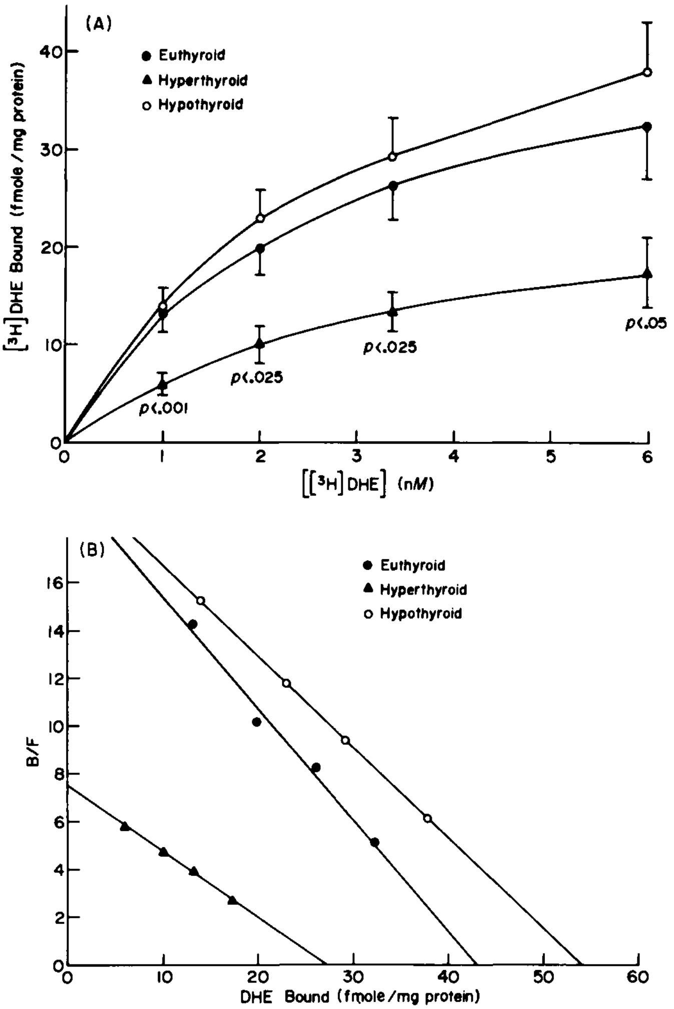

Fig. 3. (A) Specific binding of [^{3}H]dihydroergocryptine (DHE) to cardiac membranes from hypothyroid, euthyroid, and hyperthyroid rats. Each value is the mean of the results obtained from five separate experiments involving hyperthyroid animals, and from seven separate experiments involving hypothyroid and euthyroid animals, with binding assessed in triplicate in each experiment. The vertical bars represent SEM for each value. Statistical comparisons were made by Student's *t* test for unpaired samples. (B) Scatchard plots of [^{3}H]DHE binding to cardiac membranes from hypothyroid, euthyroid, and hyperthyroid rats. The points represent the means of the results obtained from five separate experiments involving hyperthyroid animals, and seven separate experiments involving hypothyroid and euthyroid animals, with binding assessed in triplicate in each experiment. The number of binding sites at saturation for each group can be determined by the intercept of each line with the abscissa, and the K_d can be calculated from the negative reciprocal of the slope of each line. Correlation coefficients for the regression lines were 0.99 for hyperthyroid membranes, 0.96 for euthyroid membranes, and 0.99 for hypothyroid membranes. With permission from R. S. Williams and Lefkowitz (1979).

TABLE II

DHE Binding to α-Adrenergic Receptors of Rat Cardiac Membranes

Study	Hyperthyroid B_{max} (% euthyroid)	Hypothyroid B_{max} (% euthyroid)	Hyperthyroid B_{max}/ hypothyroid B_{max}
Ciaraldi and Marinetti (1977)	54[a]	17	3.2*
Ciaraldi and Marinetti (1978)	106[a]	42	2.5*
Sharma and Banerjee (1978)	—[b]	—[b]	0.6 ("particulate") 0.5 ("sarcolemmal")
R. S. Williams and Lefkowitz (1979)	62	119	0.5
McConnaughey *et al.* (1979)	59	71	0.8

[a] Binding was nonsaturable.
[b] No euthyroid group reported.

hypothyroid animals, though no euthyroid control hearts were studied. R. S. Williams and Lefkowitz (1979), utilizing a crude particulate membrane preparation, observed a significant decrease in DHE binding to hyperthyroid membranes and a slight, statistically insignificant increase in binding to hypothyroid membranes (Fig. 3A). Scatchard analysis (Fig. 3B) suggested this decreased binding was due both to a decreased affinity for DHE as well as to a decreased B_{max}. McConnaughey *et al.* (1979), utilizing a different membrane preparation, confirmed this decrease in DHE binding sites in hyperthyroidism, but noted decreased binding in hypothyroid membranes as well.

The relative changes in B_{max} for DHE occurring in cardiac membranes from hyperthyroid and hypothyroid rats, expressed as a percentage of B_{max} for euthyroid membranes, are tabulated in Table II. The three more recent studies are in general agreement that α-adrenergic receptor number, calculated from B_{max} for dihydroergocryptine binding, is reduced in membranes from hyperthyroid animals when compared with either euthyroid or hypothyroid animals, the converse of the findings observed for β-adrenergic receptors. Residual controversy exists concerning the effects of hypothyroidism compared to euthyroidism on cardiac α receptors, and this point cannot be resolved on the basis of existing data.

III. EFFECTS OF THYROID HORMONE ON ADRENERGIC RECEPTORS IN ADIPOSE TISSUE

There is general agreement that alterations in thyroid state have profound effects on adrenergic modulation of adipocyte lipolysis, although there is considerable controversy over the molecular mechanisms underlying these effects.

Glycerol release from adipose tissue in tissue bath preparations or from isolated adipocytes in response to epinephrine or norepinephrine has been uniformly shown to be diminished in hypothyroidism (Deykin and Vaughn, 1963; Ichikawa *et al.*, 1971; Rosenquist, 1972; Reckless *et al.*, 1970; Malbon *et al.*, 1978a; Goswami and Rosenberg, 1978; Ohisalo and Stouffer, 1979), though the converse, an increase in catecholamine sensitivity in hyperthyroid adipocytes, has been found by some investigators (Deykin and Vaughn, 1963; Brodie *et al.*, 1966; Caldwell and Fain, 1971; Goswami and Rosenberg, 1978; Arner *et al.*, 1979), but not by others (Malbon *et al.*, 1978a). The stimulation by epinephrine or norepinephrine of cAMP accumulation in intact cells or of adenylate cyclase activity in membrane preparations is diminished in hypothyroidism (Malbon *et al.*, 1978a; Kalberg *et al.*, 1974; Grill and Rosenquist, 1973) and enhanced in hyperthyroidism (Malbon *et al.*, 1978a; Caldwell and Fain, 1971; Krishna *et al.*, 1968; Arner *et al.*, 1979). Attempts to further analyze the molecular mechanisms of these effects have led to several different hypotheses, each based on different, and sometimes conflicting, further experimental data.

Utilizing direct radioligand binding techniques similar to those previously described for the analysis of cardiac adrenergic receptors, some investigators have proposed that changes in adipocyte β-adrenergic receptor number may, at least in part, account for the changes in catecholamine sensitivity found in altered thyroid states. Ciaraldi and Marinetti (1978) reported increased DHA binding in fat cell ghosts from hyperthyroid rats, and Guidicelli (1978) reported a threefold reduction in the number of β-adrenergic receptors assessed by DHA binding in adipocytes from hypothyroid rats that was restored to normal following treatment with T_3. However, two other laboratories have failed to detect changes in DHA binding to adipocyte membranes occurring with altered thyroid state (Malbon *et al.*, 1978a; Goswami and Rosenberg, 1978). Except for the report of Ciaraldi and Marinetti (1978), whose results must be judged as inconclusive because DHA binding was assessed at only one concentration of DHA, careful analysis of methodological differences among these four reports does not yield an explanation for the contradictory results, and this issue must be considered unresolved.

Several hypotheses other than changes in the β-adrenergic receptors have been presented to account for the altered adipocyte catecholamine sensitivity occurring with changes in thyroid status. Three major mechanisms have been proposed: (1) enhancement of α-adrenergic *inhibition* of lipolysis in hypothyroidism, leading to an apparent reduction in β-adrenergic responsiveness when mixed agonists such as epinephrine or norepinephrine are tested, (2) altered coupling of agonist–receptor binding to subsequent activation of catecholamine sensitive adenylate cyclase, and (3) direct or indirect alteration in cAMP phosphodiesterase (PDE) activity.

Rosenquist (1972; Grill and Rosenquist, 1973) reported that the reduced epinephrine stimulated lipolysis or cAMP accumulation demonstrated by hypothyroid adipocytes could be restored to normal by α-adrenergic blockade with phentolamine and that, unlike the lipolytic response to the mixed agent norepinephrine, which was reduced in hypothyroidism, the lipolytic response to isoproterenol, which has high affinity for β- but negligible affinity for α-adrenergic receptors, was unchanged in the hypothyroid state. These results were largely corroborated by those of Reckless *et al.* (1976). These results are in general agreement with the hypothesis presented in Section II,B that thyroid hormones produce changes in the relative effects of α-adrenergic stimulation as opposed to β-adrenergic stimulation, with α-adrenergic effects predominating in the hypothyroid state, and β-adrenergic effects dominating the observed physiological responses in the hyperthyroid state. For the adipocyte, this hypothesis is further supported by Arner *et al.* (1979), who observed increased isoproterenol-stimulated glycerol release and cAMP accumulation in adipocytes from hyperthyroid subjects.

Another theory suggests that enhanced sensitivity to adenosine, which inhibits catecholamine sensitive lipolysis in adipose tissue, occurs in hypothyroidism and produces the apparent hyporesponsiveness to β-adrenergic stimulation of lipolysis which accompanies the hypothyroid state. Ohisalo and Stouffer (1979) reported that addition of adenosine deaminase to hypothyroid adipocytes restored the reduced lipolytic sensitivity to epinephrine previously observed in these cells to normal. Inhibitors of adenosine deaminase, or the addition of adenosine analogs that do not serve as substrates for adenosine deaminase, resulted in a much greater inhibition of epinephrine sensitive lipolysis in hypothyroid adipocytes than in normal cells.

Malbon *et al.* (1978a) have suggested that altered coupling of agonist–receptor binding to activation of adenylate cyclase occurs in adipocytes from hypothyroid rats. In a fat cell ghost preparation in which they observed no differences in DHA binding or in PDE activity, they noted equivalent fluoride-stimulated adenylate cyclase activity in hypothyroid and euthyroid ghosts, but a complete loss of catecholamine-sensitive cyclase in the hypothyroid cells. Their data did not allow further analysis of the nature of this functional "uncoupling" of the receptor–cyclase interaction. They did note, however, that the non-hydrolyzable guanine nucleotide analog Gpp(NH)p [which increases the stimulating effect of agonist occupancy of β-adrenergic receptors upon activation of adenylate cyclase in adipocytes (Rodbell, 1975) by binding to the hypothesized nucleotide regulatory site of the adenylate cyclase complex (Lefkowitz *et al.*, 1976)] did not restore the depressed catecholamine-sensitive adenylate cyclase activity in hypothyroid adipocytes to normal. It should be noted that one explanation, which was not assessed by Malbon *et al.* (1978a) for decreased coupling in hypothyroid adipocytes of receptor occupancy to adenylate cyclase activation by mixed α- and β-adrenergic agonists, would be enhanced α-adrenergic inhibition of adenylate cyclase as hypothesized by Rosenquist (1972) and others (Grill and Rosenquist, 1973).

Changes in cAMP PDE activity with altered thyroid state have also been proposed as possible mechanisms of altered adipocyte catecholamine responsiveness. Increased adipocyte PDE activity has been reported in hypothyroidism (Armstrong *et al.*, 1974), though other laboratories have not confirmed this finding (Caldwell and Fain, 1971), and altered soluble PDE activity has not been observed in hypothyroidism (Malbon *et al.*, 1978a). An indirect effect of thyroid hormones on adipocyte PDE which could potentially produce altered catecholamine sensitivity has been hypothesized by Goswami and Rosenberg (1978). These investigators observed that inhibition of epinephrine-induced lipolysis could be produced in normal adipocytes by increasing the calcium concentration in the media, or by calcium ionophores, and that this inhibition could be reversed by theophylline. In addition, they found that theophylline or EGTA corrected the reduced epinephrine-sensitive lipolysis of hypothyroid adipocytes and theorized that thyroid hormones produced alterations in cellular calcium concentrations which, by virtue of inhibitory effects on PDE activity, produced the observed changes in catecholamine sensitivity. Since increases in membrane calcium transport by cAMP-independent mechanisms have been hypothesized as the biochemical sequelae of α-adrenergic stimulation in other tissues (Exton, 1979), these observations are also compatible with the thesis that increased α-adrenergic responsiveness mediates the apparent loss of β-adrenergic responsiveness occurring in hypothyroidism. However, the reports by other laboratories (Malbon *et al.*, 1978a) that PDE inhibition by methylxanthines does not correct the decrements in catecholamine-sensitive adenylate cyclase activation or glycerol release in hypothyroid adipocytes fail to support the hypothesis of Goswami and Rosenberg.

IV. EFFECTS OF THYROID HORMONE ON ADRENERGIC RECEPTORS IN OTHER TISSUES

The effects of altered thyroid state on adrenergic modulation of cellular function have been studied, though less extensively, in a number of tissues other than adipose tissue and heart. Although many of these data await confirmation, the majority of tissues studied have demonstrated changes in adrenergic responsiveness with changes in the ambient levels of thyroid hormone. Several studies support the now familiar hypothesis that β-adrenergic responsiveness is increased, and α-adrenergic responsiveness reduced, by thyroid hormones.

Studying the well-characterized adenylate cyclase coupled β-adrenergic receptors of turkey erythrocytes, Bilezekian *et al.* (1979) noted a decreased number of β-adrenergic receptors as assessed by the binding of [^{125}I]hydroxybenzylpindolol accompanied by diminished catecholamine-sensitive adenylate cyclase activity in erythrocyte membranes, as well as a decreased accumulation of cAMP in response to β agonists in intact cells from hypothyroid as opposed to euthyroid turkeys. In hyperthyroidism, no changes in membrane β receptors or adenylate cyclase activity were noted, but a leftward shift of the dose–response curve for isoproterenol-stimulated cAMP accumulation did occur.

Popovic *et al.* (1977) noted the enhancing effect of thyroid hormone incubation *in vitro* on stimulation of erythropoiesis by isoproterenol in cultured canine marrow cells, and they expanded this work to the study of catecholamine sensitivity of marrow cells from euthyroid and hypothyroid dogs (Popovic *et al.*, 1979). In this later report these investigators found the isoproterenol-induced stimulation of erythropoiesis, which occurred in normal cells and which could be blocked by propranolol but not by phentolamine, was lost in the marrow cells obtained from hypothyroid animals. The hypothyroid cells, however, could be stimulated to form colonies by phenylephrine or norepinephrine, a response not noted in normal cells, and which could be blocked by the α-adrenergic antagonist phentolamine, but not by propranolol. Incubation of these hypothyroid cells with T_4 or T_3 *in vitro* restored the normal pattern of β-adrenergic stimulation. Other studies whose results fit this general pattern include the following. Pointen and Banerjee (1979) studied rat salivary glands and noted a fourfold stimulation in the number of β receptors, assessed as B_{max} for the binding of DHA, when T_3 was administered to thyroidectomized rats. Okajima and Ui (1978) reported that the stimulatory effect of isoproterenol on insulin secretion in normal rats (which is blocked by propranolol and presumably represents a β-adrenergic response) was enhanced in hyperthyroid rats and abolished in hypothyroid rats. They noted further that whereas the response of insulin secretion to glucose infusion was unaffected by the mixed agonist epinephrine in euthyroid animals, glucose-stimulated insulin secretion was enhanced by epinephrine in hyperthyroid rats (presumably a β-adrenergic effect) but inhibited by epinephrine in hypothyroid rats (presumably an α-adrenergic effect). Fregley *et al.* (1975) reported another β-adrenergic response, increased tail skin temperature in the rat, was abolished in hypothyroid rats, but could be restored by T_4 administration.

However, increased β-adrenergic responsiveness in hyperthyroidism does not occur in all tissues. Williams *et al.* (1979) noted no changes in either binding sites for DHA or in cAMP accumulation in response to isoproterenol when lymphocytes from human subjects with untreated hyperthyroidism were compared to lymphocytes from age and sex matched euthyroid control subjects. In addition, results of studies of changes in adrenergic responsiveness in isolated hepatocytes with altered thyroid status present a striking departure from the pattern observed in most other tissues.

Malbon *et al.* (1978b) reported increased cAMP accumulation in isolated hepatocytes from hypothyroid rats in response to adrenergic agonists, which could be blocked by propranolol but not by phentolamine and which was noted both in the presence or absence of phosphodiesterase inhibitors. Likewise, the maximum stimulation of glycogen phosphorylase by isoproterenol was enhanced in hypothyroid cells, accompanied by a leftward shift of the isoproterenol dose–response curve. Preiksaitis and Kunos (1979) noted similar findings. In their study hepatocytes from euthyroid rats demonstrated catecholamine-mediated activation of glycogen phosphorylase that suggested an α-adrenergic response in that adrenergic agonists produced the effect with the potency series epinephrine> phenylephrine> isoproterenol and the response was blocked by the α antagonist DHE but not by propranolol. In hyperthyroid cells, the sensitivity to adrenergic stimulation was enhanced but the order of agonist and antagonist potencies still suggested a response mediated through α-adrenergic receptors. In hypothyroid cells, however, a distinctly different situation was noted: agonists elicited the stimulation of glycogen phosphorylase with the potency order of isoproterenol> epinephrine> phenylephrine and the response was blocked by propranolol but not by phentolamine. Thus, in hypothyroid cells, the response appeared to be mediated through β-adrenergic receptors, as opposed to the α-adrenergic mediation of phosphorylase activation observed in hyperthyroid and euthyroid hepatocytes.

V. CONCLUSIONS

The observation that many of the clinical manifestations of thyrotoxicosis can be ameliorated by β-adrenergic antagonists, which have minimal, if any, effects on synthesis, release, or metabolism of thyroid hormones establishes the physiological importance of some type of synergistic relationship between thyroid hormones and the adrenergic nervous system. The general agreement that the changes in plasma or tissue catecholamines or in dopamine β-hydroxylase, which are noted with altered thyroid states, occur in opposite directions from the changes noted in apparent β-adrenergic activity supports the contention that thyroid hormones alter tissue sensitivity to catecholamines, rather than altering nerve traffic via sympathetic pathways or neurotransmitter release from postganglionic sympathetic neurons or from the adrenal medulla.

On the other hand, it is firmly established that many of the effects of thyroid hormones on cellular metabolism and function occur by mechanisms totally distinct from those under adrenergic modulation (Bernal and Refetoff, 1977; Sterling, 1979a,b) and, indeed, several clinical sequelae of hyperthyroidism are unaffected by adrenergic blockade (Zwillich *et al.*, 1978; Grossman *et al.*, 1971a,b; Landsberg, 1978).

In the attempt to explain the undeniable interaction between thyroid hormones and adrenergic mechanisms, it is difficult to build a consistent thesis beyond these broad conclusions on the basis of existing data. The hypothesis that thyroid hormones affect the relative numbers of α- and β-adrenergic receptors in a variety of target tissues has considerable experimental support. However, the extended hypothesis that these receptor changes lead to altered physiological responsiveness to catecholamines and produce the apparent changes in adrenergic activity observed in clinical states of thyroid hormone deficit or excess, though certainly an attractive proposal, cannot be accepted at this time due to far too many contradictory data. Whatever the true nature of the effects of thyroid hormone on adrenergic receptors and on the biochemical and physiological sequalae that are linked to agonist occupancy of those receptors, the existing data strongly suggest that these effects are tissue specific, and may not be explicable on the basis of a unifying, all-encompassing mechanism.

There are several major experimental issues about which reasonably conclusive data exist: (1) cardiac membranes from rats made hyperthyroid with short courses of T_4 or T_3 demonstrate an increase in β-adrenergic receptor number and a decrease in α-adrenergic receptor number, (2) hypersensitivity to β-adrenergic activation of cardiac glycogen phosphylase is present in experimental hyperthyroidism, (3) diminished or absent lipolytic responsiveness to β-adrenergic stimulation is present in adipocytes from hypothyroid rats or humans, and (4) although many of these data await confirmation, changes in responsiveness to adrenergic agonists with altered thyroid state appears to occur in many, though not all, adrenergically responsive tissues.

On the other hand, the frequency with which major laboratories have reported contradictory findings regarding the effects of thyroid hormones on adrenergic receptors and responses is evident from the detailed discussion of the preceding text. Although some of these discrepancies can be attributed to defects in experimental design, others remain unexplained. For the biochemical responses to adrenergic stimulation in altered thyroid states, the prevalence of discordant experimental findings serves further to illustrate the inherent difficulty of extrapolating the findings obtained from broken cell experimental preparations to physiological phenomena *in vivo,* because such results may be subject to a number of resources of unrecognized bias induced by the processing of intact tissue to produce the *in vitro* model. Also a further problem exists in the interpretation of many of the experimental data for both physiological and biochemical responses, in that "hypersensitivity" or "hyposensitivity" to adrenergic agonists may be difficult to define when the biological variable under study has a different initial value in the different thyroid states being investigated.

It is clear that much more experimental evidence is needed to clarify the complete nature of the relationship between thyroid hormones and the adrenergic nervous system. Unequivocal changes in adrenergic receptors and in the biochemical processes linked to agonist occupancy of those receptors do occur in certain tissues in experimental hyperthyroidism and hypothyroidism. However, the difficulty in linking these changes to unequivocal changes in biological responsiveness to adrenergic agonists at the organ level, coupled with the aformentioned unresolved experimental issues, means that the physiological significance of these changes remains uncertain at this time. The nature of the thyroid–catecholamine interaction remains an exciting and incompletely resolved scientific problem.

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12

Intrapituitary Mechanisms in the Control of TSH Secretion

P. R. LARSEN and J. E. SILVA

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I. INTRODUCTION

Thyroid hormone levels have a profound effect on anterior pituitary gland function. This includes not only the negative feedback of L-thyroxine (T_4) and 3,5,3′-L-triiodothyronine (T_3) on thyrotropin (TSH) secretion by the thyrotrophs, but also effects on growth hormone (Schooley *et al.*, 1966; Hervas *et al.*, 1975), prolactin (Honbo *et al.*, 1978), and perhaps gonadotropins as well (Greeley and Maheesh, 1978). It was not surprising then that early studies showed that, in the rat, the anterior pituitary gland has the highest concentration of nuclear receptor sites of any tissue (Oppenheimer *et al.*, 1974b). It is apparent that if thyroid hormone concentrations are to remain constant that a sensitive mechanism for at least the thyrotroph to respond to changes in the level of thyroid hormone must be present. With the application of radioimmunoassay technology to measurements of TSH, the inverse relationship between T_4 and this pituitary hormone were readily demonstrated (Utiger, 1965). It was also apparent that there was suppression of TSH by T_3 when this was given to hypothyroid patients. When radioimmunoassay methods for measurements of serum T_3 became available, it was again seen that, in general, there was an inverse relationship between serum T_3 and serum TSH (Larsen, 1972b). However, there were several situations in man and in animals that suggested that the relationship between serum T_4 and T_3, on the one hand, and TSH secretion, on the other, was not a simple one. Examples include the observation that, in endemic goiter areas, metabolic euthyroidism is often associated with a normal serum T_3, a reduction in serum T_4 of variable degree, and an elevated TSH (Patel *et al.*, 1973; Chopra *et al.*, 1975; Vagenakis *et al.*, 1973). Given that the normal concentration of serum T_3 is likely to be the explanation for the euthyroid metabolic status, one is confronted with the paradox of an elevated serum TSH. Similarly, in thyroidectomized rats the effect of inhibiting conversion of administered T_4 to T_3 by propylthiouracil (PTU) on the biological activity of T_4 was found to be much greater on hepatic indices of thyroid status such as mitochondrial α-glycerophosphate dehydrogenase (GPD) than on TSH suppression (Larsen and Frumess, 1977). These observations suggested that T_4 and T_3 might both independently affect TSH release.

The present chapter will review the physiological data bearing on the relationship between circulating serum thyroid hormones and TSH and the results of *in vivo* studies suggesting that nuclear binding of T_3 initiates the inhibition of TSH release following either T_4 or T_3 administration. We shall also discuss the intrapituitary conversion of T_4 to T_3 and review evidence which indicates that the 5′-monodeiodinase activity in that tissue is regulated by mechanisms different from those in the liver and kidney. Finally, the physiological and clinical implications of these observations will be summarized.

II. PHYSIOLOGICAL STUDIES ON THE RELATIONSHIPS AMONG SERUM THYROID HORMONE CONCENTRATIONS, THYROID METABOLIC STATUS, AND TSH SECRETION

A. Observations in Man

1. *Serum T_3, T_4, and TSH in Patients with Various Degrees of Primary Hypothyroidism*

In early studies of serum T_3 concentrations in various thyroid states using the then newly developed radioimmunoassay techniques, we noted that there were a significant number of patients with primary hypothyroidism in terms of serum T_4 and TSH concentrations who nevertheless had serum T_3 concentrations within the normal range (Larsen, 1972a). Accordingly, we evaluated serum T_4 and T_3 levels in 35 sequential serum samples in which serum TSH was in excess of 5 μU/ml, the upper limits of normal (Larsen, 1972b). These data are presented in Fig. 1. In the lower portion of this figure are the T_4 results, which were subnormal in all these patients. In contrast, a significant number of the same patients had serum T_3 concentrations within the normal range (69–160 ng/dl). As indicated by the regression lines, there is an inverse correlation between serum T_3 and serum TSH but this line displaced upward and to the right compared to the regression line of serum T_4 and serum TSH. In other words, many patients have an elevated TSH even though the serum T_3 is within normal limits. However, recent data on Bigos *et al.* (1978) suggest that serum TSH can be elevated and hyperresponsiveness to thyrotropin-releasing hormone (TRH) present even when serum T_4 is only modestly reduced and still within the normal range. Although available clinical techniques do not allow a precise quantitation of metabolic status, some of the patients with normal circulating T_3 concentrations, whose results were included in Fig. 1, were clearly not symptomatically hypothyroid. These and similar observations by others have provided clinical support for the concept that T_3 is the active thyroid hormone. Virtually all the effects of T_4 are explained by the fact that approximately ⅓ of the daily T_4 secreted is converted to T_3, which has 3 times the metabolic potency of the parent hormone. The large body of physiological data supporting this concept will not be reviewed here but has been summarized previously (Larsen, 1972b; Schimmel and Utiger, 1977). A more extreme example of this phenomenon has been found in infants with congenital hypothyroidism. Klein *et al.* (1976) reported that there was an excellent inverse correlation between the number of clinical features of hypothyroidism (such as retarded bone age, skin mottling, prolonged physiological jaundice, and hypothermia) and serum T_3 concentrations. A good correlation was not observed between serum T_4 levels and these clinical manifestations.

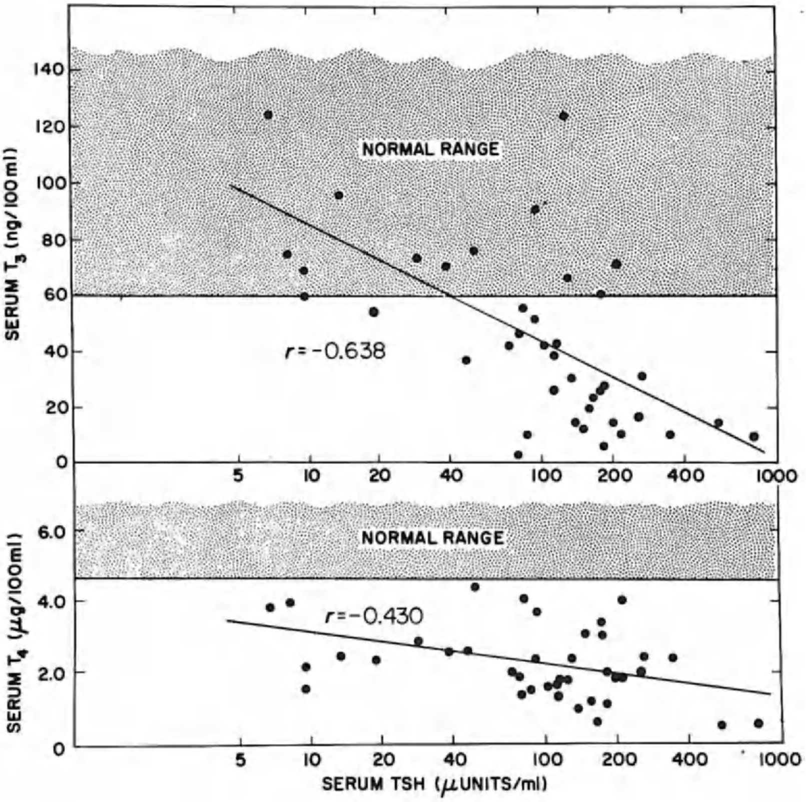

Fig. 1. Serum T_3, T_4, and TSH concentrations in 39 patients with untreated primary hypothyroidism. The samples represent all those with an elevated serum TSH (greater than 5 μU/ml) received in the laboratory over a 9 month period. The serum T_3 and T_4 concentrations for each sample are plotted directly above the corresponding TSH concentrations on the abscissa. The correlation coefficients are both significant at $p < .01$. Reproduced from Larsen (1978).

2. *Observations in Patients with Endemic Goiter*

A phenomenon similar to that in mild hypothyroidism has been reported in individuals with iodine deficiency. Several reports have shown that although serum T_3 concentrations are normal and serum T_4 reduced in endemic goiter, serum TSH is usually increased (Patel *et al.*, 1973; Chopra *et al.*, 1975; Vagenakis *et al.*, 1973). Silva and Silva (1981) found that moderately iodine-deficient pregnant women have slightly but significantly decreased serum T_4. Iodine supplementation increased serum T_4 by approximately 1.5 μg/dl ($p < .001$) and TSH decreased by 2 μU/ml ($p < .001$) with no change in serum T_3. On the other hand, patients with endemic goiter do not have metabolic hypothyroidism. This again has suggested that T_4 per se may have a more important regulatory role in

TSH production than it does in maintaining the metabolic status. Many other examples of this phenomenon have been reported though the clinical data have been difficult to interpret due to the difficulty in the precise measurement of the metabolic status in man. To obtain more precise estimates of thyroid status, animal studies are required so that more rigorous techniques can be applied.

B. Physiological Studies of the Relationships among Serum T_3, T_4, and TSH in Rats

1. *Effects of Iodine Depletion*

The acute reduction of dietary iodine results in a decrease in serum T_4 and an increase in TSH with consequent goiter (Abrams and Larsen, 1973; Fukuda *et al.*, 1975; Riesco *et al.*, 1977). Serum T_3, however, remains constant at least for 1–2 months of iodine restriction. Despite the reduction in serum T_4 and the elevation in TSH, such animals are metabolically euthyroid (Silva, 1972; Larsen and Frumess, 1977). Growth rate remains normal, oxygen consumption is not decreased, and the levels of the thyroid hormone-dependent hepatic enzyme mitochondrial α GPD are unchanged during the first two months of iodine deficiency. This is quite reminiscent of the situation in human endemic goiter and early hypothyroidism. It provided support for the concept that T_4 could have a direct role in feedback regulation of TSH, which was not explained by its conversion to T_3, at least as reflected in the concentration of T_3 in the serum. It should be emphasized that in the absence of the elevation of TSH in iodine deficiency, maintenance of serum T_3 at the normal concentrations could not occur. In iodine deficiency, virtually all the T_3 present in the rat derives directly from thyroidal secretion, whereas under normal circumstances we have estimated only about 30% of T_3 in the rat derives from this source (Abrams and Larsen, 1973).

2. *Effects of Inhibition of T_4 to T_3 Conversion by Propylthiouracil on Serum TSH*

As shown in Fig. 2, within 14 hours of a single injection of propylthiouracil, there is a decrease in serum T_3 and rise in TSH but no change in serum T_4 (Larsen and Frumess, 1977). This phenomenon is also apparent 24 hours after propylthiouracil but does not occur after administration of an equally potent dose (with respect to thyroid function) of methimazole, a drug that does not inhibit T_4 to T_3 conversion. These results indicate that there is tonic feedback inhibition of TSH by circulating serum T_3. Blockade of T_4 to T_3 conversion by propylthiouracil decreases this inhibition.

These short-term experiments do not allow comparison of the role of T_4 and T_3 in the maintenance of the metabolic status as opposed to regulation of pituitary TSH secretion. To study this question, we gave chronically hypothyroid (thyroidectomized) rats replacement doses of T_4 with or without propylthiouracil (Larsen and Frumess, 1977). Animals receiving 800 ng T4/100 g body weight for 12–13 days had restoration of serum T_4, T_3, and TSH to normal levels. In addition, weight gain resumed at its normal control rate of 5 g/day. These effects are summarized in Fig. 3 and Table I. If 1 mg/100 g body weight propylthiouracil was given with T_4, the serum T_4 concentrations were higher than those of animals receiving T_4 alone. On the other hand, the effect of propylthiouracil on T_4 to T_3 conversion in these animals was apparent in the reduction of serum T_3 to about ½ that of the control animals (Table I). In association with this, there was a reduction in hepatic mitochondrial α GPD which, when corrected for the levels in untreated controls, was proportional to the decrease in serum T_3. This confirms previous evidence of a causal relationship between T_3 production and the induction of this enzyme which has been demonstrated either directly or im-

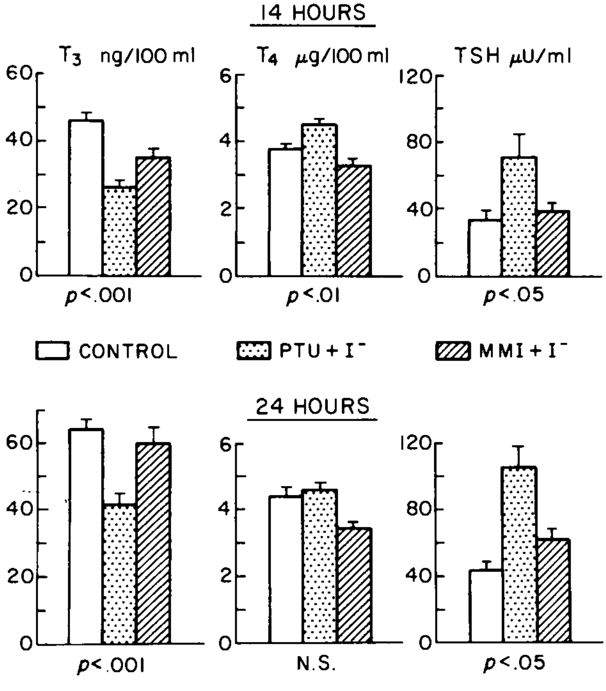

Fig. 2. Effect of injection of 1 mg propylthiouracil/100 g or 0.7 mg methylmercaptoimidazole/100 g i.p. + 10 mg/100 g NaI on serum T_3, T_4, and TSH concentrations in normal rats. Twelve animals were used at each time interval for each group. p values are calculated for differences between PTU + I⁻ and MMI + I⁻. Brackets indicate SE. Reproduced from Larsen and Frumess (1977).

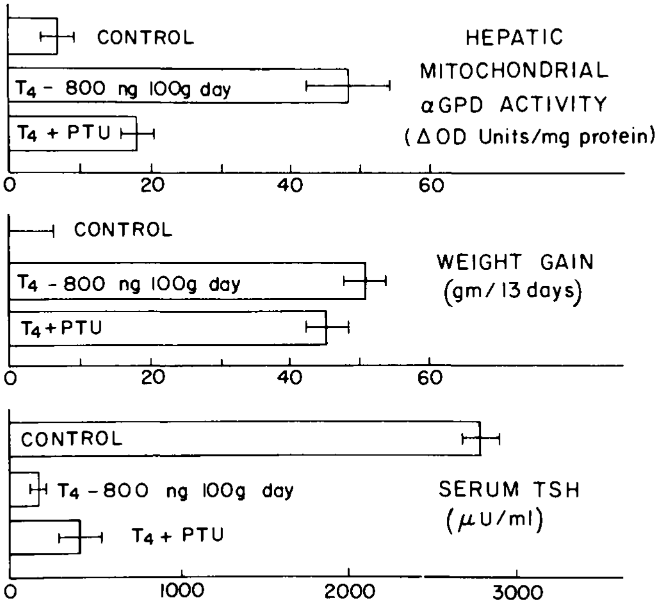

Fig. 3. Weight gain, hepatic mitochondrial α-glycerophosphate dehydrogenase activity, and serum TSH at the end of 12–13 days treatment of throidectomized rats with 800 ng T_4/100 g body weight with or without 1 mg/100 g body weight propylthiouracil. Brackets indicate SE.

plicitly in numerous studies. Thus, Morreale de Escobar and Escobar del Rey (1967) showed that the inhibition of the deiodination of T_4 and T_3 with propylthiouracil blunted the effect of T_4 only, whereas that of T_3 was not affected. Later, Oppenheimer *et al.* (1972) found that propylthiouracil decreased the rate of T_4 to T_3 conversion *in vivo*, and this was accompanied by a decrease in the rate of induction of α-GPD by T_4. An intriguing aspect of the results shown in

TABLE I

Serum T_3, T_4, and TSH Concentrations in Thyroidectomized Rats[a]

Treatment	*n*	T_4 (μg/dl)[b]	T_3 (ng/dl)[b]	TSH (μU/ml)[b]
T_4 alone	11	3.8 ± 0.2	39 ± 3	117 ± 43
T_4 + PTU	12	5.3 ± 0.3[c]	19 ± 3[c]	391 ± 114[d]

[a] The rats were given 800 ng/100 g body weight T_4 (s.c.) ± 1 mg/100 g body weight propylthiouracil (PTU) (i.p.) for 12–13 days. From Larsen and Frumess (1977).

[b] Values are means ± SE.

[c] $p < .001$ compared to rats given T_4 alone.

[d] $p < .05$ compared to rats given T_4 alone.

Table I was that while serum TSH in the T_4 propylthiouracil rats was slightly, but significantly greater than in those receiving T_4 alone ($p < .05$), it was remarkedly decreased from the levels present in thyroidectomized untreated controls. This suggested that blockade of T_4 to T_3 conversion did not result in as significant effect on T_4-induced TSH suppression as it did on induction of hepatic mitochondrial α-GPD. At this point, it seemed reasonable to suspect that T_4 could affect TSH secretion directly since administration of propylthiouracil blocks about 70% of T_4 to T_3 conversion as calculated from serum T_3 levels (Larsen and Frumess, 1977).

3. Studies of the Acute Effect of T_3 and T_4 on TSH Suppression in Chronically Hypothyroid, Thyroidectomized Rats

Because the results of the above experiments strongly suggested a direct effect of T_4, it was necessary to explore the acute effects of T_3 and T_4 on TSH suppression (Larsen and Frumess, 1977). In Fig. 4 are the results that indicated that 70 ng T_3/100 g body weight results in a rapid decrease in serum TSH to about 50% of the control value. However, this effect is short-lived and TSH has returned to its initial value by 7 hours after injection. It can be seen in the lower

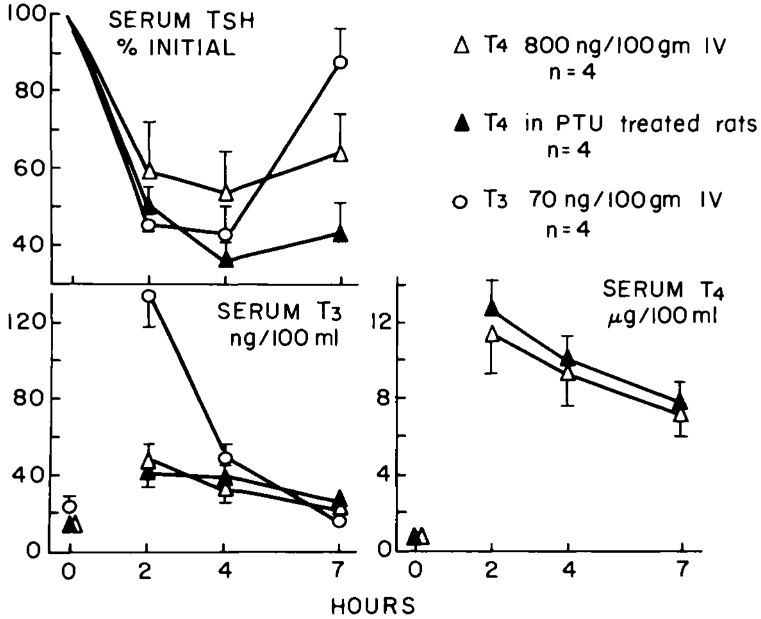

Fig. 4. Effect of i.v. infusion of T_4 or T_3 on thyroidectomized rats. One group (closed triangles) had been pretreated for 1 day with propylthiouracil 1 mg/100 g body weight. Initial serum TSH concentrations ranged from 1100 to 1300 μU/ml and were not different in the three groups. Symbols indicate the mean ± SE. Reproduced from Larsen and Frumess (1977).

TABLE II

Pituitary and Plasma TSH 1 Hour after Intravenous T_3 or T_4 Injection in Chronically Hypothyroid Rats[a]

Group	*n*	Plasma TSH (μU/ml)[b] at time 0	Plasma TSH (μU/ml)[b] at 1 hour	Paired *t* test	Pituitary TSH (mU/mg)[b] at time 0	Pituitary TSH (mU/mg)[b] at 1 hour	*p* value for comparison with vehicle
1 Base line	5	2153 ± 248	—	—	29 ± 6	—	N.S.
2 Vehicle injection	5	2348 ± 423	2135 ± 304	N.S.	—	30 ± 4	—
3 T_4 (1.5 μg/100 g bw[c])	4	2004 ± 489	1342 ± 450	< .005	—	47 ± 6	< .005
4 T_3 (0.15 ng/100 g bw[c])	4	2100 ± 338	1510 ± 282	< .025	—	37 ± 5	< .05

[a] From Silva and Larsen (1978b).

[b] Values are means ± SE.

[c] bw, body weight.

portion of the figure that serum T_3 concentrations have returned to their initial reduced levels at 7 hours, paralleling the increase in TSH. While about 10 times the dose of T_4 is required, this hormone can also rapidly suppress TSH in the serum of hypothyroid rats, but the effect lasts considerably longer and is still present at 24 hours. That this effect is due to the elevation of serum T_4 level per se is indicated by the fact that serum T_3 increased only transiently after the T_4 injection. In addition, pretreatment of rats with 1 mg/100 g body weight phenylthiouracil had no effect on the T_4-induced TSH suppression, as in the particular experiment shown in Fig. 4. TSH was suppressed to a significantly greater degree in those animals receiving T_4 and propylthiouracil than in animals given T_4 alone. Again, these results were consistent with a direct inhibitory effect of T_4 on TSH release. In subsequent studies, we provided evidence that the acute suppression of plasma TSH in chronically hypothyroid rats induced by T_3 or T_4 was associated with an increase in pituitary TSH content (Silva and Larsen, 1978b; Table II). Therefore, this decrease in TSH was presumably secondary to an inhibition of TSH release rather than to a decrease in the rate of TSH synthesis. An increase in pituitary TSH and a significant decrease in plasma TSH could be demonstrated within 1 hour after injection of physiological quantities of either T_3 or T_4. On the basis of our physiological studies, we estimated that the replacement dose in the euthyroid male Sprague–Dawley rat is about 200–210 ng T_3/100 g body weight/day. Therefore, 70 ng T_3/100 g body weight is about one-third of the 24 hour T_3 production in the rat.

III. STUDIES OF T_3 AND T_4 IN PITUITARY TISSUE

A. Pituitary Nuclear Receptors for Thyroid Hormones

Most of our knowledge about the physiocochemical characteristics of the nuclear receptor for thyroid hormones comes from studies of hepatic tissues (Oppenheimer *et al.*, 1974a). These data are reviewed elsewhere in this volume (Chapters 2, 5, and 6). Pituitary cells in culture (GH_1) and mouse thyrotroph tumor cells have also been studied *in vitro* and results of these investigations are presented in Chapters 3 and 11. The same size of the rat anterior pituitary limits physicochemical analyses but receptors in this tissue appear to be identical to those found in the liver and pituitary tumors. Schadlow *et al.* (1972) first observed that T_4 is bound much less strongly than is T_3 to pituitary tissue. The tissue/plasma ratio of T_3 was about 100 times higher than that for T_4. Even if one considers that the free fraction of T_4 may be only 5–10% that of T_3, there was still a T_3/T_4 tissue affinity ratio of 10:1. These authors were unable to show displacement of [^{125}I]T4 from anterior pituitary even by injecting as much as 12 μg T_4/100 g body weight, whereas a similar dose of T_3 produced a fourfold

TABLE III

Maximum Binding Capacity (MBC) and Apparent Dissociation Constant (K_d) for Rat Anterior Pituitary Nuclear T_3 Receptors

Study	MBC (moles/mg DNA)	Apparent K_d (M)
A. *In vivo* measurements		
Euthyroid		
Oppenheimer *et al.* (1974b)	1.2×10^{-12}	7.1×10^{-10}[a]
Silva *et al.* (1978a)	1.2×10^{-12}	8.6×10^{-10}[a]
Hypothyroid (Silva and Larsen, 1978a)	1.5×10^{-12}	1.4×10^{-9}[a]
B. Pituitary tumor		
Rat GH_1 cells (Samuels and Tsai, 1973)	6.5×10^{-13}	2.9×10^{-11}[b]
Mouse thyrotroph tumor (Gershengorn, 1978)	[c]	1.6×10^{-10}[b]

[a] T_3 concentration in rat serum.
[b] T_3 concentration in serum-free medium.
[c] 24×10^{-15} $M/10^6$ cells.

decrease in the pituitary/plasma ratio of T_3. The results suggested that there were no limited capacity binding sites for T_4 in the pituitary though evidence was strong that such sites existed for T_3. In subsequent studies, Oppenheimer *et al.* (1974b) compared binding sites in the anterior pituitary nuclei with those of other tissues of the rat. Although the binding capacity was substantially higher (1.2×10^{-12} M/mg DNA or 0.8 ng T_3/mg DNA) the apparent dissociation constant (K_d) of 7.1×10^{-10} M was comparable to that found for the thyroid hormone nuclear receptor in other tissues suggesting that these nuclear binding sites were similar (Table III). The K_d as used in this discussion refers to the total serum T_3 concentration at which there is apparent 50% occupancy of the nuclear receptors. In subsequent studies of hypothyroid rats, we found a slightly higher binding capacity for T_3 of 1.5×10^{-9} M/mg DNA (1 ng/mg DNA) in anterior pituitary and a K_d of 1.4×10^{-9} M, not significantly different from that we observed for liver and kidney in the same animals (Silva and Larsen, 1978a). In euthyroid rats, our estimate of pituitary binding capacity was identical to that reported by Oppenheimer *et al.* and the K_d was slightly lower than that we observed in hypothyroid rats (Silva *et al.*, 1978a). However, in the analyses of the data from euthyroid rats, neither we nor Oppenheimer *et al.* (1974b) have taken into account the T_3 contributed to the intracellular pool by T_4 to T_3 conversion within the pituitary cells (see Section III,D). In euthyroid rats, the quantity of T_3 from this source is approximately equal to that derived from the serum at normal T_3 concentrations. Therefore, the intracellular T_3 concentration to which the nuclear receptor is exposed is approximately twice that expected from the serum T_3

concentration. Accordingly, the affinity of the pituitary nuclear receptor is somewhat underestimated in the euthyroid rat since these sites are about 80% saturated at endogenous serum T_3 and T_4 concentrations (see Section III,E). Since the relative contribution of T_3 from T_4 to intraceullular T_3 becomes less as serum T_3 concentrations rise, this pool does not greatly influence the estimates of nuclear binding capacity (Silva *et al.*, 1978a). As explained in Section III,E below, this problem is a minor one in liver and kidney since 70% or more of intracellular T_3 derives directly from the plasma. The maximal binding capacity and K_d for pituitary tumor cells in tissue culture are also presented in Table III for comparison. If serum protein binding is taken into consideration, the apparent K_d's are similar to those found in the *in vivo* studies.

2. *Nuclear Binding of T_4 in Pituitary Tissue*

Following injection of 50–100 μCi/100 g body weight of high specific activity T_4, there is little T_4 bound specifically to the pituitary nuclear receptor. Most of the isotope injected as T_4 in the nuclei is present T_3 due to the T_4 monodeiodination and the higher binding affinity of the nuclear receptors for this particular hormone. Although formal *in vivo* comparative studies of T_3 and T_4 binding to pituitary nuclear receptors have not been reported (and would be difficult to interpret unless T_4 monodeiodination were blocked simultaneously), the low fraction of cellular T_4 bound to nuclei suggests that these receptors, like those of the liver and of GH_1 cells, may have a much higher affinity for T_3 than for T_4. The importance of T_4 to T_3 conversion to the low binding of T_4 in pituitary nuclei is confirmed by the observation that tracer T_4 binding increased approximately twofold when pituitary T_4 to T_3 conversion was completely inhibited (Larsen *et al.*, 1979; see Section III,D,2). In summary, in anterior pituitary, intracellular concentrations of T_3 and T_4 are such that virtually all the thyroid hormone specifically bound to nuclear receptors is T_3. This, of course, bears importantly on the question of why most of the effects of administered T_4 can be explained on the basis of its conversion to T_3

B. Subcellular Distribution of T_3 and T_4 in Anterior Pituitary Tissue

As mentioned, Schadlow *et al.* (1972) were able to demonstrate displaceable binding of T_3 in whole tissues only in pituitary. When liver and brain were studied under similar experimental conditions, there was no decrease in the fraction of the dose present per gram of tissue as the T_3 dose was increased. Similar data with respect to brain, pituitary, and liver were obtained by Gordon and Spira (1975). The explanation for this observation is that roughly 40–50% of intracellular T_3 is bound to the limited capacity nuclear receptors of the anterior pituitary tissue, whereas in kidney, liver, heart, and brain only 5–10% of the intracellular T_3 is found within the nucleus (Oppenheimer *et al.*, 1974b). These results indicate that over 90% of the T_3 in tissues other than anterior pituitary is bound to cytosolic proteins with either relatively low affinity or high capacity. On the other hand, since about 50% of the pituitary T_3 is bound to nuclear receptors with a K_d of approximately 2×10^{-9} *M* and a limited binding capacity, the displacement observed in whole tissue can be attributed to the displacement occurring from the limited capacity, nuclear receptor binding sites.

Nevertheless, another important consideration is whether or not there are "receptors" in other subcellular fractions. Several workers have found that pituitary cytosolic proteins can bind thyroid hormone (Sufi *et al.*, 1973; Galton, 1977). The K_d of such proteins for T_4 is of the order of 10^{-9} to 10^{-8} *M* and even greater for T_3, and their binding capacity is extremely high. Therefore, the biological effects of T_3 on anterior pituitary take place over concentrations during which there is no significant change in the saturation of such proteins. These pituitary intracellular binding proteins thus resemble more closely the circulating plasma thyroid hormone-binding proteins than they do the nuclear receptor proteins. In confirmation of these predictions are data shown in Fig. 5 derived from unpublished observations of J. Joffe, J. E. Silva, and P. R. Larsen. When hypothyroid rats were given [^{131}I]T3 with or without 400 ng T_3/100 g body weight, there was displacement of isotope only in the nuclear fraction. No displacement of label was found in mitochondria, microsomes, or cytosolic fractions as indicated. These results are similar to those reported for liver by Oppenheimer *et al.* (1974a) and suggest strongly that there are no subcellular

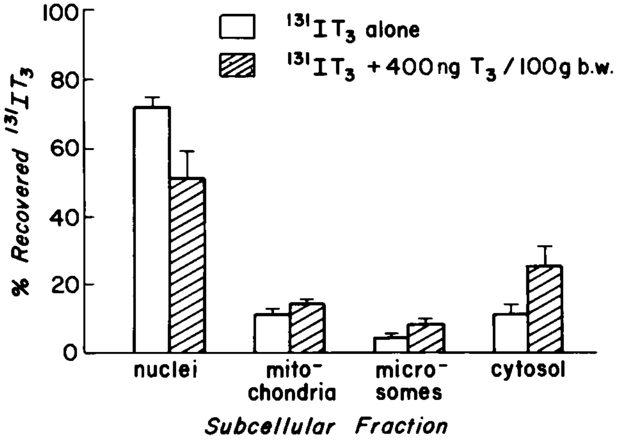

Fig. 5. Distribution of [^{131}I]T_3 in subcellular fractions of anterior pituitary 2 hours after i.v. injection. Animals received tracer T_3 ± 400 ng T_3/100 g body weight. The brackets indicate the SD.

fractions other than the nucleus, which contain receptors with a high enough affinity to show changes in saturation over the physiological range for serum T_3 concentrations.

Only a small and inconstant fraction of injected tracer T_4 is present in the nucleus of hypothyroid rats as outlined above. The interpretation of the physiological significance of a small degree of T_4 binding is further clouded by the fact that even a minute contamination of pituitary homogenate with plasma can artifactually increase the apparent quantity of nuclear T_4. Aside from studies of T_4 binding to the nucleus, there are no further studies of the subcellular distribution of T_4 within the pituitary.

C. Time Course of Nuclear T_3 Binding and Correlation with the Suppression of TSH Release

Oppenheimer *et al.* (1974b) found that tracer T_3 in the pituitary nuclei of euthyroid rats reaches a peak about 90 minutes after intravenous injection. This time, denoted t_m by these investigators, is the time that can be shown kinetically to be that at which the specific activity of T_3 in the cytoplasm plasma compartment and that fraction of the nuclear T_3 derived from plasma T_3 are identical (Oppenheimer *et al.*, 1974a). Because the specific activity of these two compartments is the same, the nuclear/plasma ratio for tracer T_3 at the t_m can be used to estimate the gravimetric nuclear T_3 contribution from the plasma T_3 RIA. In our studies of euthyroid rats, we have found that we are not generally able to identify a single t_m, but find that nuclear T_3 in pituitary is relatively constant between 1 and 2 hours after T_3 administration (Silva *et al.*, 1978a).

When 70 ng T_3/100 g body weight were injected into chronically hypothyroid rats in experiments similar to those discussed earlier (Section II,B,3), we observed the results presented in Fig. 6 (Silva and Larsen, 1977). Thyrotropin suppression occurred rapidly, reached a nadir between 2 and 4 hours and by 7 hours had returned to the initial elevated level. These results are identical to those obtained several years earlier (Larsen and Frumess, 1977). Nuclear T_3 rises rapidly after T_3 injection reaching a peak between 1 and 2 hours and falls over the next 4–5 hours at a rate which is parallel to the decrease in plasma T_3. The amount of nuclear T_3 specifically bound at the time of peak occupancy was equivalent to 0.45 ng T_3/mg DNA occupying approximately 45% of the available binding sites in the hypothyroid pituitary. The nadir in plasma TSH occurred about 60 minutes after the peak of nuclear occupancy was reached. There were two aspects of these results which were of extreme interest: the obvious chronological inverse relationship between nuclear T_3 binding and the suppression of TSH release and (2) the integrated saturation of the nuclear receptors by T_3 (about 45%) closely approximated the degree to which TSH release was inhibited.

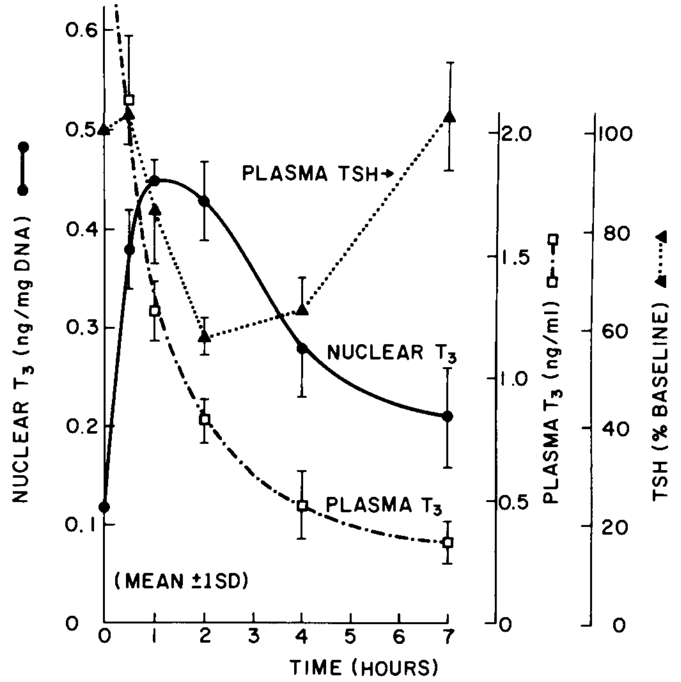

Fig. 6. Time course of pituitary nuclear T_3 and plasma T_3 and TSH after administration of a single intravenous dose of 70 ng T_3/100 g to chronically hyopthyroid rats. Each point is the mean ± SD for four rats. The mean ± SD of plasma TSH at time 0 was 1236±558 μU/ml (normal range 50–150 μU/ml). Reproduced from Silva and Larsen (1977).

To clarify whether there was a quantitative relationship between nuclear T_3 binding in the pituitary and the degree of suppression of TSH release, we performed another series of experiments using increasing quantities of injected T_3 (Silva and Larsen, 1978a). Rats were killed 3 hours after T_3 injection, this time being chosen because TSH was at its nadir. Because the specific activity of the T_3 was known, it was possible to calculate the gravimetric quantities of nuclear T_3 based on the plasma T_3 as measured by radioimmunoassay. When the degree of suppression of TSH as a percentage of the basal TSH and the estimated nuclear occupancy were plotted as a function of the plasma T_3, the curves were superimposable (Fig. 7). Because there is a short (~60 minute) delay between nuclear occupancy and plasma TSH suppression and nuclear occupancy did not change dramatically between 1 and 3 hours (Fig. 6), the results indicate that nuclear occupancy and TSH suppression bear a linear relationship. This is shown graphically in Fig. 8 where the calculated integrated occupancy between 0 and 3 hours for each dose and the 3 hour occupancy is plotted as a function of the TSH decrement.

It is tempting to speculate from these results that the rapid suppression of TSH release in the hypothyroid rat induced by T_3 occurs via a mechanism that requires binding of T_3 to the nuclear receptor. Such a mechanism would require that there be a protein (or proteins) synthesized as a result of changes in messenger RNA and that this protein somehow interferes with the release of TSH from the thyrotroph. This mechanism would be consistent with previous studies that have demonstrated that inhibitors of protein synthesis or of DNA-directed RNA synthesis appear to block the *in vitro* suppression of TSH release by T_4 or T_3 at least as manifested by a system in which TRH is employed as the stimulating factor (Bakke and Lawrence, 1978; Schally and Redding, 1967; Bowers *et al.*, 1968; Vale *et al.*, 1968). Alternatively, T_3 could directly repress the synthesis of some proteins as shown recently by Ivarie *et al.* (1981) and Seelig *et al.* (1981). This possibility is intriguing because T_3 depresses the overall incorporation of [^{3}H]uridine into RNA in the anterior pituitary and thyroidectomy enhances it (Lee *et al.*, 1967). There are, however, objections to this hypothesis. First, the effect of T_3 on the pituitary is extremely rapid, probably beginning within 30 minutes after injection. This is a very short time for the complete synthesis of a

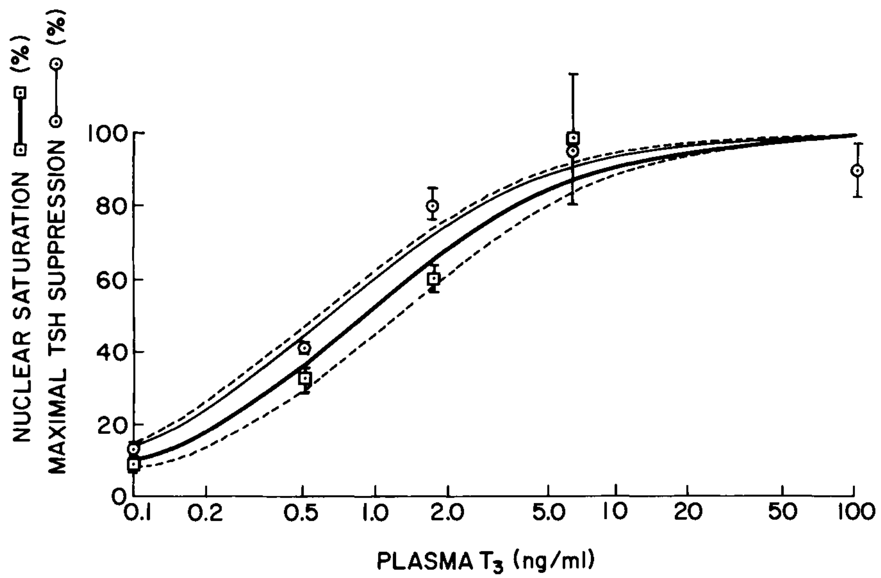

Fig. 7. Pituitary nuclear saturation and maximal TSH suppression plotted as a function of plasma T_3 concentration in chronically hypothyroid rats injected with increasing quantities of T_3 3 hours previously. The heavy line (open squares) is the curve describing nuclear saturation vs. plasma T_3, and the dotted lines indicate the 95% confidence limits of the regression coefficient. The thin line (open circles) relates TSH suppression to plasma T_3 in the same animals. Reproduced from Silva and Larsen (1978a).

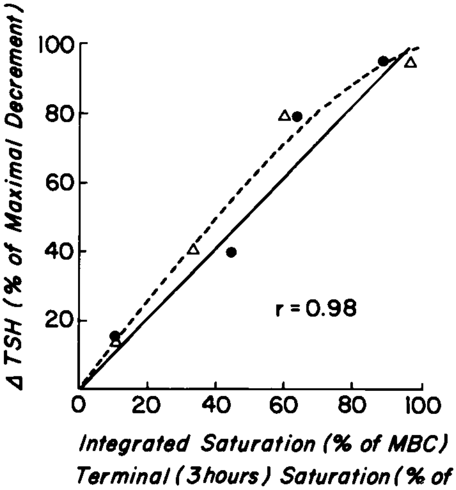

Fig. 8. Correlation between the integrated or terminal saturation of pituitary nuclear receptors with the change in TSH after infusion of increasing doses of T_3 3 hours earlier. Using the time course of nuclear T_3 from Fig. 6, the saturation at each point up to 3 hours was plotted as a fraction of the maximal binding capacity (MBC). The area under the curves was graphically determined and expressed as a function of maximal saturation (100%) over the 3 hour time period. This is the "integrated" saturation on the abscissa and is plotted as a solid line (closed circles). The "terminal" saturation of the nuclear receptors is also plotted for comparison. This is the percent saturation observed at the time the animals were killed. There is a linear relationship between the decrement in TSH and either the integrated or the terminal saturation with the slope of both lines not significantly different from 1.0.

protein starting at the transcriptional level. Second, it is well known that when T_3 is given on a chronic basis the synthesis of TSH in the anterior pituitary is suppressed. This effect is not apparent at the early time intervals that we are concerned with in these experiments, but its presence would imply a diphasic effect, both mechanisms operating through binding of T_3 to a nuclear receptor. It is, of course, possible that the same protein could suppress TSH release as well as suppressing TSH synthesis but this seems unlikely.

Possible alternatives that may not be mutually exclusive should be mentioned. Studies of pituitary fragments showed that TRH binding to this tissue was reduced by exposure to T_4 (DeLean *et al.*, 1977). Recent studies of Gershengorn (1978) have indicated that T_3 decreases the number of TRH receptors in thyrotroph tumors, an effect that is rapidly reversible upon removal of T_3 from the culture medium (see Chapter 11). This has also been shown for a prolactin producing rat pituitary tumor cell line (GH_3) by Perrone and Hinkle (1978). However, the latter investigators have more recently shown that the concentration of T_3 required to reduce TRH receptor levels to less than 50% of their initial number in a GH_1 tumor cell line occupies only 2–3% of the nuclear receptors and is 45-fold lower than the quantity of T_3 required to induce half-maximal growth hormone synthesis in the same cells (Hinkle *et al.*, 1979). The latter process correlated quite well with nuclear receptor occupancy. Taken together, these results suggest that depletion of TRH receptor sites in this tumor cell line may occur via mechanisms that do not necessarily involve nuclear receptor binding of T_3. However, because much higher concentrations of serum free T_3 are necessary to suppress TSH release *in vivo,* as shown above, one should be cautious in interpreting these *in vitro* data.

In addition to suppression of the numbers of TRH receptors as a mechanism for the T_3 effect, the possibility that there are alterations in the TRH secretory rate itself should be mentioned. Several investigators have found that anti-TRH antibody given intravenously to hypothyroid rats leads to an acute decrease in serum TSH (Harris *et al.*, 1978; Szabo *et al.*, 1978; Chihara *et al.*, 1978; Mori *et al.*, 1978). It should be noted that the degree to which TSH is decreased in such experiments is rarely more than 50% and may be considerably less. In euthyroid rats, similarly treated, the decrease is more impressive. These results point to the pituitary as opposed to the hypothalamus as a major feedback site regulating TSH secretion in the hypothyroid animal, though significant suppression of TSH in hypothyroid monkeys has been achieved with intrahypothalamic T_3 injections (Belchetz *et al.*, 1978). Despite these possible explanations, the quantitative and chronological results provide strong, albeit circumstantial, support for the hypothesis that there is a causal relationship between nuclear T_3 binding and suppression of TSH release.

D. Metabolism and Biological Effects of T_4 Given *in Vivo*

1. T_4 to T_3 Conversion, Nuclear T_3 Binding, and TSH Suppression after T_4 Injections

In Section II,B,3, it was shown that T_4 can suppress TSH release as rapidly as can T_3. To test the hypothesis that nuclear receptor binding of iodothyronine was necessary for suppression of TSH, this acute effect of T_4 was studied. As mentioned, there is little binding of T_4 to the anterior pituitary nuclear receptor. To discover the events occurring in the pituitary nucleus subsequent to T_4 injection, the experiments shown in Fig. 4 were repeated. Eight hundred ng T_4/100 g body weight of T_4 was given together with 100–250 μCi $[^{125}I]T_4$ (Silva and Larsen, 1977). Nuclei were isolated, iodothyronines extracted, and the radioactivity chromatographed in various systems. $[^{131}I]T_3$ was given simultaneously to allow recovery studies of the $[^{125}I]T_3$ contaminating the T_4 and also as a marker for any $[^{125}I]T_3$ that might be generated during the experiment. A typical chromatograph of pituitary nuclear radioactivity is shown in Fig. 9. It was apparent that while there was specifically bound $[^{125}I]$iodothyronine in the nucleus, vir-

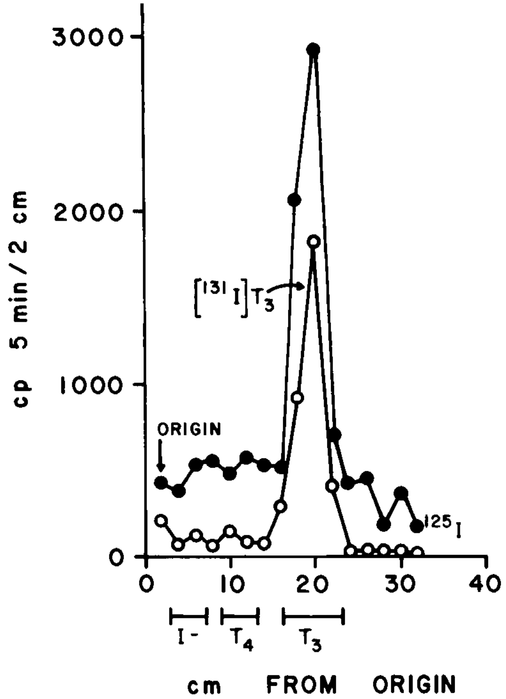

Fig. 9. Representative chromatograph of pituitary nuclear radioactivity 24 hours after $[^{125}I]T_4$ and 2 hours after $[^{125}I]T_3$ injection into a euthyroid rat. The nuclear radioactivity was extracted with acid–butanol and applied to 3 mm Whatman chromatography paper. Strips were developed for 24 hours in tertiary amyl alcohol–hexane–2 *N* NH_4OH for 24 hours. Carrier I^-, T_3, and T_4 were also added to the origin and identified by color reactions. The observed peaks accounted for all of the radioactivity added to the strip. Reproduced from Silva *et al.* (1978a).

tually all of this was T_3. The results shown in this figure are from a euthyroid rat but are identical to what was observed in chronically hypothyroid animals. In Table IV are the results of the quantitative analysis of nuclear iodothyronines in animals given 70 ng T_3 vs. those given 800 ng T_4/100 g body weight. Since the specific activity of the administered T_4 was known, it was possible to calculate the quantities of T_3 present from the counts present in the nucleus. The amount of nuclear T_3 after 800 ng T_4/100 g body weight was identical to the quantity found after 70 ng T_3/100 g body weight. In the last column of this table is presented evidence that equal quantities of nuclear T_3 result in identical suppression of TSH release. Because the nuclear T_3 after T_4 injection could derive either from the plasma or from intrapituitary T_4 to T_3 conversion, it was necessary to have a precise estimate of plasma T_3 in these animals. Obtaining the latter was not a simple matter since it can be determined that the ratio of $[^{125}I]T_4$ to $[^{125}I]T_3$ might be as high as 100–300/1 at these short intervals after T_4 injection. Because

TABLE IV

Specifically Bound Nuclear and Plasma T_3 in Rat Anterior Pituitary 2 Hours after T_3 or T_4 Injections[a]

Treatment	Nuclear T_3 (ng/mg DNA)[b]	Plasma T_3 (ng/ml)[b]	Plasma TSH (% of basal)[b]
70 ng T_3/100 g bw[c]	0.43 ± 0.04	0.73 ± 0.07	55 ± 13
800 ng T_4/100 g bw[c]	0.40 ± 0.04	< 0.06	67 ± 12
p value for difference between T_3 and T_4	N.S.	$p < .001$	N.S.

[a] From Silva and Larsen (1977).
[b] Values are means ± SE.
[c] bw, body weight.

it is recognized that paper chromatography of T_4 alone can lead to the generation of 0.5–1% T_3 (Larsen, 1971), simple extraction of the serum and chromatography would not be a satisfactory method for precise quantitation of the T_3 present. We therefore employed a method developed in our laboratory that employs the principles of affinity chromatography to achieve this separation (Zimmerman *et al.*, 1978). Using anti-T_3 antibody–agarose conjugates, the ratio of T_4 to T_3 in the sera can be reduced about 100-fold. Subsequently, the agarose-bound iodothyronines are extracted and chromatographed. Since recent studies have shown that at relatively short time intervals after tracer T_3 injection, 10–30% of the tracer is TCA precipitable but not T_3, the agarose method is now also used to estimate the residual plasma $[^{131}I]T_3$. The data presented in Table IV show that the quantity of T_3 present in the serum after T_4 is at least 10-fold lower than that required to account for the quantity of T_3 present in the nuclei of the anterior pituitary tissue. Thus, we concluded that T_4 was being converted to T_3 were rapidly by the pituitary tissue and a portion of this T_3 was bound to the nuclear receptor. Although our results at the time were at variance with two careful investigations that yielded no evidence of T_4 to T_3 conversion in pituitary tissue (Galton, 1976; Chopra *et al.*, 1978), our subsequent studies have established beyond question that this reaction occurs *in vitro* as well as *in vivo* (see Section IV). Thus, the same degree of acute suppression of TSH release was induced by identical quantities of nuclear T_3 whether this T_3 derived from the injection or was generated largely within the pituitary from injected T_4. The possibility that T_4 facilitated the transport of T_3 into the nucleus could be ruled out since injected unlabeled T_4 did not alter the nuclear/plasma ratio for tracer T_3 (Silva and Larsen, 1977). In further studies in hypothyroid rats, it was shown that the distribution of the T_3 derived from T_4 in the pituitary tissue was not significantly different from the T_3 derived from the plasma (Silva and Larsen, 1978a). It appeared that the T_3 generated from T_4 mixed completely in the intracellular T_3 pool and did not appear to have preferential access to the nuclear receptor.

2. *Contribution of Intrapituitary T_4 to T_3 Conversion to Nuclear T_3 in Euthyroid Rats in Tracer Studies*

In later experiments, we determined the contribution of intrapituitary T_4 to T_3 conversion to the total T_3 in anterior pituitary tissue (Silva *et al.*, 1978a). To accomplish this, we determined the equilibrium ratio between the nuclear T_3 derived from T_4 by intrapituitary conversion of T_4 to T_3, which we have denoted $T_3(T_4)$, to the plasma T_4. Multiplication of this ratio by the plasma T_4 concentration gave an estimate of the contribution of this source to nuclear T_3. Between 12 and 18 hours were required for nuclear $T_3(T_4)$ to peak following tracer T_4 injection. The ratio of locally generated nuclear $T_3(T_4)$ (% dose/mg DNA) to plasma T_4 (% dose/ml) was constant after this time and was 13 ± 2 (SE) $\times 10^{-3}$ ng T_3/mg DNA/ng T_4/ml serum. Using this ratio, we determined that intrapituitary T_4 to T_3 conversion contributed approximately 50% of the nuclear T_3 and that the nuclear (or intracellular) T_3 in the pituitary was roughly twice the concentration that one would anticipate from the plasma T_3 alone. This indicated that since approximately 40–50% of the nuclear receptor binding sites are occupied by T_3 derived from plasma, about 80% of the nuclear receptor sites in the pituitary gland are occupied by T_3 at endogeneous T_3 and T_4 concentrations. Comparison of pituitary tissue with liver, kidney, and heart with respect to the sources in intracellular T_3 is discussed in Section III,E. It would appear that the high degree of saturation of the receptors in the normal pituitary could help explain the 10- to 15-fold lower rate of TSH secretion in the euthyroid as opposed to the chronically hypothyroid rat (Silva and Larsen, 1978b; Surks and Lifschitz, 1977).

3. *Evidence Indicating That T_4 to T_3 Conversion Is Required for the Biological Effect of T_4*

As previously stated, pretreatment of hypothyroid rats with propylthiouracil did not blunt the TSH suppression which followed T_4 injection. This suggested a direct role for T_4 in this effect. When $[^{125}I]T_4$ was given to rats after propylthiouracil, there was no inhibition of intrapituitary T_4 to T_3 conversion (Silva and Larsen, 1978a). Since the quantity of nuclear T_3 at these short times after T_4 injection was identical whether or not propylthiouracil was given, the identical suppression of TSH release was therefore explained but another agent was required to evaluate the role of T_4 to T_3 conversion in this response. To attempt to inhibit conversion in the pituitary, we pretreated rats with iopanoic acid (Telapague), an iodinated contrast agent used clinically for visualization of the gallbladder. It had been suggested by Bürgi *et al.* (1976) that this compound could inhibit T_4 to T_3 conversion in patients, and Kaplan and Utiger (1978a) later

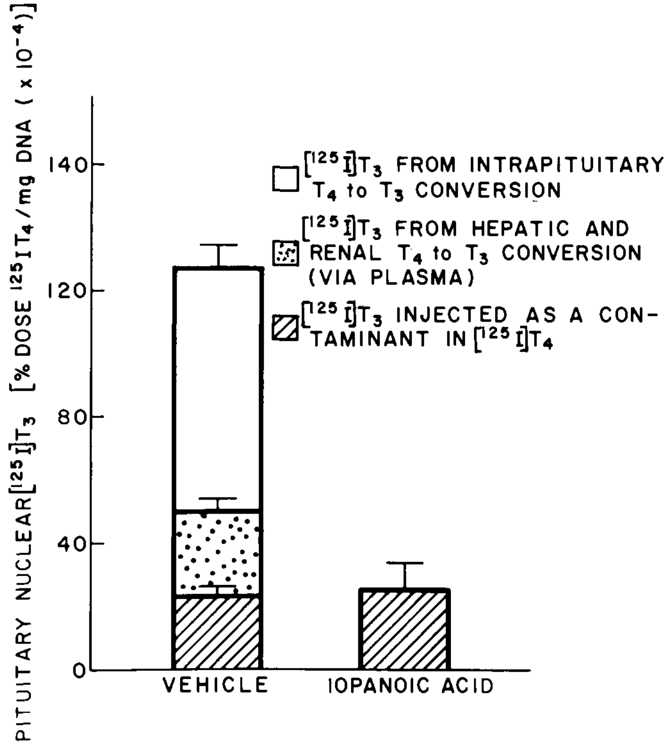

Fig. 10. Effect of iopanoic acid treatment on pituitary nuclear T_3 3 hours after $[^{125}I]T_4$ injection. The quantity of T_3 derived from contaminant was determined by recovery of simultaneously injected $[^{131}I]T_3$. The contribution of newly generated plasma $[^{125}I]T_3$ to nuclear $[^{125}I]T_3$ was determined by multiplication of the nuclear/plasma ratio for T_3 at 90 minutes in hypothyroid rats by the quantity of plasma $[^{125}I]T_3$ not accounted for by the $[^{125}I]T_3$ contaminating the injected $[^{125}I]T_4$. The open portion of the "vehicle" bar indicates the residual nuclear $[^{125}I]T_3$ which was derived from intrapituitary T_4 to T_3 conversion. Treated animals received 5 mg/100 g body weight iopanoic acid 24, 16, and 1½ hours prior to $[^{125}I]T_4$.

demonstrated that in liver homogenates, it was a potent inhibitor of iodothyronine 5′-monodeiodination. Administration of this agent to rats caused complete inhibition of *in vivo* T_4 to T_3 conversion, not only in liver and kidney but also in the pituitary gland. In Fig. 10, the effect of iopanoic acid on the nuclear $[^{125}I]T_3$ after $[^{125}I]T_4$ is shown (Larsen *et al.*, 1979). It is apparent that the quantity of nuclear $[^{125}I]T_3$ present after $[^{125}I]T_4$ injection is reduced to an amount that can be attributed to the $[^{125}I]T_3$ contaminant in the $[^{125}I]T_4$. Thus, this agent was an ideal tool for testing the hypothesis that conversion to T_3 was required for the T_4 effect in the pituitary. When TSH suppression by T_3 or T_4 was evaluated in animals pretreated with iopanoic acid, the results shown in Fig. 11 were obtained (Larsen *et al.*, 1979). Eight hundred ng of T_4/100 g body weight caused approximately 50% suppression of TSH release in control animals whereas in animals pretreated with iopanoic acid, there was no suppression of TSH release. On the other hand, neither the nuclear T_3 binding nor the effect of T_3 on TSH suppression was influenced by iopanoic acid pretreatment. This indicated that generation of T_3 in the pituitary was necessary for the biological effect of T_4. Obregon *et al.* (1979b) have recently reported identical results in experiments in which induction of growth hormone synthesis was used as the biological end point of T_4. This suggests that, in both thyrotrophs and somatotrophs, conversion to T_3 is required for biological activity of physiological

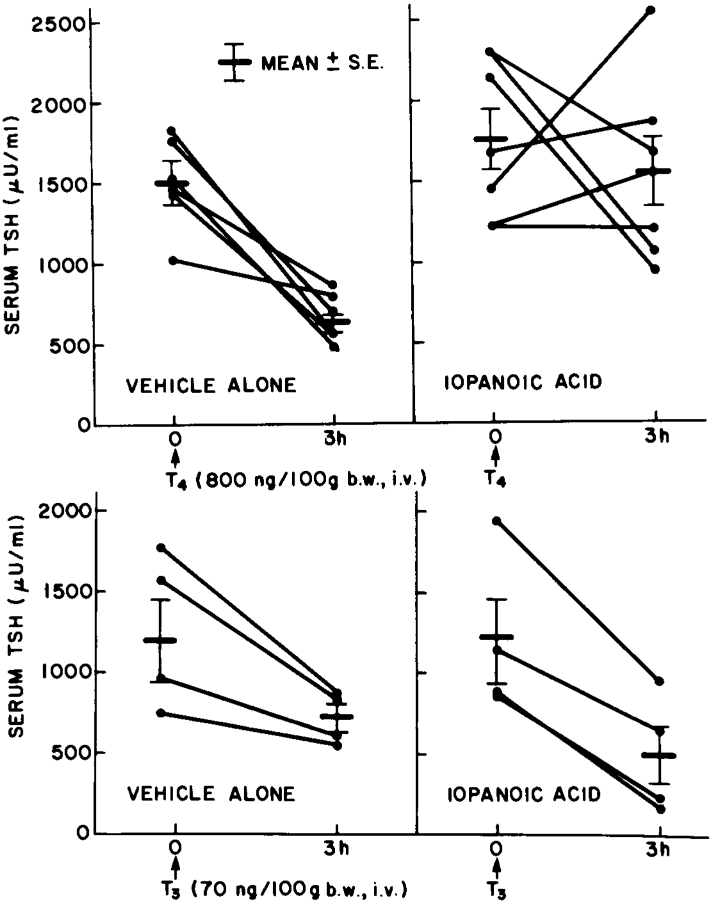

Fig. 11. Acute response of chronically hypothyroid rats to 800 ng T_4 or 70 ng T_3/100 g bw i.p.; one group received vehicle alone (alkaline 50% solution of propylene glycol) and the other group received three injections of iopanoic acid, 5 mg/100 g bw, i.p. 24, 16, and 1½ hours before injection of iodothyronines. Reproduced from Larsen *et al.* (1979).

TABLE V

Effect of Iopanoic Acid-Induced Blockade of Pituitary T_4 to T_3 Conversion on the Nuclear Binding of $[^{125}I]T_3$ and $[^{125}I]T_4$ Injection[a]

	$\left(\frac{\text{Nuclear } [^{125}I]T_3}{\text{Nuclear } [^{125}I]T_3 + [^{125}I]T_4}\right) \times 100$ (mean ± SE)		
	Exp. A	Exp. B	Exp. C
Control	71 ± 2	82 ± 2	76 ± 4
Iopanoic acid	43 ± 3	38 ± 2	31 ± 7
p value	< .005	< .001	< .005

[a] From Larsen *et al.* (1979).

quantities of T_4. Inspection of Fig. 11 shows that there is a heterogeneous response in the animals given T_4 plus iopanoic acid. In two, there was suppression of TSH, whereas in the rest there was not. When larger doses of T_4 (1.6 μg T_4/100 g body weight) were given to iopanoic acid-treated animals, suppression of TSH release does occur but always to a lesser extent than occurs in vehicle-treated rats. Therefore, iopanoic acid shifts the T_4 dose–response curve to the right. The explanation for this phenomenon may lie in the fact that when conversion to T_3 is blocked by iopanoic acid, nuclear binding of T_4 increases. Table V shows that in control experiments 70–80% of the nuclear $[^{125}I]$iodothyronine was T_3, whereas only 40% of the $[^{125}I]T_3$ iodothyronine was T_3 in iopanoic acid-treated rats. Nuclear T_4 binding approximately doubled in these experiments due to a decrease in the intracellular concentration of the competing ligand, T_3. If sufficient numbers of receptor sites were occupied by T_4, instead of T_3, a response could occur. Papavisiliou *et al.* (1977) have previously shown in experiments with GH_1 cells that reverse T_3 or even 3,3′-diiodothyronine can induce growth hormone synthesis if sufficient quantities are present in the medium to saturate nuclear receptor sites. Thus, it would appear that any iodothyronine analog capable of binding to the specific nuclear receptor site can activate thyroid hormone-dependent nuclear mechanisms, and it is no surprise that this is the case for T_4 as well. However, it would appear that under the normal physiological circumstances and at the concentrations of T_4, which obtain in normal rats, T_4 acts via its conversion to T_3. It is of interest that Obregon *et al.* (1979a) found the total content of pituitary T_3 and T_4 to be equal. Therefore, if the intracellular binding of these two hormones were exactly the same in the pituitary, one would expect roughly 10 times as much T_3 as T_4 to be present in the nucleus. Because T_4 is more firmly bound in cytosol than is T_3, an even higher nuclear T_3 to T_4 ratio would be anticipated. Although we have found as much as 20–30% of the nuclear iodothyronine is T_4, it should be reemphasized that a small amount of plasma contamination will artifactually increase the apparent nuclear T_4.

In the above experiments with T_4, there was no question that deiodination to T_3 had to occur in order to produce an effect at the pituitary level. The data are also consistent with the hypothesis that it is the nuclear receptor binding of T_3 that initiates the events leading to suppression of TSH release since there is an identical quantitative suppression of TSH release regardless of the source of T_3. This provides added support for the putative function of the nuclear receptor in the acute TSH suppression process. However, the relationship remains unproved until such time as the intermediate steps between T_3 entrance into the pituitary thyrotroph and inhibition of TSH release are identified.

E. Comparison of T_4 to T_3 Conversion as a Source of Nuclear T_3 in Pituitary as Opposed to Other Tissues of the Rat

If the physiological discrepancy between TSH concentrations, on the one hand, and metabolic status, on the other, in situations where T_3 and T_4 are altered independently is to be explained by intrapituitary T_4 to T_3 conversion, then local T_4 to T_3 conversion could not make a substantial contribution to intracellular T_3 in every tissue. Otherwise, metabolic abnormalities would be observed when serum T_3 is normal and serum T_4 is depressed. We have shown that the hepatic content of mitochondrial α-GPD is proportional to serum T_3, not T_4. We therefore predicted that, in hepatic and perhaps renal tissue as well, nuclear T_3 would derive largely from the serum. This prediction was substantiated in parallel experiments to those in which anterior pituitary was evaluated. Locally derived $T_3(T_4)$ accounted for 28% of nuclear T_3 in the liver and only 14% of the renal nuclear T_3 (Silva *et al.*, 1978a). In earlier studies of Surks and Oppenheimer (1977), excellent agreement was observed between the hepatic and renal nuclear T_3 by RIA and that predicted from either isotopic equilibration studies or use of the nuclear/serum T_3 ratio and serum T_3 concentrations. Although our studies would predict that a discrepancy should have been seen in the liver nuclei because of the 25–30% $T_3(T_4)$ contribution in this tissue, both results indicate a much less important role for $T_3(T_4)$ in the liver and kidney than in the pituitary. In more recent studies, cardiac muscle and brain have been evaluated. The former appears to depend virtually entirely on serum T_3 for nuclear T_3 (Larsen *et al.*, 1979). In contrast, both in cerebral cortex and cerebellum most of the nuclear T_3 may well derive from intracellular T_4 monodeiodination (Crantz and Larsen, 1980). As in the anterior pituitary, nuclei from cerebral cortex are highly saturated (≃90%) and ⅘ of the T_3 found comes from intracellular 5′-monodeiodination of T_4 (Crantz *et al.*, 1980). The mechanism responsible for these differences between tissues are not known. Furthermore, in hypothyroid rats there is a striking increase in T_4 to T_3 conversion and a decrease of T_3 degradation (Kaplan and Yaskoski, 1980), whereas in the liver of hypothyroid rats there is a decrease in T_3 generation from T_4. Altogether these findings suggest intracellular mechanisms constitute another level of regulation of intracellular concentration of T_3.

The studies referred to have all been performed using tracer injections with certain assumptions required regarding the specific activity of T_3 in various tissue compartments. While these assumptions are reasonable, it would be desirable to confirm these results using independent estimates of the quantity of nuclear T_3. The radioimmunoassay approach employed by Surks and Oppenheimer (1977) referred to above is one such method we have recently applied to anterior pituitary (Larsen *et al.*, 1980). In these experiments, euthyroid rats were given tracer doses of T_3. The serum T_3 was measured and the nuclear/serum ratio for T_3 was calculated. In addition, in the same animals, nuclear T_3 was extracted and quantitated by radioimmunoassay. The measured nuclear T_3 was compared with the expected nuclear T_3 which was based on the nuclear/serum ratio and serum T_3 concentration. The expected nuclear T_3 (plasma T_3 contribution) was 0.23 ±0.02 ng T_3/mg DNA whereas the observed quantity of nuclear T_3 by radioimmunoassay was 0.63±0.04 ng T_3/mg DNA. Thus, we estimated that the contribution of locally generated $T_3(T_4)$ to nuclear T_3 was about 65% whereas the tracer studies cited above had suggested a figure of approximately 50%. Given the difficulties of both methods, this agreement is excellent. The estimated anterior pituitary nuclear T_3 by radioimmunoassay, 0.63 ng/mg DNA, is precisely that which was calculated from the tracer studies in euthyroid rats. The total anterior pituitary T_3 content determined by RIA was 92 pg (10.5 pg/mg wet weight) since 30% of the tracer T_3 within the anterior pituitary was recovered in the nuclear pellet. These estimates are in reasonably good agreement with earlier total pituitary T_3 measurements of Obregon *et al.* (1979a) of 70 pg and of Gordon and Spira (1975) of 6.8 pg/mg wet weight.

Both lines of investigation point to marked differences between pituitary (and brain) on the one hand, and the liver, kidney, and heart, on the other, in the importance of tissue T_4 to the generation of intracellular T_3. The reasons for the differences between the various tissues are not understood at present. There are no reliable measurements of endogenous T_4 to T_3 conversion in any tissue *in vivo* which obviates comparisons between them. Because the intracellular distribution of locally generated $T_3(T_4)$ and $T_3(T_3)$ are identical and since about 50% of the T_3 in the anterior pituitary is in the nucleus, 5 times as much of the T_3 generated from T_4 in the pituitary will be bound to the nuclear receptor than would be the case in the liver or kidney where only 5–10% of intracellular T_3 is present in the nucleus. Anatomical differences in the location of the T_4 5′-monodeiodinase in the two types of tissues such that more ready access to plasma is available for the T_3 formed from T_4 in the liver than is the case in the pituitary is another possible explanation.

Figure 12 shows a schematic diagram comparing the sources of T_3 in the

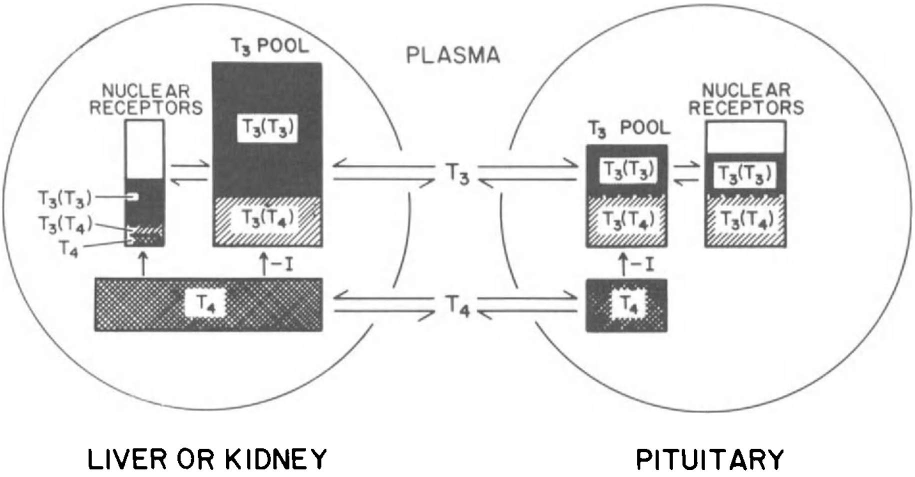

Fig. 12. Schematic comparison of the sources of intracellular and nuclear iodothyronines in liver or kidney as opposed to anterior pituitary. The relative sizes of the T_3 compartments within each cell are proportional to the pool sizes according to our present understanding. The height of the bar symbolizing the nuclear receptors represents the maximal binding capacity for T_3 of these receptors in the two types of tissues. The T_3 derived from plasma, $(T_3)T_3$, and the T_3 derived from intracellular T_4 to T_3 conversion, $T_3(T_4)$, are thought to mix completely within the cell based on results of Silva and Larsen (1978a).

pituitary with those in the liver or kidney cell. The relative sizes of the T_3 pools within each tissue are proportional to the size of the respective rectangles. Although we have concentrated on nuclear T_3 in this discussion because it seems most likely that this is the physiologically active hormone pool, this structure samples indiscriminately the total intracellular T_3. Thus, the present arguments apply to any mechanism requiring intracellular T_3 for its activation. Figure 12 shows that only 20–30% of intracellular T_3 in the liver derives from T_4 to T_3 conversion. As can be appreciated from this figure, the discrepancy between total radioimmunoassayable nuclear T_3 and that derived from plasma T_3 alone would likely be so small as to be undectectable in the radioimmunoassay studies, whereas a considerable difference should be apparent in the nuclear T_3 in pituitary between that found by radioimmunoassay and that predicted on the basis of plasma T_3. Little, if any, T_4 is found within the nucleus of the pituitary whereas there appears to be a small quantity of T_4 specifically bound to the nuclear receptor of the liver. Precise quantitation of this T_4 nuclear binding is quite difficult due to the problems encountered in eliminating cytosolic proteins from the liver nuclear preparations. The diagram illustrates why a reduction in serum T_4 with maintenance of a constant serum T_3 would cause a significant reduction in intracellular T_3 in anterior pituitary, but not in the liver of kidney.

IV. STUDIES OF PITUITARY T_4 TO T_3 CONVERSION *IN VITRO*

A. Experiments Using Intact Anterior Pituitary Gland Fragments

The studies cited clearly indicated that T_4 was rapidly converted to T_3 *in vivo* and suggested that the anterior pituitary was the site of this conversion. Pituitary T_4 to T_3 conversion had been suspected on the basis of early *in vivo* studies in the rabbit, mouse, rat, and man in which significant quantities of T_3 were found in anterior pituitary or thyrotopic pituitary tumor tissue at relatively short intervals after tracer T_4 injection (Ford and Gross, 1958; Werner *et al.*, 1961; Volpert *et al.*, 1962; Grinberg *et al.*, 1963; Reichlin *et al.*, 1966). These quantities were thought to be in excess of that which could be attributed to circulating plasma tracer T_3 though no quantitation of this was attempted. With the subsequent recognition of the high T_3 binding capacity of the anterior pituitary nuclear receptors, it was assumed that this phenomenon might only be a reflection of the high avidity of pituitary nuclei for T_3. This impression was further strengthened by the fact that neither Chopra *et al.* (1978) using pituitary fragments nor Galton (1976) using homogenates could find evidence of T_4 to T_3 conversion.

To evaluate the possibility of T_4 to T_3 conversion *in vitro* in tissue fragments we applied techniques similar to those used in the *in vivo* system. We took advantage of the fact that the anterior pituitary nuclei bind T_3. Accordingly, we incubated quartered pituitary fragments in the presence of $[^{131}I]T_3$ and $[^{125}I]T_4$ (Silva *et al.*, 1978b). The nuclei were then isolated, and the iodothyronines chromatographed, and the media subjected to the affinity chromatographic system previously described to quantitate $[^{131}I]T_3$ and $[^{125}I]T_3$. As was the case *in vivo*, the ratio $[^{131}I]T_3/[^{125}I]T_3$ in the nucleus was identical to that in the cytosol (Cheron *et al.*, 1979). It was thus possible to determine the nuclear/medium T_3 ratios for both isotopes. The concentrations of $[^{125}I]T_3$ in the medium did not increase over 3–4 hours of incubation with pituitary fragments. Therefore, if T_4 to T_3 conversion were occurring, the T_3 formed remained largely within the tissue. Additionally, this indicated that the only $[^{125}I]T_3$ present in the medium was that present as a contaminant in the added $[^{125}I]T_4$. Demonstration of T_4 to T_3 conversion required that the nuclear/medium ratio of $[^{125}I]T_3$ exceed that for $[^{131}I]T_3$. As a convenient way of expressing these results, we calculated a value R which was the nuclear/medium (N/M) ratio for $[^{125}I]T_3$ ÷ the N/M ratio for $[^{131}I]T_3$. A value significantly in excess of 1 indicated T_4 to T_3 conversion. That T_4 to T_3 conversion occurs is demonstrated in Table VI which shows the N/M ratios for $[^{131}I]T_3$, $[^{125}I]T_3$, and R in several studies. By knowing the fraction of tissue T_3 in the nucleus (20%), it is possible to calculate the net T_4 converted to T_3 which varied from 0.4 to 1% in 3–4 hours. It is the use of the nuclear receptor

TABLE VI

Intrapituitary Monodeiodination in Euthyroid, T_4-Treated and Hypothyroid rats[a]

Experiment	Euthyroid	T_4-Treated[b]	Hypothyroid
A	3.65 ± 0.5	—	9.66 ± 0.48[c]
B	3.49 ± 0.35	2.01 ± 0.25[d]	9.84 ± 0.79[c]
C	4.5 ± 059	2.02 ± 0.05[d]	—

[a] Mean ± SE of *R* values for triplicate samples. R = nuclear:medium ratio for T_3 derived from medium T_4 ÷ nuclear:medium ratio for T_3 derived from medium T_3.

[b] 10 μg T_4/100 g body weight/day × 5 days s.c.

[c] $p < .005$ for difference from euthyroid by unpaired *t* test.

[d] $p < .025$ for difference from euthyroid by unpaired *t* test.

as a relatively specific T_3 binder and the successful use of the affinity chromatographic analysis of the medium for $[^{125}I]T_3$ content which accounted for the sensitivity of this technique. Further experiments demonstrated the following physiological influences on T_4 to T_3 conversion (Cheron *et al.*, 1979). Thyroxine at concentrations of 25–75 ng/ml (in 1.0% BSA) decreased *R* whereas similar concentrations of T_3 in the incubation medium had no effect. In pituitaries removed from rats treated with 10 μg T_4/100 g body weight for 5 days, T_4 to T_3 conversion is markedly reduced (Table VI). On the other hand, in pituitary tissue obtained from hypothyroid rats, T_4 to T_3 conversion is about three- to four-fold greater than that in control rats (Table VI). This suggests the possibility that T_4 to T_3 conversion may be a more important phenomenon in thyrotrophs than in other tissues since the metabolic effects of these alterations in thryoid status in the liver are exactly opposite to those that have been observed in pituitary (Kaplan and Utiger, 1978b). It is well known that fasting in both man and animals is associated with a decrease in the rate of T_4 to T_3 conversion in the liver. In man this is manifested by a fall in peripheral T_3, whereas in the rat both T_4 and T_3 fall due presumably to a hypothalamically mediated decrease in TSH secretion (Kaplan and Utiger, 1978a). We evaluated pituitary T_4 to T_3 conversion in pituitaries from rats who had been fasted for 3–5 days and found that it was not significantly different from controls receiving an *ad lib* diet. Thus, the pituitary appeared to be affected little, if at all by fasting, whereas hepatic conversion in the same animals was reduced to 50% of control. This could explain, in part, the failure of TSH to rise during fasting or stress-induced decreases in T_4 to T_3 conversion. However, since serum T_3 falls, an increase in TSH secretion should occur analogous to the events after acute propylthiouracil administration, and this does not occur. This suggests that there may be also changes in hypothalamic input which occur during fasting which provide a negative (or lack of positive) stimulus to TSH secretion.

Because of our interest in the effects of propylthiouracil, fragments were incubated with 29 μM concentrations of this agent. This concentration of propylthiouracil caused 90% inhibition of hepatic T_4 to T_3 conversion but did not inhibit pituitary conversion. It is not clear whether this is due to the fact that the drug does not enter the pituitary cells or that the conversion mechanism itself is not sensitive to this agent. Nevertheless, these *in vitro* results are quite consistent with those *in vivo* observations cited in Section III,D,3. On the other hand, iopanoic acid, a potent inhibitor of hepatic T_4 to T_3 conversion, caused a marked inhibition of pituitary fragment T_4 to T_3 conversion at a concentration of 1.3×10^{-5} M. This is again in agreement with the *in vivo* findings outlined above. Finally, we have also shown that T_4 to T_3 conversion in the pituitary of neonatal rats is more rapid than that in the adult rat (Cheron *et al.*, 1980). Conversion rates approximately 3–4 times that of adult rats were found in 2-day-old rats and the rate fell slowly reaching adult levels by about 3 weeks of age. Similar conclusions were reached in a preliminary report by El-Zaheri *et al.* (1980) using pituitary homogenates. This observation may have considerable physiological relevance in that in newborn rats serum thyroid hormone levels are reduced and yet TSH is not elevated. The accelerated pituitary T_4 to T_3 conversion would result in higher intracellular T_3 concentrations in the pituitary than would be predicted from the circulating thyroid hormone levels. Accordingly, TSH secretion would be more suppressed in this age group than it would be in an adult rat with comparable levels of circulating serum thyroid hormones. There is an obvious parallel in the human fetus where serum TSH concentrations are only slightly increased despite a marked reduction in serum T_3 and normal serum free T_4 concentrations.

B. Studies in Homogenates of Anterior Pituitary

Kaplan (1980) has evaluated T_4 to T_3 conversion in anterior pituitary homogenates and these studies provide additional information about the conversion process in this tissue. In this system, T_4 5′-monodeiodination is evaluated by radioimmunoassay of T_3 in the presence of 1.3 μM concentration of T_4. It was not possible to demonstrate T_4 to T_3 conversion in homogenates without the addition of a source of reduced sulfhydryls such as dithiothreitol (DTT) or reduced glutathione (GSH). DTT has been shown to be a potent stimulator of hepatic T_4 to T_3 conversion, probably acting like the endogenous SH-containing cofactor (Visser *et al.*, 1976). At maximum DTT concentrations (50–100 μM) T_3 production was 78 fmol T_3/mg protein/minute. As is the case with pituitary fragments, T_4 to T_3 conversion is saturable and the apparent K_m is approximately 8.8 nM roughly 500- to 1000-fold lower than that found in the liver homogenate. Thyroxine 5′-monodeiodinase activity was increased three-fold in hypothyroid rats and is lower in rats after 5 days of 10 μg T_4/100 g body weight. Conversion in homogenates is not inhibited by propylthiouracil but is by iopanoic acid. All these results parallel those described in studies of fragments indicating that the effects of these various treatments are due to changes in enzyme content rather than in the SH-containing cofactor. In studies of homogenates from fasted animals, Kaplan demonstrated about a 20% inhibition of T_4 to T_3 conversion, which was statistically significant though still less than the 50% inhibition in hepatic conversion that was found in the liver homogenates in fasting rats.

These results from both intact cells and homogenates confirmed the *in vivo* findings that normal anterior pituitary tissue converts T_4 to T_3. There is an excellent correlation between the observations *in vivo* and those *in vitro* with respect to the influences of various pharmacological agents on this conversion. It is intriguing to speculate on the specific pituitary cell type involved in the conversion process. The increased enzyme activity in hypothyroidism, and the decrease in hyperthyroidism suggests the possibility that thyrotrophs might be specifically involved in this conversion. Similar conclusions could be drawn from the earlier *in vivo* results of Reichlin *et al.* (1966) in hypothyroid rats and from the fact that thyrotrophic, but not adrenotrophic, tumors did show indirect evidence of converting activity (Werner *et al.*, 1961). In initial studies using a mouse thyrotroph tumor, Gershengorn (1978a) did not find T_4 to T_3 conversion but in a second tumor strain, this conversion was demonstrated (Gershengorn *et al.*, 1979). These results indicate there is biological variation within these such tumors. Both Samuels *et al.* (1979) and Melmed *et al.* (1979) have recently demonstrated T_4 to T_3 conversion in GH_1 and GH_3 rat pituitary tumors, respectively. These studies, together with the recent report of Obregon *et al.* (1979b) that iopanoic acid pretreatment inhibits the induction of growth hormone synthesis by T_4 in hypothyroid rats, indicate that the somatotroph is also capable of converting T_4 to T_3 and that this conversion is required for T_4 biological activity. Comparative data in normal pituitary cell types will be of considerable interest.

V. PHYSIOLOGICAL AND CLINICAL IMPLICATIONS OF PITUITARY T_4 TO T_3 CONVERSION

A. The Role of Pituitary T_4 to T_3 Conversion in the Physiological Response to Changes in Circulating T_4 or T_3

It is clear from the above studies that the contribution of T_4 to T_3 via intrapituitary conversion is significant. Thus, the pathophysiological observations discussed in Section II can be explained by the fact that a reduction in the serum T_4 concentration would be associated with a decrease in intrapituitary T_3 and desaturation of the nuclear T_3 receptors. TSH synthesis and release would thereby be increased, which would result in an increase in the T_3/T_4 ratio in thyroidal secretion and a return of serum T_3 to normal. This would, in turn, normalize intracellular T_3 in those tissues such as liver, kidney, and heart, which depend primarily on serum T_3 for intracellular T_3. Despite a normal serum T_3, intracellular T_3 in anterior pituitary would remain subnormal due to the decrease in serum T_4. Thus, in early hypothyroidism, endemic goiter, and experimental iodine deficiency, the serum TSH concentration can remain elevated even though serum T_3 is normal. The observation that acute administration of propylthiouracil to intact rats leads to a decrease in serum T_3 concentration, no change in serum T_4, but an increase in serum TSH can also be explained since approximately 50% of the intracellular T_3 in the pituitary derives from this source.

B. Clinical Implications of the Present Observations

There are several diagnostic and therapeutic clinical implications from the results of these studies. In the diagnosis of thyroid failure, serum T_4 and TSH are much better discriminators with respect to thyroidal function than is serum T_3. All the compensatory processes in the hypothalamic–pituitary–thyroid axis would appear to be attempting to maintain serum T_3 at normal concentrations. Since even modest elevations in TSH (between 5 and 10 μU/ml) in man may be associated with thyroidal stimulation and goiter, it is important to quantitate TSH as precisely as possible to determine whether the quantity of T_4 present in the serum is adequate for an individual patient. While one would not necessarily anticipate a clinical response to thyroid hormone replacement in a patient with such a modest degree of hypothyroidism, because by definition most of the body tissues remain euthyroid if plasma T_3 is normal, goiter in and of itself may be, or become, a symptomatic problem. If sensitive TSH assays are not available, then detection of mild degrees of thyroid dysfunction could be made by use of TRH infusions, since the studies of Bigos *et al.* (1978), and recent studies in the Marshallese population (Larsen *et al.*, 1978) have demonstrated that hyperresponsiveness to TRH is invariably observed with even modest TSH elevation.

Perhaps the most important clinical implication of these studies is that they demonstrate that one cannot extrapolate from the concentration of T_3 in the serum to that of the cell interior. In the pituitary gland, both T_3 and T_4 must be normal to achieve a normal intracellular T_3 under most circumstances. Recent studies have indicated that, in the brain, intracellular T_4 to T_3 conversion may be the predominant source of T_3 (Crantz and Larsen, 1980). Since the brain and pituitary appear to be similar in this respect, serum TSH or T_4 concentrations may be the best indirect reflections of the intracellular T_3 content in the brain.

It follows from these arguments that ideal replacement therapy for patients with hypothyroidism must duplicate the physiological situation as closely as possible if abnormalities in intracellular T_3 are to be remedied. A strong case can then be made for the exclusive use of T_4 as a replacement hormone because it duplicates most closely the situation found in normal individuals (Stock *et al.*, 1974). The use of thyroid extract, T_3–T_4 combinations, and T_3, per se, for chronic replacement therapy will all lead to deviations of intracellular T_3 away from normal in one group of tissues or another because the ratio of T_3 to T_4 in these preparations is significantly higher than normal (Rees-Jones and Larsen, 1977). Because we are unable to monitor intracellular T_3 concentrations directly in patients, a normal serum T_3 and T_4 would appear to provide the best evidence that the goal of thyroid hormone replacement has been achieved.

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13

Thyroid Hormone Regulation of Thyrotropin Production and Interaction with Thyrotropin Releasing Hormone in Thyrotropic Cells in Culture

MARVIN C. GERSHENGORN

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I. INTRODUCTION

The importance of the hypothalamic–pituitary–thyroid axis in maintaining a normal rate of thyroid hormone secretion and euthyroid metabolic state in the intact animal is well known (Reichlin *et al.*, 1972). Production of pituitary thyrotropin (thyroid stimulating hormone; TSH), the major modulator of secretion of L-triiodothyronine (T_3) and L-thyroxine (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 which appears to be mediated by hypothalamic thyrotropin releasing hormone (TRH) and somatostatin, which has been shown to inhibit TSH release (Vale *et al.*, 1977). Although multihormonal regulation of TSH synthesis and secretion has been documented in numerous studies, the loci within the thyrotroph which may mediate these actions are incompletely understood.

In order to study the molecular mechanisms involved in regulation of TSH synthesis and secretion by thyroid hormones and TRH, 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. In this review, I describe (1) a thyrotropic cell culture system that is responsive to physiological concentrations of thyroid hormones and to TRH, (2) the characteristics of nuclear binding sites for thyroid hormones and the evidence for these sites being receptors for thyroid hormone action, and (3) the interaction between thyroid hormones and TRH.

II. THYROTROPIC TUMOR (TtT) CELL CULTURE SYSTEM

A. Development of Cell Culture System

Pituitary thyrotropic tumors were induced in mice of the LAF_1/J strain (Jackson Laboratories, Bar Harbor, Maine) by ablation of the thyroid gland with radioiodine (200 μCi ^{131}I intraperitoneally) as originally described by Furth and his colleagues (1973). The pituitary tumors were then serially transplanted into similarly radiothyroidectomized mice; however, it is important to note, that after several animal-to-animal passages a large proportion of tumors became unresponsive to thyroid hormones and TRH (see below) and it has been necessary to induce continuously new tumors. Short-term suspension cell cultures of thyrotropic (TtT) cells were established after enzymatic dispersion and selective attachment techniques (Gershengorn *et al.*, 1978). Cells were dispersed by enzymatic digestion with collagenase, hyaluronidase, and Pancreatin N. F. according to the method of Vale *et al.* (1972), resuspended in growth medium (recently, this has been Ham's F-12 medium supplemented with 15% horse serum

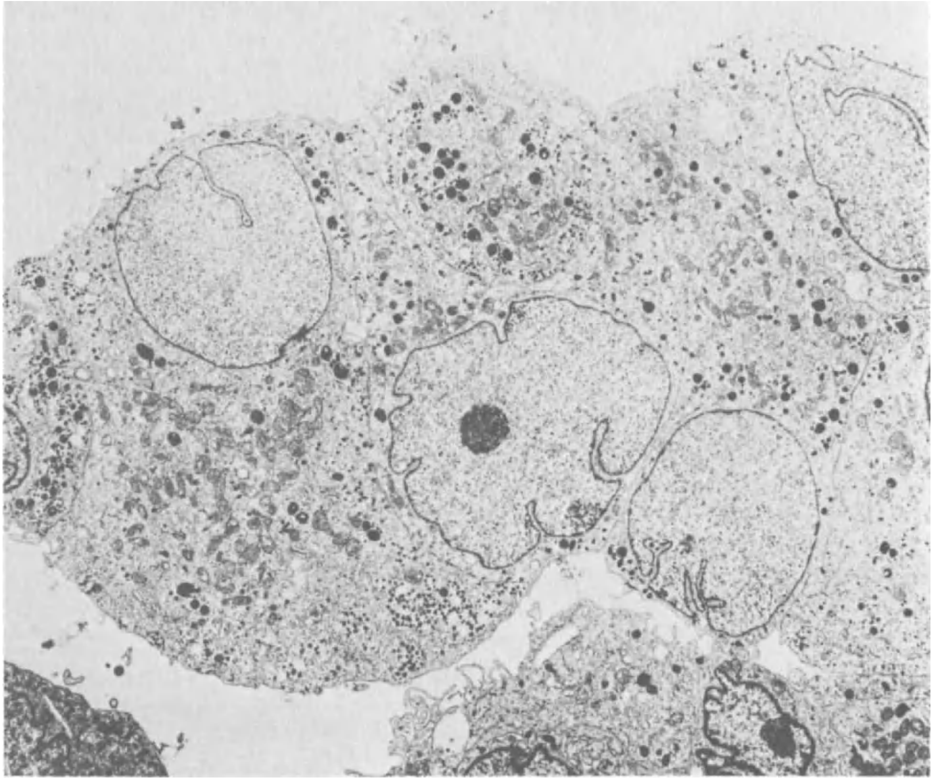

Fig. 1. TtT cells from suspension culture (×2700) containing 80–120 nm secretory granules present mainly at the cell periphery (curved arrows). From Gershengorn *et al.* (1978).

and 2.5% fetal bovine serum), placed in culture flasks, and incubated at 37°C in a moist atmosphere of 5% CO_2–95% air. After 4 and 24 hours, the nonadherent cells were collected and inoculated into new flasks. After the second transfer, there were no adherent cells in the suspension cultures which were composed exclusively of clusters of spherical cells, 14–18 μm in diameter.

Electron micrographs of cells in suspension culture showed that they were closely entwined, forming strands and cords (Fig. 1). Many had morphological traits characteristic of thyrotrophs in hypothyroid animals (Tixier-Vidal, 1975). Most were polygonal in shape with numerous electron dense granules of two types. One granule was small, round to oval in profile, and approximately 80–120 nm in diameter. The other was variable in shape and size but the smallest was 2–3 times larger than the small granules and sometimes contained myelin figures typical of secondary lysosomes. The rough endoplasmic reticulum was slightly dilated and contained a moderate amount of fine granular material. The nuclei were large, pale, deeply indented, often with two or more nucleoli. Electron lucent vacuoles were seen, many of which contained a single small granule adherent to their inner surface. Most importantly, TSH production by cells derived from the same tumor was increased from 24±1.5 ng-equiv of rat TSH/10^6 cells/48 hours in monolayer culture to 710 ± 42 ng-equiv in suspension cultures. Hence, the suspension cultures were greatly enriched in functional thyrotropic cells and appeared morphologically to be comprised of a homogeneous population of epithelial cells containing characteristic TSH secretory granules.

B. Production of TSH and Its Subunits

TSH is composed of two noncovalently bound subunits, an α subunit, which is virtually identical to that in luteinizing hormone, follicle-stimulating hormone, and chorionic gonadotropin, as well as a unique β subunit (TSH-β) (Pierce, 1971). Several studies had demonstrated secretion of free subunits in addition to complete TSH from the pituitary gland in man and that release of the subunits, like TSH, was inhibited by thyroid hormones and stimulated by TRH (Kourides *et al.*, 1973, 1975). In addition, excess secretion of free α subunits had been observed in patients with thyrotropic tumors (Kourides *et al.*, 1976, 1977).

In order to measure production of TSH and its subunits by TtT cells in culture, specific, heterologous radioimmunoassays for mouse tumor TSH, α subunit and TSH-β were developed (Blackman *et al.*, 1978). For the TSH assay, using purified rat TSH tracer and anti-bovine TSH serum, the displacement curve generated by an extract of mouse thyrotropic tumor was not parallel to the curve generated by pure rat TSH. Hence, tumor extracts were used as the primary standards and, using the cross-reactivity at the 50% displacement point, all data were converted from microliters of mouse tumor extract to nanogram-equivalents (ng-equiv) of pure rat TSH. The TSH assay was highly specific; cross-reaction of rat α subunit with respect to rat TSH was only 0.2%, whereas that of rat TSH-β was 13%. In the α subunit assay, in general, displacement curves generated with mouse tumor extract and rat α subunit were parallel, that is, there was immunological similarity between mouse and rat α subunits, and rat α subunit was used as standard. Cross-reaction of rat TSH with respect to rat α subunit was 14% and that of TSH-β was less than 0.5%. In the TSH-β assay, displacement curves generated by mouse tumor extract and rat TSH-β were not parallel. Hence, the tumor extract was used as the standard and the data were calculated as in the TSH assay. Cross-reaction of rat TSH was 6% and that of rat α subunit was less than 0.6%. The specificity and sensitivity of these immunoassays allowed for measurement of TSH, α subunit, and TSH-β in incubation medium and within TtT cells even in the presence of greater amounts of the other two glycopeptides.

Accumulation of TSH, free α subunits, and TSH-β into the incubation medium was measured by immunoassay during a 72 hour incubation in control cultures and in cultures exposed to maximally effective concentrations of TRH or T_4. For control cultures, TSH and TSH-β accumulation rates were nearly constant up to 48 hours, 0.67 ng-equiv/10^6 cells/hour, and 0.15 ng-equiv, respectively (Fig. 2). There was little if any additional accumulation during the next 24 hours. In contrast, the rate of free α subunit accumulation was constant throughout the 72 hours of incubation, 5.0 ng-equiv/10^6 cells/hour, nearly 7.5-fold greater than that of TSH and 33-fold greater than that of free TSH-β. The accumulation of all three glycopeptides was inhibited by T_4 and stimulated by TRH (Fig. 2). At 72 hours, TRH stimulated the accumulation of TSH, free α subunit, and TSH-β to approximately the same degree (175–185% of controls). In contrast, T_4 inhibited accumulation to varying degrees: 19% of control for TSH, 68% for α subunit, and 58% for TSH-β. Of note was the greater inhibitory

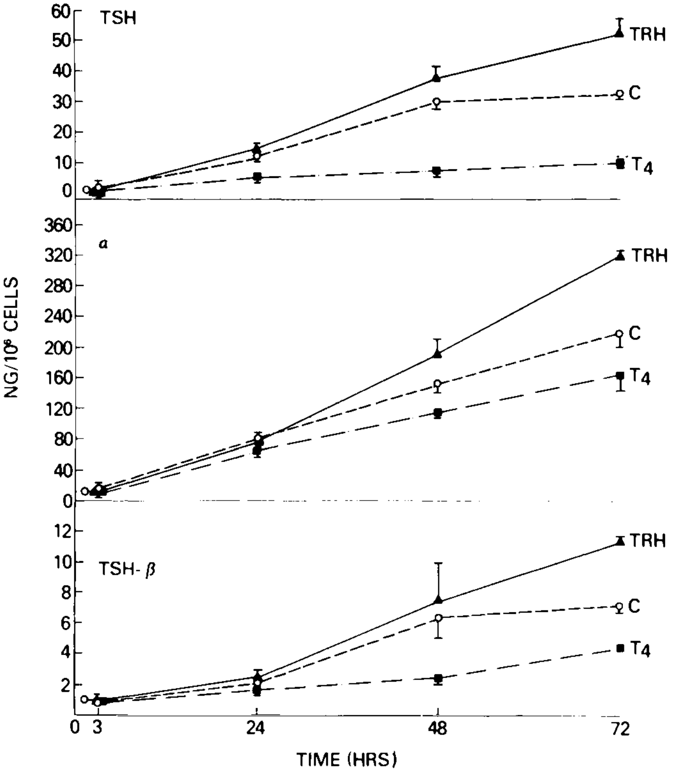

Fig. 2. Accumulation in the medium of TSH, free α subunit, and TSH-β from TtT cells during 72 hours of incubation. Control cultures were not exposed to hormones. T_4 was added at 0 time. TRH was added at 0 time and then every 12 hours. From Blackman *et al.* (1978).

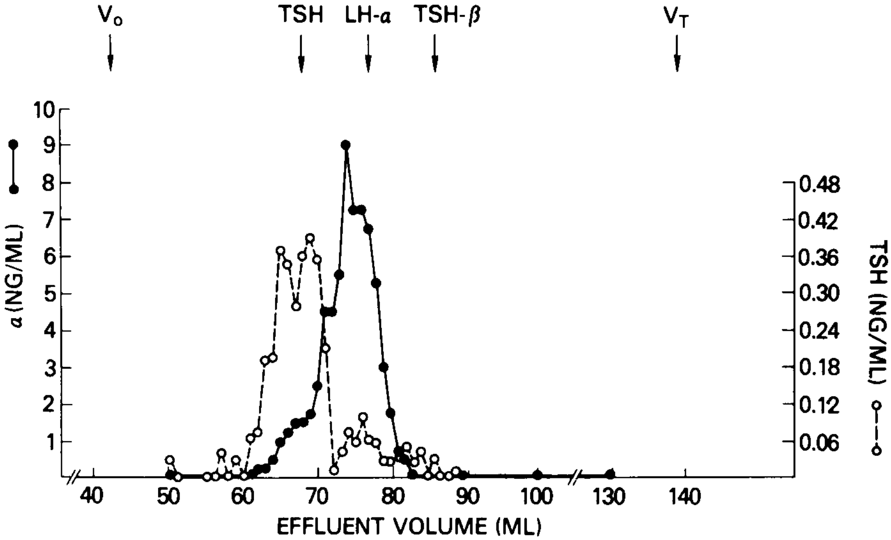

Fig. 3. Gel chromatography on a column (1.5 × 90 cm) of Sephadex G-100 of a pool of incubation medium from TtT cell cultures. Elution positions of rat [^{125}I]iodo-TSH, [^{125}I]iodo-α subunit (LH-α) and [^{125}I]iodo-TSH-β (TSH-β) used as internal column marks are shown. V_0, void volume; V_T, total column volume. From Blackman *et al.* (1978).

effect of T_4 on the accumulation of TSH than of its free subunits, especially α subunit. This was manifest as an increase in the ratio of α subunit to TSH accumulated from 6.2 to 16. Other tumors ("autonomous variants") were not responsive to thyroid hormone, that is, TSH production was not inhibited by T_3 or T_4 (see Section II,D).

Gel chromatographic analysis of incubation medium from TtT cell cultures was employed to validate the measurements of TSH and free α subunit (Fig. 3). The majority of the TSH and α subunit immunoactivity eluted in separate peaks. The major peak of TSH immunoactivity, although biphasic (K_{av} = 0.26, 0.31), eluted in a position similar to that of standard rat TSH (K_{av} = 0.30) and different from rat α subunit (K_{av} = 0.40) and rat TSH-β (K_{av} = 0.49). Another peak of TSH immunoactivity (K_{av} = 0.37), representing 12% of the total, eluted in a position similar to α subunit. The peak α subunit immunoactivity eluted identically to this latter TSH peak (K_{av} = 0.37) and similar to rat α subunit. As with TSH there was heterogeneity of the α immunoactivity.

The total production of each glycopeptide, the sum of the medium and intracellular contents, was measured by radioimmunoassay after 72 hours of incubation. In control cultures and in those exposed to maximally effective concentrations of TRH, there was proportionally greater production of α subunit than intact TSH and of TSH-β in a molar ratio of approximately 30 to 2.5 to 1, respectively. Maximally effective concentrations of T_4 inhibited TSH and

TSH-β production by only 29%. The different responses of TSH-β and α subunit to thyroid hormone and the excess production of α subunit suggested that the subunits of TSH were snythesized independently. More recently, in a series of very elegant studies, three groups of investigators have provided more direct evidence of the independent nature of the biosynthesis of α subunit and TSH-β by mouse thyrotropic tumor cells (Weintraub and Stannard, 1978; Chin *et al.*, 1978; Vamvakopoulos and Kourides, 1979). In addition, Marshall *et al.* (1981) and Cacicedo *et al.* (1981), using incorporation of radiolabeled amino acids, have demonstrated that TRH stimulated and thyroid hormones inhibited *de novo* biosynthesis of TSH by these cells.

C. Regulation of TSH Production by Physiological Levels of Thyroid Hormones and by TRH

In order to study regulation of TtT cell function by physiological levels of T_3 and T_4, it was necessary to culture cells under thyroid hormone-free conditions. This was accomplished by using defined medium supplemented with 10% serum obtained from a hypothyroid calf as originally described by Samuels *et al.* (1973). Hypothyroid calf serum contained less than 20 ng/dl T_3 and less than 0.5 μg/dl T_4. Because both T_3 and T_4 are bound avidly by serum proteins and the

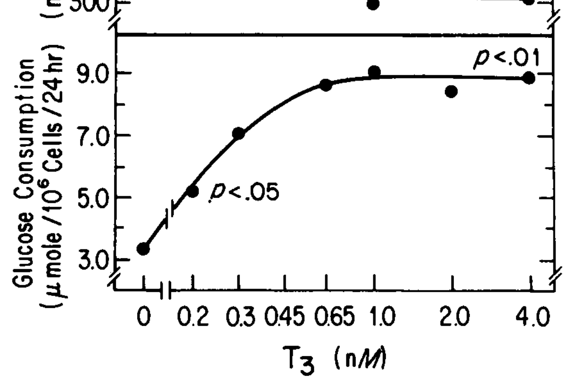

Fig. 4. Effect of T_3 between 0.2 and 4.0 n*M* on TSH accumulation in the medium and glucose consumption in TtT cell cultures. From Gershengorn (1978a).

free or unbound fraction correlates best with biological activity, the fraction of thyroid hormones present in the unbound form in culture medium with 10% hypothyroid calf serum was determined by equilibrium dialysis. The unbound fraction was 4.0±0.1% of the total for T_3 and 0.92±0.05% for T_4 and the free hormone concentrations in this culture medium were less than 10 p*M* T_3 and less than 50 p*M* T_4. Figure 4 illustrates the effects of 0.2–4.0 n*M* T_3 on cellular metabolism, measured as glucose consumption, and accumulation of TSH in the medium during a 24 hour incubation. Mirror-image dose–response relationships were observed for glucose consumption and TSH accumulation. Glucose consumption, 3.3 μmol/10^6 cells/24 hours in control cultures, was stimulated half-maximally by 0.2 n*M* T_3, and maximally (2.6-fold) by 0.65 n*M* T_3. TSH accumulation was inhibited half-maximally by 0.2 n*M* and maximally by 0.65 n*M* T_3 with no further inhibition up to 4.0 n*M*. Hence, in contrast to previous investigators (Vale *et al.*, 1972; Eto and Fleischer, 1976), our TtT cell culture system was regulated by physiological concentrations of T_3.

In other experiments, the effect of T_3 on TSH accumulation in the medium was studied over a wider range of hormone concentrations and compared to that of T_4. There was a biphasic dose–response relationship for T_3 and T_4 (Fig. 5). TSH accumulation in cultures incubated in the absence of thyroid hormones was

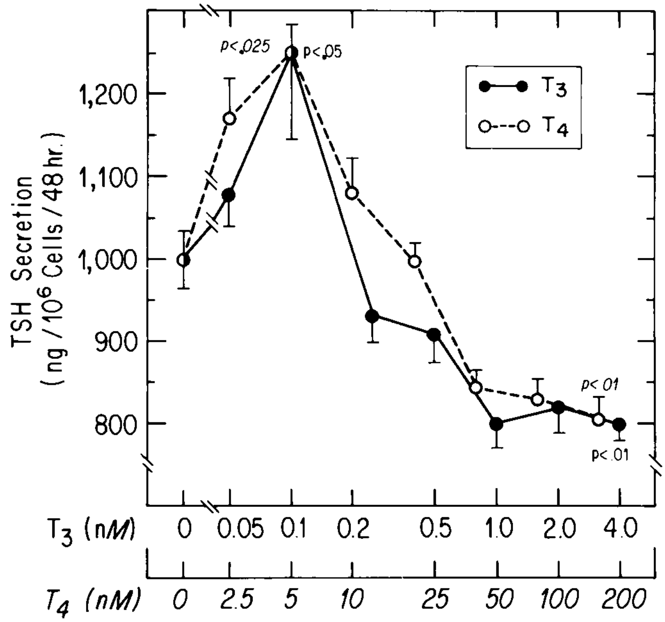

Fig. 5. Dose–response comparison of T_3 and T_4 on TSH accumulation in the medium in TtT cell cultures. From Gershengorn (1978a).

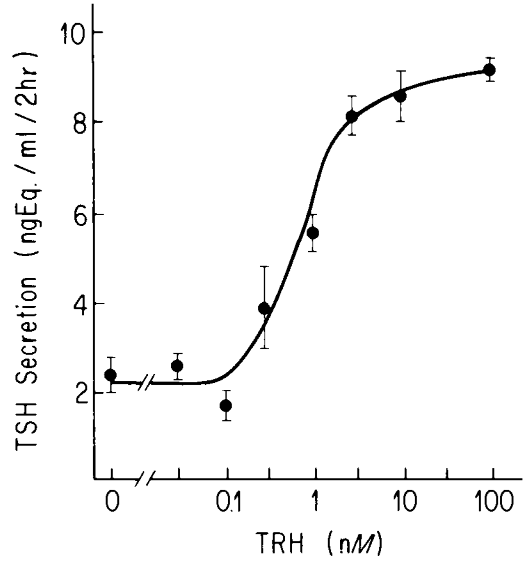

Fig. 6. Dose–response effect of TRH on TSH secretion by TtT cells.

enhanced progressively up to 0.1 n*M* T_3 and 5 n*M* T_4. With higher concentrations of T_3 and T_4, there was a progressive decrease in TSH accumulation. Half-maximal inhibition occurred at 0.2 n*M* T_3 and 15 n*M* T_4 and maximal inhibition occurred above 1.0 n*M* T_3 and 40 n*M* T_4. The biphasic nature of the response was observed also when total TSH production was measured. Stimulation of TSH production by very low levels of thyroid hormones had been suggested by D'Angelo *et al.* (1976), who observed an increase in pituitary content of TSH in excess of that accounted for by inhibition of TSH release in profoundly hypothyroid rats given T_4 intravenously prior to sacrifice, and by Connors and Hedge (1981), who found an increase in serum TSH in hypothyroid rats given 5 μg T_4/100 g/day, and an increase in TRH stimulated TSH at 1 and 2 μg T_4/100 g/day. We demonstrated a similar biphasic response to T_4 of production of thyroxine-binding globulin by rat hepatocarcinoma cells *in vitro* (Gershengorn *et al.*, 1976).

TRH stimulated secretion and production of TSH by TtT cell cultures in a concentration-dependent manner. Figure 6 illustrates the effect of TRH on TSH release during a 2 hour incubation. There was a 4.5-fold stimulation of TSH release by maximally effective concentrations of TRH; half-maximal stimulation of TSH release occurred with approximately 0.5 n*M*. TRH stimulation of TSH production, measured by radioimmunoassay as total TSH in the cultures (intracellular + medium) during a 24-hour incubation, displayed a similar concentration dependence; half-maximal stimulation of TSH production occurred with approximately 0.3 n*M* TRH. These half-maximal stimulatory concentrations were about one-tenth that of the equilibrium dissociation constant we obtained for the interaction of TRH with plasma membrane receptors on TtT cells (see Section IV,A) and suggest that fewer than 10% of the available receptors need be occupied to achieve a half-maximal effect on TSH secretion or production.

Hence, the TtT cell culture system was responsive to physiological concentrations of thyroid hormones and to TRH and appears to be a valid model in which to study the mechanism of physiological regulation of TSH secretion and synthesis.

D. Intracellular Monodeiodination of T_4 to T_3

Although T_4 appears to have intrinsic biological activity (see Section III,B) and need not serve simply as a prohormone for T_3, rapid intracellular monodeiodination of T_4 to T_3 may be the pathway through which a significant portion of the activity of T_4 is exerted in the pituitary thyrotroph. Much of the data supporting this hypothesis has come from a series of elegant studies in rats given T_4 intravenously prior to sacrifice and isolation of anterior pituitary cells by Larsen *et al.* (1981). Thyrotropic tumor cells have been shown to possess an increased capacity relative to extrapituitary tissues in the mouse to monodeiodinate T_4 to T_3 (Werner *et al.*, 1961; Volpert *et al.*, 1962). However, this activity has been variable in different tumors. It has been suggested that there may be a correlation between the monodeiodinating activity and the autonomy from suppression of tumor growth by thyroid hormones (Volpert *et al.*, 1966); however, none has been proved. We have also observed variable T_4 monodeiodinating activity in different TtT cell cultures (Gershengorn, 1978a; Gershengorn *et al.*, 1979a) (see Section III). The variable nature of this activity had been initially suggested by our finding that T_4 displayed a variable potency in inhibiting TSH production relative to T_3. In some TtT cultures, the monodeiodinating activity has been so great that after a 3 hour incubation with $[^{125}I]T_4$, 62±1.0% of the radioactivity in the cell cytosol was $[^{125}I]T_3$ and only 36±1.5% was still present as $[^{125}I]T_4$. This activity was shown to be intracellular, since no $[^{125}I]T_3$ was detected in the culture medium. However, it is important to note, that in tumors in which no monodeiodination of T_4 was detected, inhibition of TSH production by T_4 still occurred although higher concentrations of T_4 were required.

III. CHARACTERIZATION OF PUTATIVE NUCLEAR RECEPTORS FOR THYROID HORMONES IN TtT CELLS

A large body of data has accumulated that supports the contention that nuclear binding sites for thyroid hormones in several tissues may serve as physiologically relevant receptors for the initiation of at least some thyroid hormone actions.

Three characteristics of the interaction between thyroid hormones, and the binding sites within the nucleus are considered to be of primary importance in deciding whether a binding site may serve a receptor function. First, the binding sites should exhibit sufficiently high affinities for T_3 and T_4 that a portion are occupied at physiological free hormone concentrations. Second, the number of binding sites should be limited because biological responses to thyroid hormones usually demonstrate maximal effects. Third, there should be a good correlation between the binding affinities for various thyroid hormone analogs and their thyromimetic activities. It must be emphasized, however, that fulfillment of these criteria in a particular system constitutes only indirect evidence which is necessary but not sufficient to conclude that nuclear binding sites are receptors for initiation of thyroid hormone action. Direct and conclusive evidence of this function would be achieved only by the demonstration that addition of a purified binding moiety to a broken cell preparation would allow for thyroid hormone action not present in its absence.

A. Binding of T_3 in Intact Cells

Figure 7 illustrates the time course of specific binding of $[^{125}I]T_3$ to nuclear and cytosolic fractions of TtT cells after incubation of intact cells in medium with

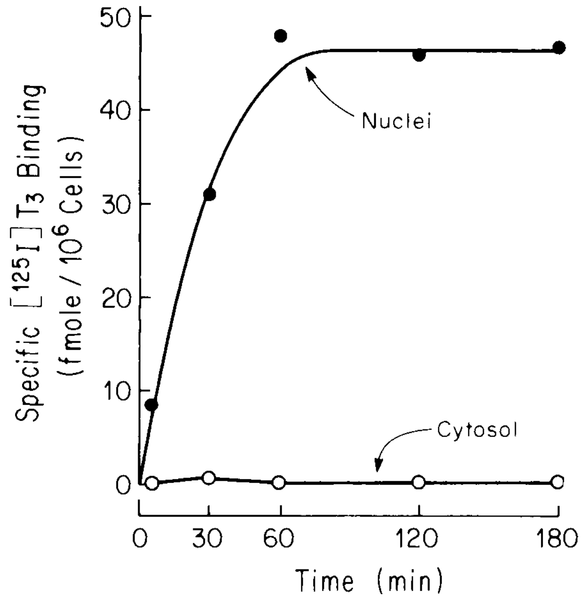

Fig. 7. Time course of specific binding of $[^{125}I]T_3$ to nuclear and cytosolic fractions of TtT cells. Specific binding was defined as the component of bound $[^{125}I]T_3$ that was inhibitable by 200-fold molar excess of nonradioactive T_3.

1 nM [^{125}I]T_3 without serum proteins. Specific binding was defined as the component of bound [^{125}I]T_3 that was inhibitable by a large excess of nonradioactive T_3, i.e., binding sites of limited capacity. T_3 binding to nuclei achieved an equilibrium level after 60 minutes, which was maintained for at least an additional 120 minutes. In contrast, although the total [^{125}I]T_3 present in the cytosolic fraction was approximately 2.5-fold that in the nuclear fractions, no specific binding was measured in the cytosol. It must be noted, however, that a very limited capacity, specific set of binding sites in the cytoplasm may have been obscured by a large component of unsaturable sites and we, therefore, cannot exclude the presence of specific cytosolic sites.

The specific binding of [^{125}I]T_3 to TtT cell nuclei was further characterized by incubating cells in medium with or without serum proteins containing varying concentrations of [^{125}I]T_3 for 2.5 hours. Figure 8 illustrates analysis of these data by the method of Scatchard (1949) from which an estimation of the apparent equilibrium dissociation constant (K_d) and of the maximal binding capacity could be made. The finding of a linear plot of these data suggested that T_3 binds to a single class of noninteracting binding sites in the nuclei of TtT cells as has been observed with other cell types. The apparent K_d for T_3 was 0.16 nM in medium without serum proteins and 2.9 nM in medium with 10% hypothyroid calf serum. The difference in the apparent K_d's in the presence and absence of serum was totally attributable to the binding by proteins in the medium with 10% hypothy-

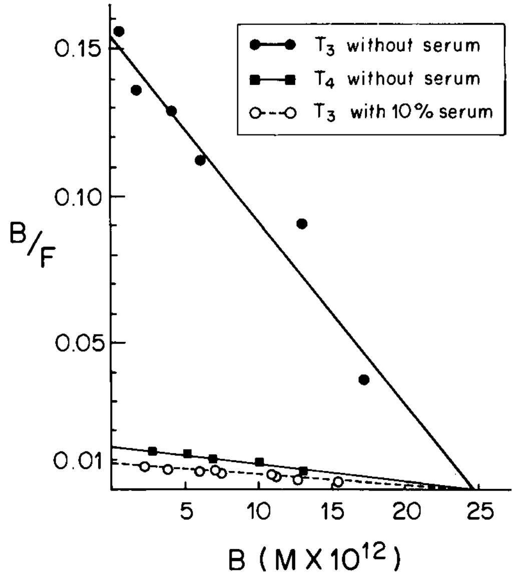

Fig. 8. Scatchard analysis of nuclear binding of T_3 and T_4 in serum-free medium and T_3 in medium containing 10% hypothyroid calf serum. From Gershengorn (1978a).

roid serum of 96% of the $[^{125}I]T_3$. The maximal binding capacity was similar under all conditions studied. There were 24 fmoles of T_3 bound per 10^6 cells at saturation which represented approximately 14,000 available binding sites per cell nucleus, a value similar to that described in other pituitary cells (Samuels, 1978). Hence, nuclear binding sites for T_3 in TtT cell nuclei were similar to T_3 binding sites in other target tissues.

B. Binding of T_4 to Nuclei in Intact Cells

As discussed in Section II,D, TtT cells derived from different pituitary tumors have differing capacities to monodeiodinate T_4 to T_3. It would be expected, therefore, that a variable proportion of the thyromimetic activity of T_4 would be attributable to intracellular generation of T_3 in different cell cultures. If the nuclear binding sites for T_3 described above were thyroid hormone receptors, we anticipated that these sites would also bind T_4, and that in TtT cells in which the activity of the monodeiodinating system was great, both T_4 and T_3 would be bound after exposure to T_4. We first studied binding of $[^{125}I]T_4$ to nuclei of TtT cells that had no measurable monodeiodinating activity (Gershengorn, 1978a). The time course of binding of $[^{125}I]T_4$ was very similar to that for $[^{125}I]T_3$, nonradioactive T_3 could completely inhibit $[^{125}I]T_4$ binding, and the number of $[^{125}I]T_4$ molecules bound at saturation was identical to that for $[^{125}I]T_3$. We concluded, therefore, that T_3 and T_4 bound to the same nuclear sites. The apparent K_d for T_4 measured in medium without serum proteins after 2.5 hours was 1.7 n*M*. Hence, the nuclear binding sites had approximately a 10-fold lower affinity for T_4 than for T_3, a relative binding similar to that found in other cell types (Samuels, 1978). In order to show directly that the radioactivity bound to nuclei of these TtT cells after incubation with $[^{125}I]T_4$ was not $[^{125}I]T_3$ formed by monodeiodination during the incubation, a nuclear extract containing 93% of the total nuclear bound radioactivity was examined by ion-exchange chromatography (Fig. 9). The bound radioactivity consisted of more than 96% $[^{125}I]T_4$, 2% $^{125}I^-$ and less than 1% $[^{125}I]T_3$. Because TSH production by these cells was inhibited by T_4 (Fig. 5), and the only iodothyronine found bound to the nuclear sites was T_4, it may be concluded that T_4 itself has intrinsic biological activity and that T_4 need not be monodeiodinated to T_3 to exert its thyromimetic activity in TtT cells. Intrinsic biological activity of T_4 has been shown also in GH cells, a rat pituitary cell line that produces growth hormone (Samuels, 1978). However, as discussed in Section II,D, a major portion of the thyromimetic activity of T_4 in the pituitary of the intact animal may be exerted by T_3 after intracellular T_4 monodeiodination (Larsen *et al.*, 1981).

Because the affinity of nuclear binding was 10-fold higher for T_3 than for T_4, it would be anticipated that in TtT cells in which monodeiodination of T_4 occurred a significant proportion of the iodothyronines bound to the nucleus after incubation

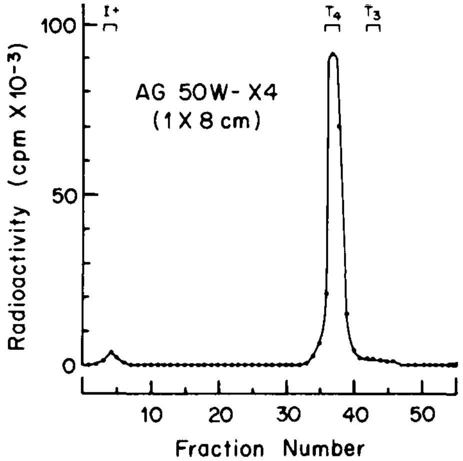

Fig. 9. Ion-exchange resin chromatography (AG 50W-X4) of an extract of nuclear bound radioactivity after incubating TtT cells that had no apparent monodeiodinating activity with [^{125}I]T$_4$. From Gershengorn (1978a).

with T$_4$ would be T$_3$. We analyzed the nuclear bound radioactivity of TtT cells with a high level of monodeiodinating activity incubated with 5 n*M* [^{125}I]T$_4$ (Gershengorn *et al.*, 1979a). After 3, 24, and 48 hours, the nuclear associated radioactivity was extracted and analyzed by ion-exchange chromatography. It was apparent that within 3 hours the major fraction of nuclear bound iodothyronines was [^{125}I]T$_3$, which increased during the 48 hours of incubation. [^{125}I]T$_3$ made up 70% of the total nuclear bound [^{125}I]iodothyronines after 3 hours, 89% after 24 hours, and 91% after 48 hours. Because the [^{125}I]T$_4$ used in those experiments contained ^{125}I only at one of the two chemically equivalent outer ring positions (3′ or 5′), it was likely that one-half of the generated T$_3$ was nonradioactive. Therefore, it could be calculated that T$_3$ comprised 82, 94, and 95% of the total nuclear bound iodothyronines at 3, 24, and 48 hours, respectively. In contrast to the large amount of [^{125}I]T$_3$ present intracellularly, only [^{125}I]T$_4$ was found in the medium at all times. Hence, the monodeiodination of T$_4$ to T$_3$ almost certainly occurred intracellularly and provided the major fraction of the biologically active thyroid hormone in these TtT cell cultures.

C. Comparison between Nuclear Binding of Thyroid Hormone Analogs and Their Relative Biological Potencies

A comparison of the relative affinities of nuclear binding of four thyroid hormone analogs and their relative biological potencies was performed. Since the binding studies and the measurements of biological effects were both performed with TtT cells incubated under identical conditions, a direct comparison of the concentration dependence of these functions for the analogs could be made. Estimation of the relative affinity of the nuclear sites for T_3, T_4, D-triiodothyronine (D-T_3), and triiodothyroacetic acid (triac) was made by incubating cells in medium with 10% hypothyroid calf serum, 3.5 n*M* [^{125}I]T_3, and varying concentrations of unlabeled analogs for 2.5 hours. Relative effectiveness of the analogs on TSH production and TRH receptor depletion (see Section IV) was made after a 48 hour incubation; TSH production was measured as the amount of TSH accumulated in the medium during the second 24 hour period of exposure to the analogs. Figure 10 illustrates the competition for nuclear binding in intact cells of [^{125}I]T_3 and nonradioactive T_3, T_4, triac, or D-T_3. Triac was equally effective, D-T_3 was one-sixth as effective, and T_4 was one-fortieth as effective as T_3 in displacing [^{125}I]T_3. Figure 11 illustrates concentration-dependent inhibition of TSH production by the thyroid hormone analogs. These analogs effected nearly parallel inhibition of TSH production. In all experiments, T_3 and triac were equally potent in inhibiting TSH production (half-maximal inhibition occurred with 0.25 n*M*) and D-T_3 was one-sixth as potent (half-maximal inhibition occurred with 1.6 n*M*). Hence, for these analogs, their relative potencies in inhibiting TSH production was in excellent agreement with their relative binding to the nuclear sites. T_4 exhibited a somewhat variable potency in inhibiting TSH production in TtT cells with monodeiodinating capacity: half-maximal inhibition occurred with between 3.2 and 5.6 n*M* as in Fig. 11. Therefore, the relative potency of T_4 in inhibiting TSH production, although variable, was always greater than its relative binding to nuclear sites: 4.5 to 7.7% as effective as T_3 in

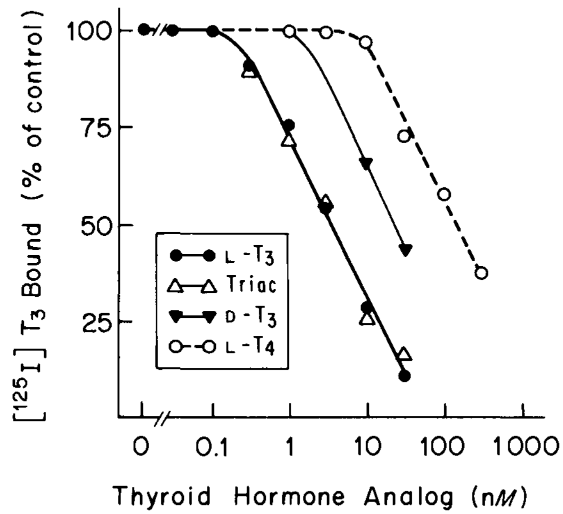

Fig. 10. Competition of specific [^{125}I]T_3 binding to nuclear sites by T_3, T_4, D-T_3, and triac. From Gershengorn *et al.* (1979a).

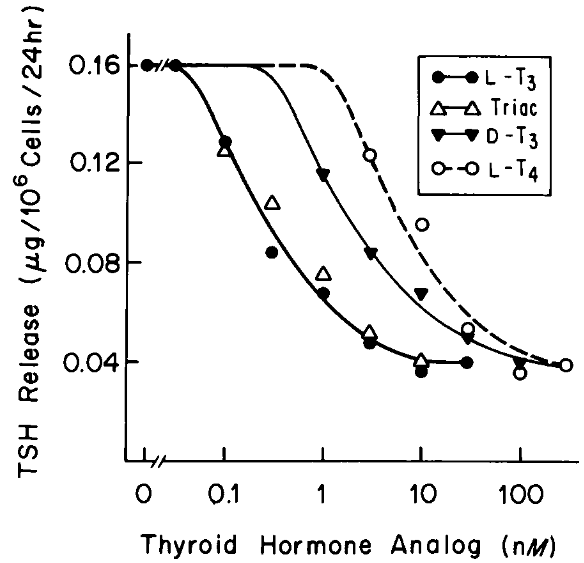

Fig. 11. Concentration-dependent inhibition of TSH production by T_3, T_4, D-T_3, and triac. From Gershengorn *et al.* (1979a).

inhibiting TSH production versus 2.5% as effective in competing for nuclear binding. The increased biological potency of T_4, which was measured during a 48 hour incubation, as compared to its binding to nuclear sites, measured after 2.5 hours, was shown to be due to intracellular monodeiodination of T_4 to the biologically more potent T_3.

As mentioned above, a major portion of the evidence suggesting that nuclear binding sites for thyroid hormones are physiologically relevant receptors is based on the very close agreement between the relative affinity for binding of hormonal analogs and their relative biological potencies. In GH cells in culture, Samuels (1978) demonstrated a parallel order of potency for several analogs in binding to the receptor and in affecting two biological responses: (1) increasing the growth hormone production and (2) decreasing the nuclear thyroid hormone receptor number. Binding to isolated hepatic nuclei *in vitro* has been shown also to correlate well with *in vivo* biological effects of thyroid hormones: antigoiter activity, increasing oxygen consumption, and induction of α-glycerophosphate dehydrogenase activity (Oppenheimer and Dillman, 1978). We compared the binding of four thyroid hormone analogs (T_3, T_4, triac, and D-T_3) to the nuclear receptor and their effectiveness in eliciting two biological responses: inhibition of TSH production and depletion of TRH receptor number (see Section IV). With the exception of L-T_4, there was an identical order of potency for the analogs in binding to the receptor and in affecting these biological functions. The concentration of T_3 necessary to induce a half-maximal biological response (0.25–0.32 n*M* in medium containing 10% serum from a thyroidectomized calf) was in good agreement with the values reported in two other *in vitro* systems in GH cells by Samuels (1978) and in rat myocardial cells by Tsai and Chen (1976). Thus, certain biological functions in several different cell types in culture are regulated by almost identical concentrations of thyroid hormones apparently acting through nuclear receptors.

IV. EFFECTS OF THYROID HORMONES ON TRH ACTION IN TtT CELLS

The dual regulation of TSH secretion and synthesis by thyroid hormones and TRH is well known. Although the effects of thyroid hormones and TRH in most circumstances oppose one another, under conditions of severe thyroid hormone deficiency both TRH and small amounts of T_4 and T_3 have been observed to stimulate TSH production (see Section II,C). Previous investigators (Vale *et al.*, 1972; Eto and Fleischer, 1976) had demonstrated that thyroid hormones inhibited the increase in TSH release from cells derived from thyrotropic tumors caused by TRH. We extended these studies in an attempt to determine the site(s) of interaction of thyroid hormones and TRH. Because it had been shown that the concentration of plasma membrane receptors for a variety of hormones (Kahn, 1976) including TRH on prolactin-producing cells (Hinkle and Tashjian, 1975; Perrone and Hinkle, 1978) could be modulated and it was suggested that this may serve as an important site of regulation of hormone action, we studied the regulation of putative receptors for TRH on TtT cells.

We first demonstrated that TRH action in TtT cells could be opposed by physiological concentrations of thyroid hormones (Gershengorn, 1978b). Basal and TRH-stimulated TSH accumulation in the medium was inhibited by physiological levels of T_3. TRH (10 n*M*) stimulated accumulation of TSH to 170% of control. Both stimulated and unstimulated TSH accumulation was progressively inhibited by T_3; half-maximal inhibition occurred with approximately 0.2 n*M* as shown previously. TRH stimulation of TSH accumulation was completely abolished by T_3 concentrations of 1 n*M* or greater. Hence, this effect in cells derived from thyrotropic tumors did not require pharmacological levels of thyroid hormones as previously employed (Vale *et al.*, 1972; Eto and Fleischer, 1976), and these data supported the concept of an important physiological interaction between TRH and thyroid hormones.

A. TRH Receptor

Putative receptors for TRH on cells derived from thyrotropic tumors were originally described by Grant *et al.* (1972, 1973) and Eddy *et al.* (1973). We studied binding of $[^3H]$TRH to intact TtT cells incubated in medium without serum at 37°C. Nonspecific binding, that is, $[^3H]$TRH bound in the presence of a

200-fold molar excess of unlabeled TRH, was subtracted from each value to yield specific binding. [^{3}H]TRH added to the medium bound rapidly to the cells. The specific binding was 47% of maximum after 10 minutes, 59% after 15 minutes, and 89% after 30 minutes, maximum after 60 minutes, and remained constant for longer than 3 hours. Therefore, in all other binding studies incubation with [^{3}H]TRH was for 90 or 120 minutes. To demonstrate reversibility of binding, aliquots of cells were incubated with 25 n*M* [^{3}H]TRH for 90 minutes, the cells were washed, and the amount of specifically bound radioactivity was measured. The remaining cell aliquots were resuspended in medium containing the same concentration of [^{3}H]TRH, unlabeled TRH, or no TRH and the amount of [^{3}H]TRH bound to the cells was measured at 30 minute intervals for 2 hours. The amount of bound [^{3}H]TRH remained constant in the cells reexposed to [^{3}H]TRH. In contrast, the radioactivity rapidly dissociated from cells exposed to no TRH or unlabeled TRH. The rate constant of dissociation of [^{3}H]TRH from cells was greater in the presence of unlabeled TRH (2.3×10^{-4}/second) than in its absence (1.3×10^{-4}/second). The ^{3}H radioactivity that dissociated from the cells was able to bind to other cells to the same extent as authentic [^{3}H]TRH, suggesting that it was unaltered [^{3}H]TRH. We also compared the rates of dissociation of [^{3}H]TRH from receptors on TtT cells after incubation with 5 n*M* or 100 n*M* [^{3}H]TRH for 2.5 hours. The rate of dissociation of [^{3}H]TRH was independent of the initial level of bound hormone and was best approximated by a single rate constant of 1.4×10^{-4}/second in this experiment. The enhanced dissociation of [^{3}H]TRH from its binding site in the presence of unlabeled TRH may be a result of prevention of reassociation of [^{3}H]TRH. DeMeyts *et al.* (1973, 1976), using insulin binding to lymphocytes as a model, suggested that enhanced dissociation of labeled ligand in the presence of unlabeled ligand is consistent also with negatively cooperative site–site interaction within a homogeneous population of binding sites. However, since there was no difference in the rates of dissociation of [^{3}H]TRH from its receptor after binding at medium concentrations of 5 and 100 n*M*, levels that would occupy approximately 60 and 95% of the receptors, there appeared to be no site–site interactions among TRH receptors.

The characteristics of binding of TRH to its receptors at equilibrium were defined by incubating cells with increasing concentrations of [^{3}H]TRH, up to 100 n*M*, for 90 minutes. Figure 12 shows the analysis of [^{3}H]TRH binding to intact TtT cells by the method of Scatchard (1949). A linear plot of the data was found consistent with a single class of noninteracting binding sites. The dissociation constant was 3–5 n*M*. There were 1.1 pmol [^{3}H]TRH bound per mg cell protein at saturation, corresponding to 99,000 binding sites per cell. These characteristics of the interaction of TRH with its receptors differ from those reported by Grant *et al.* (1972, 1973). They concluded that there were two receptors for TRH with apparent equilibrium dissociation constants of 20 and

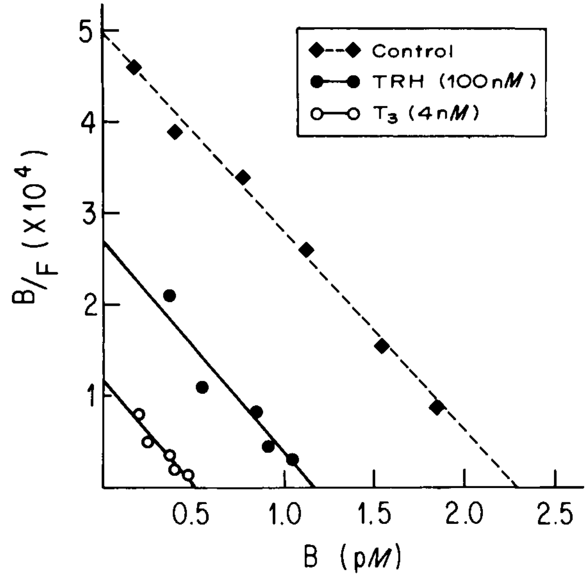

Fig. 12. Scatchard analysis of binding of [^{3}H]TRH to intact TtT cells incubated with TRH or T_3 for 48 hours. From Gershengorn (1978b).

approximately 500 n*M*. Subtle differences in the kinetics of [^{3}H]TRH displacement by a series of TRH analogs were interpreted as further evidence in support of two binding sites. The number of TRH receptors per cell could not be calculated from the data presented in their report. In contrast, we have observed only a single class of binding sites for TRH, using up to 100 n*M* [^{3}H]TRH, with a lower apparent dissociation constant. Although we are unable to offer a conclusive explanation for the differences in binding characteristics of TRH to these cells observed in these two studies, there were major differences in the conditions under which the binding experiments were performed, in that Grant *et al.* (1972, 1973) employed half-isotonic medium at 0°C, whereas we used the more physiological conditions of incubation in isotonic medium at 37°C.

B. Regulation of TRH Receptors by Thyroid Hormones and TRH

We next determined whether TRH receptors on TtT cells could be regulated by thyroid hormones and by TRH as had been demonstrated for TRH receptors on prolactin-producing cells in culture (Hinkle and Tashjian, 1975; Perrone and Hinkle, 1978). Determining whether TRH receptors on thyrotropic cells were modulated in a fashion similar to or different from those on mammotropic cells would allow for better interpretation of observations of changes in TRH receptors in heterogeneous cell populations, such as data from studies employing cells derived from whole pituitary glands. We, therefore, determined the time- and concentration-dependent effects of thyroid hormones and TRH on TtT cell TRH receptors.

Before studying the binding of [^{3}H]TRH to cells exposed to TRH or T_3, it was necessary to show that these hormones did not interfere with the subsequent binding reaction under the experimental conditions employed. Binding of [^{3}H]TRH (25 n*M*) to control cells was performed in triplicate in the presence of 10 n*M* T_3 and compared to that in medium alone (control). The amount of [^{3}H]TRH bound in the presence of T_3 was 94±12% of contol. In a parallel set of experiments, the effect of preincubation with TRH for 90 minutes was determined. Equal aliquots of cells in triplicate were incubated with 100 n*M* unlabeled TRH, 25 n*M* [^{3}H]TRH, or medium alone (control). The cells were washed 3 times. After washing, the amount of [^{3}H]TRH bound was decreased to 63±5% of the initial level. Binding of [^{3}H]TRH (25 n*M*) for 90 minutes was then performed with the washed cells. The cells previously exposed to unlabeled TRH bound 93±3% of the amount of [^{3}H]TRH bound by controls. Therefore, it was possible to accurately measure [^{3}H]TRH binding to cells exposed to T_3 or TRH.

Because TRH may be degraded during incubation, its stability was determined by incubating 20 n*M* [^{3}H]TRH at 37°C in medium. After 48 hours, the incubated [^{3}H]TRH was able to bind specifically to TtT cells to the same extent as fresh [^{3}H]TRH (104±3% of control). Therefore, there was virtually no degradation of TRH in the medium under the conditions of incubation.

TtT cells were incubated with 100 n*M* TRH or 4 n*M* T_3 for 48 hours; control cultures were incubated with no added hormone. After 48 hours, the cells were washed and binding of [^{3}H]TRH was studied. There was no difference in binding affinity of the TRH receptor after incubation with TRH or T_3 as compared to controls (Fig. 12). In contrast, there was a marked decrease in the number of available receptors to 50 and 22% of control after a 48 hour exposure to TRH and T_3, respectively. When cells were incubated with 100 n*M* TRH plus 4n*M* T_3, the number of available receptors decreased further to 12% of control ($p < .01$).

The time course of depletion of available TRH receptors was studied in cells exposed to 100 n*M* TRH or 4 n*M* T_3. The number of TRH receptors was estimated after 24, 48, and 72 hours by incubating the cells with 25 n*M* [^{3}H]TRH, a concentration sufficient to saturate 83–89% of the receptors. The number of receptors available to bind [^{3}H]TRH was 70% of control after 24 hours ($p < .05$), decreased further to 50% after 48 hours ($p < .01$), and to 45% after 72 hours ($p < .001$ versus control; $p > .1$ versus 48 hours) exposure to 100 n*M* TRH. The depletion of TRH receptors after incubation with 4 n*M* T_3 was more profound. After 24 hours there were 52% ($p < .001$) of the control number of receptors, after 48 hours the level decreased further to 20% ($p < .001$) and after 72 hours was 17% of control ($p < .001$ versus control: $p > .1$ versus 48 hours).

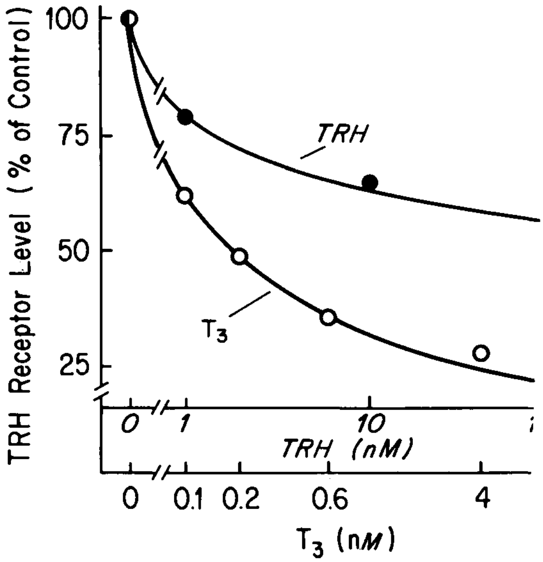

Fig. 13. Concentration-dependent depletion of TRH receptors by TRH or T_3. TtT cells were incubated with medium alone (control), with 1–100 n*M* TRH, or with 0.1–10 n*M* T_3 for 48 hours prior to determining the TRH receptor level. From Gershengorn (1978b).

As shown in Fig. 13, depletion of the number of available TRH receptors was dose dependent for TRH and T_3. After 48 hours, there were 79% of the control level of receptors present in cells exposed to 1 n*M* TRH ($p < .05$), 65% in cells exposed to 10 n*M* TRH and 55% in cells exposed to 100 n*M* TRH or greater; half-maximal depletion occurred at approximately 2 n*M* TRH. One-tenth n*M* T_3 lowered the number of receptors to 62% of control ($p < .05$), and maximal inhibition was achieved with T_3 concentrations greater than 4 n*M*; half-maximal inhibition occurred at approximately 0.15 n*M* T_3 in this experiment.

In order to gain insight as to whether T_3-induced depletion of the TRH receptor levels was mediated via the thyroid hormone nuclear receptor, we determined the relative potencies of thyroid hormone analogs. Figure 14 illustrates dose-dependent depletion of TRH receptor number by thyroid hormone analogs after a 48 hour exposure. As for inhibition of TSH production (see Section III,B), T_3 and triac were equally effective (half-maximal depletion occurred at 0.32 n*M*) and D-T_3 was one-fifth as effective (half-maximal depletion occurred at 1.6 n*M*) in causing a decrement in the number of TRH receptors. T_4 was the least effective with a potency of between one-tenth and one-fifteenth that of T_3 (half-maximal depletion occurred between 3.2 and 4.8 n*M*). The similar rank order of potency of these analogs and their relative affinities for binding to nuclear sites (Table I) suggests that this action also is mediated at the nucleus.

To determine the reversibility of the loss of TRH receptors, cells were incu-

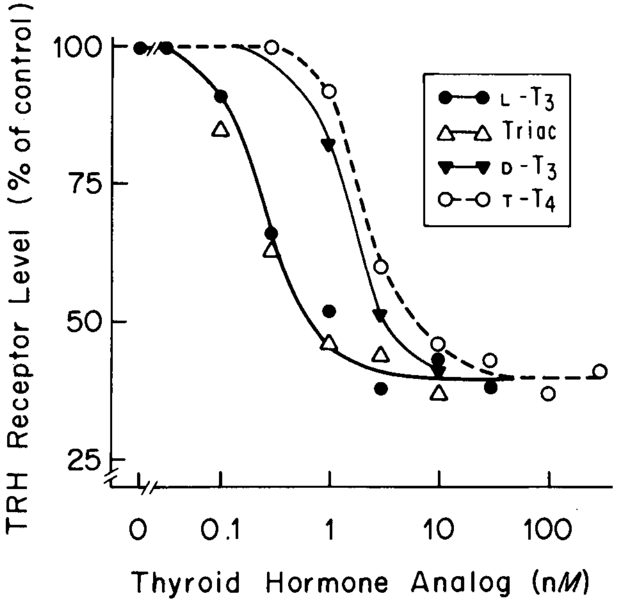

Fig. 14. Concentration-dependent depletion of TRH receptors by T_3, T_4, D-T_3, and triac. TtT cells were incubated with various concentrations of thyroid hormone analogs for 48 hours prior to determining the TRH receptor level. From Gershengorn *et al.* (1979a).

bated with 100 n*M* TRH and 4 n*M* T_3 for 48 hours, which reduced the TRH receptor number to 52 and 21%, respectively. After 48 hours, the cells were washed 3 times, resuspended in fresh TRH- and T_3-free medium, and incubated for up to 120 hours. During this experiment cell protein increased less than 20%. After removal of TRH, progressive repletion of the TRH receptor level occurred. After 24 hours, the level had returned to 65% of control ($p < .05$), after 48 hours to 74% of control, and by 72 hours to control levels. In contrast, there was a much more delayed repletion of TRH receptor number after removal of T_3. There

TABLE I

Relative Potencies of L-T_3, Triac, D-T_3, and L-T_4 in Binding to the Thyroid Hormone Nuclear Receptor and in Affecting TSH Release and TRH Receptor Depletion

	Binding to nuclear receptor	Inhibition of TSH release	Depletion of TRH receptor
L-T_3	100	100	100
Triac	100	100	100
D-T_3	16	16	20
L-T_4	2.5	4.5	10

was no change in the number of receptors after 24 hours ($p > .1$), an increase to 30% of control after 48 hours ($p < .05$), followed by a gradual rise to only 49% of control after 120 hours.

To determine whether the differences in the rates of repletion of the TRH receptor after removal of TRH and T_3 from the medium was a result, in part, of differences in the amount of residual hormone remaining bound to the respective receptor, the time course of disappearance of [^{3}H]TRH from the cell was compared to the disappearance of [^{125}I]T_3 from the nucleus. The half-life of the receptor–TRH complex was approximately 85 minutes (see Section IV,A). Therefore, after 9 hours there was virtually no TRH remaining on the receptor. T_3 disappearance from its nuclear receptor was much slower. After a rapid decline to approximately 50% of its initial level within 1.5 hours, there was a more gradual decrease of bound T_3 ($t_{1/2}$ = 7 hours). Therefore, even 12 hours after exposure to 4 n*M* T_3 there was still as much T_3 associated with the nuclear receptor as was initially present after incubation with 0.2 n*M* T_3, a level that yields a half-maximal T_3 effect. The more prolonged binding of T_3 to its receptor after removal of T_3 from the medium may be a result of the high concentration of T_3 found in the cytoplasm of the cell, a compartment with which the nucleus equilibrates. It seems, therefore, that the differences in the hormone bound after removal of TRH and T_3 may account, to some extent, for the different durations of their effects on TRH receptor number. However, there was still a profound effect on the number of TRH receptors 120 hours after removal of T_3 or greater than 72 hours after virtually no T_3 would be bound to its receptor. Therefore, T_3 must induce a long-lived message for this effect.

Modulation of the number of TRH receptors may be an important mechanism by which thyrotrophs autoregulate their sensitivity to TRH and control TSH release. For example, in the intact animal an increase in the circulating level of thyroid hormones would decrease the number of TRH receptors. Thyrotrophs would then be less sensitive to TRH. TSH release would decline, thyroidal secretion of T_3 and T_4 would diminish, and the level of circulating thyroid hormones would return toward normal. Because the receptors for several other polypeptide hormones have been shown also to be regulated under physiological conditions, it has been suggested that modulation of the number of hormone receptors might be a common mechanism by which endocrine target cells autoregulate their sensitivity to tropic hormones

V. SUMMARY AND CONCLUSIONS

We have developed a short-term culture system (TtT cells) comprised of a homogeneous propulation of TSH-producing (thyrotropic) cells derived from mouse pituitary tumors. These cells in culture synthesize and secrete large amounts of TSH, free α subunit, and TSH-β. In TtT cells, TSH production and cellular metabolism are regulated by physiological concentrations of thyroid hormones. Nuclear binding sites for thyroid hormones are present in TtT cells which display high affinities for T_3 and T_4 and are of limited capacity. Evidence that these binding sites may be receptors for initiation of thyroid hormone action is supported mainly by the excellent correlation between the rank order of biological potency of several thyroid hormone analogs and the relative affinities of the nuclear sites for these analogs. Synthesis and secretion of TSH by TtT cells is stimulated by TRH, which appears to initiate its action after binding to a receptor on the plasma membrane. Because the effects of thyroid hormones and TRH on TSH synthesis and secretion oppose one another under most conditions, it is very interesting that thyroid hormones down regulate the concentration of TRH receptors on TtT cells. Although this has not been proved, it is possible that thyroid hormone-induced depletion of TRH receptors serves to decrease cellular responsiveness to TRH and is, at least in part, a mechanism for inhibition of TRH response by thyroid hormones.

Similar interactions between thyroid hormones and TRH are well known to occur in intact animals and in humans. For example, Wilber and Seibel (1973) and DeLean *et al.* (1977) observed a decrement in pituitary TRH binding sites in cells derived from T_4-treated rats as compared to controls. However, since at least two cell types in the normal anterior pituitary gland possess receptors for TRH, one can not be certain whether the effect *in vivo* occurred in both or only one of these cells or even whether opposite effects may have occurred on thyrotrophs and mammotrophs. In a series of elegant studies, Hinkle, Perrone, and their colleagues (Perrone and Hinkle, 1978; Hinkle *et al.*, 1979, 1981) have demonstrated that thyroid hormones decrease the TRH receptor concentration on GH_3 cells, clonal strains of pituitary tumor cells that produce prolactin and we have confirmed their findings (Peck and Gershengorn, 1980). Hence, it appears that thyroid hormones cause qualitatively similar depletions in TRH receptor concentrations on thyrotropic and mammotropic cells. Similar decrements in TRH receptor concentrations by TRH itself have been observed on both cell types also (Hinkle and Tashjian, 1975; Gershengorn, 1978b). In contrast, however, we have observed differential regulation of TRH receptor concentration on TtT and GH_3 cells by estrogen (Gershengorn *et al.*, 1979b) and hydrocortisone (Tashjian *et al.*, 1977; Peck and Gershengorn, 1980). We found that estrogens and hydrocortisone increase the TRH receptor level on GH_3 cells but have no effect in TtT cells. Whether these differences in regulation of TRH receptor are peculiar to these rodent tumor-derived cells in culture remains to be elucidated. At the present time, however, it seems prudent to consider the possibility of discordant regulation of receptors in studies in intact animals. For example, the increment in pituitary binding sites observed in estrogen-treated rats (DeLean *et al.*, 1977) may have reflected a change in receptor level on mammotrophs only.

The TtT cell culture system described herein, in addition to having the advantage of being responsive to physiological concentrations of thyroid hormones and to TRH, has the important attribute that it is comprised of a homogeneous population of cells. As evidenced by our findings with regard to regulation of TRH receptor concentration, conclusions drawn from observations in heterogeneous cell populations need not be valid for all cell types. The homogeneous nature of the TtT culture system should allow it to be useful in defining intracellular mechanisms of thyroid hormone and TRH action in thyrotropic cells in a manner similar to the usefulness of GH cell culture systems as models of mammotropic cells.

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14

Effect of Thyroid Hormone on Growth and Development

HAROLD L. SCHWARTZ

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I. INTRODUCTION

Research into the role of the thyroid hormone in growth and development has been most active during the last 50 years. However, a link between goiter and cretinism was apparently already known to the people of ancient Rome. According to Benda (1949), the word "cretin" may derive from the Latin word *creta*, which means chalk, suggesting the pasty complexion associated with the condition. Benda also credits Curling (1850) with the first autopsy data suggesting the absence of the thyroid as the cause of the condition that he named "sporadic cretinism." During the latter part of the nineteenth century, the association of

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congenital hypothyroidism and the cretinous condition was further confirmed through the efforts of a number of the leading clinicians of the period, including Gull, Ord, Osler, and Charcot. Nevertheless, the fact that thyroid deficiency is rarely fatal has led to an almost total lack of autopsy examinations in neonates. This has led to a paucity of data, especially on the effect of thyroid hormones on the growth and maturation of the human brain during fetal development. Since the condition is generally not recognized at birth, it is difficult during later examinations to determine those abnormalities that were present in the fetus and those that arose during later development. The recognition that the rat pup is born in a relatively premature condition in comparison to that of the human neonate, especially insofar as development of the central nervous system is concerned, has fostered increased research in this area. In this chapter, I will review the current state of knowledge of the role of thyroid hormones in growth of the organism and in the development and maturation of several organ systems. At the same time, we will attempt to relate the action of the thyroid hormones to that of growth hormone and other growth factors in this process.

II. GROWTH

In our discussion of the role of thyroid hormone in development, I shall distinguish between growth (increased mass and length) and maturational processes. The latter term relates to the complex of processes that lead to alterations in cellular function. Although thyroid hormones play a role in both aspects of development, their effect on maturation of a variety of tissues and the organism as a whole appears to be unique. The role of thyroid hormone in fostering growth is less certain and may, in large measure, be dependent on interaction with growth hormone or other growth factors such as the somatomedins.

It is probable that intrauterine growth of the fetus takes place independent of the hormonal status of either the mother or fetus, so long as that status does not interfere with the nutritional requirements for fetal growth. Jost (1954) showed, both in rabbits and rats, that decapitation of the fetus *in utero* does not interfere with the ultimate size and weight reached at birth. Pennell and Kukral (1946) described a case of a child born at 7½ months of gestation with complete absence of the head, which was of normal size and weight for age. All the abdominal organs were of normal size and in proper position. Further, reports of normal birth weight and size of anencephalics (Greenberg *et al.*, 1974) also argue against any role of pituitary hormones, including growth hormone, in fetal growth. Since the human fetal thyroid does not produce detectable amounts of hormone prior to the tenth week of gestation (Shephard, 1967; Greenberg *et al.*, 1970), it is likely there is no role for them in early development either.

The lack of effect of thyroid hormone in later fetal growth is also evident from many observations of normal birth weight and length of children born with evidence of intrauterine hypothyroidism (Greenberg *et al.*, 1974; Anderson, 1961; Benda, 1949; Hurxthal, 1948). Greenberg *et al.* (1974) reported a mean birth weight of 7.6 lb for 66 such cases as compared with 7.5 lb for the normal. However, the distribution of weight appeared to be skewed toward the heavier end of the normal curve. This observation has been reported by the other authors as well and is unexplained. Geloso *et al.* (1968) have also reported that thyroidectomy of rabbit fetuses did not interfere with the attainment of normal weight and size at birth. Because maternal thyroid hormones do not cross the placental barrier to any significant extent (Grumbach and Werner, 1956; Fisher *et al.*, 1964; Raiti *et al.*, 1967; Dussault *et al.*, 1969), it is unlikely that the fetal deficit can be ameliorated by maternal hormone. At this point, nothing is known of the possible role of somatomedin-like factors in fetal growth. If they are present and active, they must quite obviously be independent of pituitary or thyroid hormone regulation.

Growth after birth is clearly thyroid hormone dependent. The literature is replete with reports of marked retardation of growth in all mammalian species deprived of thyroid hormone. Rats thyroidectomized soon after birth lag behind their normal counterparts in rate of gain of body weight and length and the ultimate size attained is significantly diminished (Scow *et al.*, 1949; Ray *et al.*, 1950; Scow, 1951; Scow and Simpson, 1945). Children suffering from congenital or acquired hypothyroidism also grow at markedly diminished rates (Hurxthal, 1948; Benda, 1949; Pickering and Fisher, 1958).

Administration of growth hormone to neonatally thyroidectomized or hypophysectomized–thyroidectomized rats stimulates a significant gain in growth as measured by increased body weight and length (Scow and Marx, 1945; Scow, 1951, 1959; Ray *et al.*, 1950, 1954). Thyroid hormone administration causes only a minimal stimulation of growth in the absence of growth hormone (Scow, 1959; Thorngren and Hansson, 1973). However, there is evidence to suggest that growth hormone is significantly more effective in the presence of thyroid hormone (Scow, 1959; Evans *et al.*, 1939, Scow *et al.*, 1949; Ray *et al.*, 1954; Thorngren and Hansson, 1973). Animals given both hormones grow at a much greater rate than those given maximal doses of either one alone (Tables I and II). Moreover, prolonged growth hormone administration to thyroidectomized rats could not produce gigantism, although its administration to hypophysectomized or intact rats did produce such results (Asling *et al.*, 1965).

Further evidence suggesting a possible synergistic relationship in the actions of growth hormone and thyroid hormones with respect to growth derives from several studies of children with growth hormone deficiency. Martin and Wilkins (1958) and Van den Brande *et al.* (1973) demonstrated that thyroid hormone administration has little effect on the growth rate of these children. On the other hand, the presence of thyroid hormone was required for an optimal response to

TABLE I

Effect of Thyroxine and Growth Hormone on Thyroidectomized–Hypophysectomized Rats[a]

Treatment	Body weight (g)	
	At autopsy	Gain in 30 days
None	34	−9
	±1	±4
Thyroxine	64	21
	±8	±8
Growth hormone	69	30
	±16	±15
Thyroxine and growth hormone	122	78
	±19	±17

[a] Rats were thyroidectomized at birth, hypophysectomized at 20 days of age. Treatment with thyroxine (2.5 μg/day) and/or growth hormone (0.1–2.0 mg/day) was carried out from 30 to 60 days of age. Data taken from Ray *et al.* (1954).

growth hormone. In addition, recent reports suggest that growth hormone administration may induce a hypothyroid state in these children, possibly by inhibition of synthesis and/or secretion of pituitary TSH. The condition is manifested by decreased thyroidal uptake of radioiodine (Root *et al.*, 1970) as well as lowered concentrations of plasma T_3, T_4, and TSH (Root *et al.*, 1970, 1973; Lippe *et al.*, 1975; Porter *et al.*, 1973). In addition, the response of pituitary TSH to TRH is severely blunted (Root *et al.*, 1973; Porter *et al.*, 1973; Lippe *et al.*, 1975). Accompanying the decline in serum thyroid hormone levels was a marked decrease or complete absence of growth response to continued growth hormone administration (Root *et al.*, 1970; Lippe *et al.*, 1975).

The mechanism by which thyroid hormones increase the responsivity of the animal to growth hormone is unknown. Recently, it has been shown that thyroid hormones can modulate the concentration of prolactin and other plasma membrane receptors (Duran-Garcia *et al.*, 1979; Marshall *et al.*, 1979; Sharma and Banerjee, 1978; Williams *et al.*, 1977). Although plasma membrane receptors for growth hormone have been identified in lymphocytes (McGuffin *et al.*, 1976), adipocytes (Fagin *et al.*, 1980), and hepatocytes (Ranke *et al.*, 1976; Postel-Vinay and Desbuquois, 1977), there is no evidence to date of modulation of receptor concentration in these tissues by thyroid hormone. In any case, biochemical studies of liver indicate that in this tissue growth hormone and thyroid hormone act in an additive fashion to stimulate synthesis of total and poly(A)$^+$-containing RNA (Simat *et al.*, 1980) as well as the activity of RNA polymerase (Widnell and Tata, 1966). These data, however, do not necessarily rule out such a mechanism in bone. An alternate pathway might, in fact, be more indirect and involve potentiation of the action of growth hormone-dependent somatomedins rather than that of growth hormone itself. Studies of the relationship of thyroid hormone and somatomedin action would be of interest.

Lack of growth due to hormone deficiency is also reflected in reduced excretion of urinary hydroxyproline. Hydroxyproline is present in significant amounts only in collagen, and urinary hydroxyproline is derived from the metabolic degradation of collagen. Excretion of hydroxyproline is related to the degree of collagen fiber maturation and to growth. Urinary excretion of this product increases from birth to a peak at adolescence and then falls to significantly lower levels in the adult. Excretion of hydroxyproline is decreased both in hypopituitary dwarfs and in hypothyroid children (Smiley and Ziff, 1964; Graystone and Cheek, 1968). The latter are also deficient in growth hormone (Solomon and Greep, 1959; Contopolous *et al.*, 1958; Hervas *et al.*, 1975). Although replacement with the appropriate hormone increases hydroxyproline excretion in both groups (Table III), the level attained in the hypothyroid patients given thyroxine is 2 times as great as that induced by growth hormone alone in the group of hypopituitary dwarfs. Moreover, growth hormone administration or its absence did not lead to any alteration in the urinary hydroxyproline/creatinine ratio. This ratio was reduced in the hypothyroid children and was markedly increased by

TABLE II

Effect of Thyroxine and Growth Hormone on Length of Growth Cartilage of Proximal Tibia in Hypophysectomized Rats[a]

Group	Thyroxine (μg/kg)	GH (μg)	Accumulated growth (μm)
1	0	0	43 ± 33[b]
2	5	0	128 ± 45
3	10	0	276 ± 91
4	20	0	333 ± 145
5	40	0	304 ± 109
6	0	25	578 ± 134
7	5	25	772 ± 94
8	10	25	960 ± 156
9	20	25	1144 ± 135
10	40	25	1075 ± 271
11	0	100	795 ± 133
12	20	100	1903 ± 214
13	40	100	1750 ± 179

[a] Rats were hypophysectomized at 60 days of age. Thyroxine and growth hormone were administered alone or in combinations, as indicated, for 20 days (days 15–35 postoperatively). Data taken from Thorngren and Hansson (1973).

[b] Mean ± SD.

TABLE III

Hydroxyproline Excretion in Hypothyroid and Hypopituitary Dwarfs[a]

Subjects	n	Hydroxyproline excretion (mg/24 hours)	Hydroxyproline/creatinine
Hypopituitarism	9	38.6 ± 20.5[b]	0.12 ± 0.05
Hypopituitarism after HGH	9	68.2 ± 22.7	0.15 ± 0.05
Hypothyroidism	8	16.0 ± 16.3	0.04 ± 0.02
Hypothyroidism after treatment	8	136.3 ± 84.1	0.34 ± 0.11

[a] Data taken from Graystone and Cheek (1968).
[b] Mean ± SD.

administration of thyroid hormone. This would suggest that although both hormones may increase collagen through an effect on protein synthesis, thyroid hormone may also increase the rate of degradation of collagen.

Not only are body size and weight affected by thyroid hormone deficiency, but organ weights may be reduced as well (Scow and Marx, 1945; Scow, 1951, 1959). In thyroidectomized or hypophysectomized–thyroidectomized neonatal rats, the organ/body weight ratios of heart, liver, kidney, and skeletal muscle are reduced. But, whereas administration of growth hormone stimulates organ growth that is proportional to the renewed gain in body weight (resulting in maintenance of a reduced ratio), thyroxine causes growth of the organ in excess of body weight gain sufficient to normalize the ratio.

It is likely that reduced growth in thyroid hormone deficiency is, for the most part, secondary to decreased growth hormone secretion. Some authors have reported small increases in the rate of growth of hypophysectomized rats given thyroxine (Scow, 1959; Thorngren and Hansson, 1973), whereas several groups have reported the absence of any growth in response to thyroid hormone even after 74 weeks of treatment (Eartley and Leblond, 1954; Bielschowsky *et al.*, 1962; Goodall and Gavin, 1966). Improved linear growth after thyroid hormone administration to hypothyroid children has been reported to be related to a return of normal growth hormone secretion (MacGillivray *et al.*, 1968). Lastly, as already noted, thyroid hormone administration to growth hormone deficient children does not correct the deficit in linear growth (Van den Brande, *et al.*, 1973). It is likely, then, that linear growth in both laboratory animals and humans is primarily stimulated by growth hormone but that thyroid hormone is essential both for growth hormone synthesis and secretion from the pituitary and for its optimal peripheral action.

III. SKELETAL GROWTH AND MATURATION

In the skeleton, growth is measured by an increase in size, particularly in length of the long bones, whereas differentiation (maturation) is indicated by establishment of centers of ossification and their subsequent fusion. Ordinarily, these proceed concurrently and under hormonal control. However, although growth of the skeleton in the postnatal period does require the presence of growth hormone (Simpson *et al.*, 1950), there appears to be no need for growth hormone during the fetal period. Pennell and Kukral (1946) reported that in their case of a 7½ month fetus born with complete absence of the head (and pituitary) skeletal development was perfectly normal.

Whereas skeletal growth during intrauterine life is independent of thyroid hormones, skeletal maturation is not. Congenital hypothyroidism is invariably associated with delayed bone age (Hurxthal, 1948; Benda, 1949; Pickering and Fisher, 1958). Intrauterine deficiency of thyroid hormone leads to lack of ossification of the distal femoral epiphysis (normally present at 35–40 weeks of age) and/or proximal tibial epiphysis (normally present at 40 weeks of gestation). Wilkins (1941) also pointed out that the epiphyseal centers of the knee and foot, which normally appear at 7 to 9 months of gestation, are frequently calcified or are dysgenetic in newborn cretins. In the rat, Geloso *et al.* (1968) reported abnormal skeletal development of thyroidectomized fetuses. Hamburgh (1968), on the other hand, reported that propylthiouracil-induced thyroid hormone deficiency caused no abnormality in skeletal development. However, it is difficult to determine the degree of hypothyroidism in such animals, despite the reported presence of enlarged thyroid glands.

Postnatal skeletal development has clearly been shown to be thyroid hormone dependent. There are a number of reports detailing the radiological evidence of delayed skeletal maturation in children with acquired hypothyroidism (Wilkins, 1941; Hurxthal, 1948; Benda, 1949; Anderson, 1961). Wilkins (1941), in a classic report, described in detail the absence or delayed appearance of new epiphyseal centers. In the normal child, ossification of the epiphyseal cartilage appears as a single small area near the center. The center normally grows by outward extension until it assumes the shape of the mature end of the bone. In the absence of thyroid hormone, this process does not occur. Eventually, with or without treatment, there appear multiple irregular islets of calcification. These eventually grow and coalesce but have a stippled appearance. Wilkins (1941) has shown that the "stippled epiphyses" are specific indicators of hypothyroidism. After institution of therapy, those centers that ossify first continue to show this dysgenetic pattern; those that ossify later develop normally. The epiphyses that show dysgenesis are those that normally would have ossified during the period of untreated hypothyroidism. This suggests that those processes of cartilage formation, which ordinarily precede formation of bone, were abnormal, not merely delayed, in the absence of thyroid hormone. In addition to delayed maturation of the skeleton, linear growth in height is markedly slowed in comparison with normal children. Delayed development will be manifested by immature skeletal and height age as long as treatment is withheld. Administration of thyroid hormone leads to the advancement of both height and bone age.

Evans and his colleagues produced a large series of studies that detailed the role of thyroid hormone in skeletal development in the neonatal rat and its relationship to growth hormone (Scow *et al.*, 1949; Ray *et al.*, 1950; Becks *et al.*, 1950; Simpson *et al.*, 1950). In these studies, both bone length and histological maturation were studied. In the absence of thyroid hormone both growth and maturation were retarded. Whereas in the hypophysectomized rat, the epiphyseal and diaphyseal cartilages became sealed by bony plates, this did not occur in thyroidectomized rats, even after prolonged periods. The epiphyses do not fuse in the thyroidectomized rat at any time. In rats made thyroid hormone deficient, whether due to thyroidectomy or secondary to hypophysectomy, skeletal age advanced only 18–20 days beyond the age at surgery in the absence of hormone replacement no matter how long the period of observation. Growth hormone replacement was able to induce a marked increase in body weight and bone length but was without effect on skeletal maturation (Table IV). On the other hand, thyroid hormone given to hypophysectomized animals caused marked differentiation of bone with little increase in body weight or bone length. Excess thyroid hormone given to normal neonates causes premature epiphyseal closure with consequent stunting of growth. Excess growth hormone, on the other hand, does not effect epiphyseal maturation but does induce gigantism (Asling *et al.*, 1965).

Observations in children who suffer congenital or acquired hyperthyroidism

TABLE IV

Skeletal Ages of Rats Thyroidectomized at Birth[a]

	Skeletal age at various chronological ages (days)		
Treatment	31	45	61
Growth hormone	15	15	18
Thyroxine	15	30	Between 40 and 56
Thyroxine and growth hormone	15	30	Between 40 and 56
Uninjected control	15		18

[a] Rats were thyroidectomized at birth and treatment carried out from 30 to 60 days of age. Data taken from Scow *et al.* (1949).

also indicate that excess thyroid hormone can accelerate skeletal development (Hung *et al.*, 1962). Hollingsworth and Mabry (1976) reported four cases of their own and reviewed 75 others of children born with evidence of congenital Graves' disease. All showed advanced skeletal age. A common finding in neonatally hyperthyroid children is cranial synostosis, in some cases leading to increased intracranial pressure (Hollingsworth and Mabry, 1976; Johnsonbaugh *et al.*, 1978). Mental retardation reported in a significant number of these cases, as in those studied by Riggs *et al.* (1972), is likely a result of this process. Penfold and Simpson (1975) also reported cranial synostosis as a complication of excessive thyroid hormone replacement in cretinism.

IV. CENTRAL NERVOUS SYSTEM

A. Morphology

Of all the organs studied, the effects of thyroid hormones on the development and maturation of the central nervous system have received the most detailed attention. There have been a few reports of histological studies of human brain from cases of congenital hypothyroidism (Benda, 1949; Marinesco, 1924; Marie *et al.*, 1920; Lotmar, 1929). However, the advanced age of the patients at the time of autopsy makes it difficult to be certain whether the observed abnormalities were present at birth. Nevertheless, the findings generally include hypoplasia of the cerebral and cerebellar cortices, edema, poor myelination, a decrease in the extent of the capillary beds, and vascular dilation.

Eayrs and his colleagues began the first systematic investigations into the pattern of normal and abnormal morphological development of the rat sensorimotor cortex (Eayrs and Goodhead, 1959). It was noted by several groups that early thyroidectomy only minimally affected growth of the brain although body weight was markedly inhibited (Eayrs and Taylor, 1951; Eayrs and Horne, 1955; Scow *et al.*, 1949) (Table V). At 24 days of age, hypothyroid neonates attained an average body weight of 20.8 g, compared with 31.8 g for normal controls, a difference of 35%. Brain weight, however, was only modestly less, 0.83 g compared to 1.08 g, resulting in an increase in the ratio of brain to body weight. It was also noted that the length of the brain was reduced in relation to its width. Consistent with earlier findings in the brains of cretins, Eayrs (1954) observed that although the proportion of cortical tissue occupied by capillaries was reduced, the numbers of vessels exceeding 5 μm in diameter were more numerous. This would lead to a decrease in the capillary surface area available for metabolic exchange. It is uncertain whether these changes are primary events leading to further alterations in cerebral architecture or are themselves due to the same primary stimulus.

TABLE V

Comparison of Body Weight, Brain Weight, and Linear Dimensions of Hypothyroid and Euthyroid Rats[a]

Measurement	Control	Experimental	p
Body wt (g)	31.8 ± 2.23[b]	20.8 ± 1.43	< .001
Brain wt (g)	1.08 ± 0.24	0.83 ± 0.020	< .001
Brain wt/body wt × 100	3.60 ± 0.21	4.22 ± 0.25	< .05
Brain width (cm)	1.34 ± 0.014	1.23 ± 0.017	< .001
Brain length (cm)	1.19 ± 0.016	1.06 ± 0.015	< .001
Width/length	1.123 ± 0.0067	1.161 ± 0.0060	< .001

[a] Neonatal rats received methylthiouracil from the day of birth and were studied at 24 days of age. Data taken from Eayrs and Taylor (1951).

[b] Mean ± SD.

In the hypothyroid rat neonate, detectable divergence from normal histological and biochemical development of the brain is not observed until after the tenth day of age. The period of 10–15 days of age in the rat appears to be a critical one and thyroid hormones must be available prior to this period for normal development to proceed. Replacement therapy begun after this age is only minimally effective in restoration of normal development (Legrand, 1967a,b; Rabie and Legrand, 1973; Hamburgh and Flexner, 1957). It is of interest in relation to these findings that Schwartz and Oppenheimer (1978b) have reported a sharp rise in the concentration of specific nuclear receptors for T_3 in brain at birth. The increased concentration is maintained during the critical first weeks of development (Fig. 1).

The outstanding histological feature of the cerebral cortex in the hypothyroid animal is the lack of proper development of the neuropil. In the normal rat, by 24 days of age there is a measurable reduction in the number of neurons in a unit area of tissue as compared to that in newborns, whereas the size of the cell body remains unchanged. The wider spacing of the cell bodies is accounted for by increased development of intervening components including the axonal– dendritic network. In the hypothyroid animal, the cross-sectional area of pyramidal cell bodies is approximately one-third less than normal by 15 days of age, and there is little reduction in the number of cells per unit area (Eayrs and Taylor, 1951). As a result, these authors proposed that there must be a reduction in interneuronal connections which, in turn, might affect behavior in such animals. Later studies (Eayrs and Horne, 1955; Eayrs, 1955), demonstrated, in fact, that axonal density is reduced by 30–40%. Although the number of primary dendrites is unaltered in experimental animals, branching is severely reduced and the individual processes are shorter. This was observed to be most severe in the internal granular layer of the cortex, which is dominated by the axonal plexus associated with the outer band of Baillarger and suggests some specificity of thyroid hormone effect in areas receiving significant thalamic projections.

It has also been reported that the number of spines on the apical shafts of pyramidal neurons is severely reduced in hypothyroid neonates (Sanchez-Toscana *et al.*, 1977; Ruiz-Marcos *et al.*, 1979). Schapiro and co-workers (1973) also tested the effect of the administration of excess thyroxine to normal neonates and demonstrated that development of dendritic spines could be advanced by 1–2 days, although there was no apparent difference in the ultimate level of development obtained.

In the cerebellum, lack of thyroid hormone during the critical period of neonatal development causes a marked reduction in dendritic arborization of the Purkinje cells and delayed migration of the external granule cells toward the center of the cerebellar cortex (Legrand, 1967a,b; Hamburgh *et al.*, 1971; Clos *et al.*, 1974). In normal animals, the external granular layer disappears by 24 days of age (Hamburgh *et al.*, 1971; Nicholson and Altman, 1972a). Nicholson and Altman (1972a) reported that in hypothyroid neonates the kinetics of labeling of DNA with [^{3}H]thymidine suggested extension of the period of cell proliferation, whereas excess hormone caused this phase to be condensed in time. This is

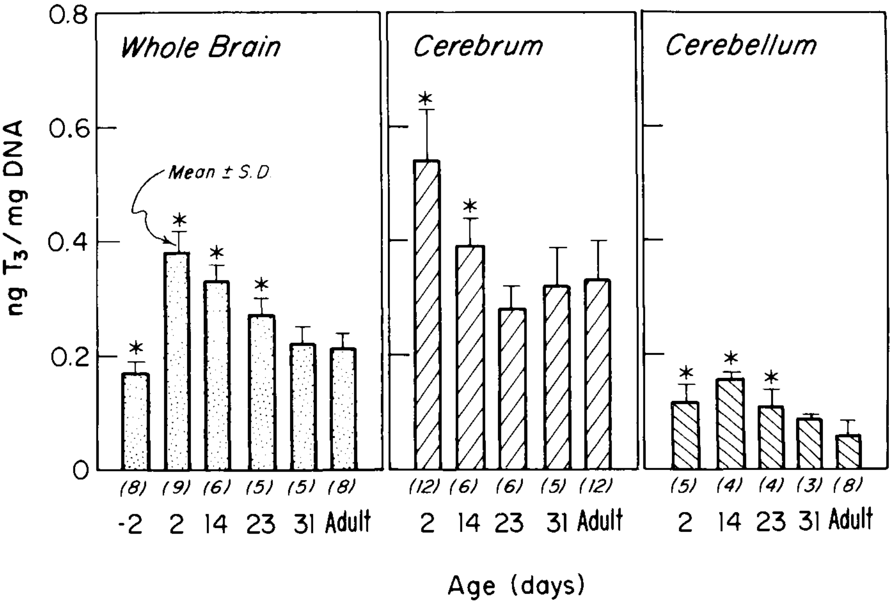

Fig. 1. Binding capacity of nuclear T_3 receptor in whole brain, cerebrum, and cerebellum of neonatal rats. (*) Values that are statistically different ($p < .025$) from adult values. Numbers in parentheses represent numbers of pools of tissue studied.

consistent with the histological finding of Hamburgh *et al.* (1971) that the germinal centers appear to persist beyond the normal period in hypothyroid animals and with the report by Brasel and Boyd (1975) that DNA polymerase activity in cerebellum is not reduced to adult levels until 35 days of age, a week later than in normal neonates. This led Hamburgh and colleagues to propose that the role of thyroid hormone is to switch development from a proliferative phase to one of differentiation (Hamburgh *et al.*, 1971). The bulk of evidence suggests that there is in the hypothyroid neonate only a transient decrease in cerebellar cell number, which ultimately reaches normal levels despite continued hormonal insufficiency. However, administration of excess hormone appears to cause early differentiation with the result that granule cell numbers are reduced. Legrand *et al.* (1976) have also reported that excess hormone leads to an overall decrease in the size and weight of the cerebellum.

Cerebellar synaptogenesis is also severely reduced in hypothyroid neonates (Nicholson and Altman, 1972b; Rebiere and Dainat, 1976; Rabie and Legrand, 1973). By 10 days of age, synaptosomal protein content is noticeably reduced. There is a reduction in the total number of synaptic profiles as well as in their density (profiles/cell). Treatment with thyroxine, if begun after 15 days of age, was without effect in restoring the process to normal. Clos and colleagues (1974) found that propylthiouracil caused a reduction in branching of Purkinje cell dendrites. The primary dendrites, however, grew to be twice as long as those in controls.

As discussed above the lack of thyroid hormone appears to lead only to a transient decrease in maturation of granule cells. Pesetsky (1973), however, reported that development of cerebellar astrocytes is abnormal as late as 3 months of age. Because studies were not carried out beyond this point, it is unclear as to whether the abnormality was permanent. Bergmann cells, on the other hand, appeared normal throughout the period of study. This would suggest that development of these Bergmann cells is independent of thyroidal state. However, Seress *et al.* (1978) have since reported that daily administration of triiodothyronine to neonates speeded the formation of these cells. It is assumed that the migration of granule cells from the external granular layer is guided by Bergmann fibers. The accelerated development of these latter cells may then in some manner trigger the early migration of granule cells reported by Nicholson and Altman (1972a) after excess hormone administration to neonates.

B. Behavior

The gross anatomical deficits observed in the brains of cretins and in experimental animals must obviously lead to significant neurological and mental disturbance. At birth there is little to distinguish the severely hypothyroid baby from normal. However, after several months, if untreated, it becomes more and more lethargic. The EEG in very young hypothyroid children may show a slow α rhythm but some show normal patterns of activity (Anderson, 1971).

Although there is general agreement that early treatment of children with congenital hypothyroidism can forestall severe mental retardation (Smith *et al.*, 1957; Raiti and Newns, 1971; Maenpaa, 1972; Klein *et al.*, 1972), controversy continues as to whether such treatment leads to full normalization of mental development. In all these reports, at least 80% of the patients demonstrated I.Q. scores above 85% if treatment were begun prior to 3 months of age. Frost *et al.* (1979) compared the I.Q. scores attained by patients with those of their sibs rather than that of the general population and found that in six of these, for whom treatment was begun after 3 months of age, the mean difference in I.Q. was −25 points. In four patients, for whom hormone replacement was begun before 3 months of age, there was no difference in score when compared with their sibs. Of significant interest is a recent study by Money and colleagues (1978). After 16–23 years, they retested the I.Q. level of a group of treated congenitally hypothyroid patients who had originally been tested at ages 5 and 6. They found that during the extended follow-up period the I.Q. scores had increased an average of 21 points (range −5 to 43). The data suggest that intelligence, or whatever it is that is measured by I.Q. tests, is not a fixed quantity even in mentally retarded individuals and that a low score at a young age need not be indicative of a failure of therapy.

There is, however, some evidence to suggest that early treatment may not fully overcome the fetal deficit in hormone-dependent development. Anderson (1971) reported that despite early treatment (within the first 3 months) one-half of his series of 50 patients had signs of cerebellar dysfunction, i.e., ataxic gait, general clumsiness, and dysdiadochokinesis, for extended periods of time. McFaul *et al.* (1978) reported that 23 of their 30 patients had at least one sign of impaired neurological function including clumsiness, squint, speech, learning, and behavioral disorders. These signs were as common in those for whom treatment was begun as early as 4–10 weeks after birth as in those treated later. Frost *et al.* (1979) also reported neurological disturbances in their series of patients which were independent of the time of onset of therapy. These reports are disturbing since great efforts have recently been organized for the mandatory screening of all newborns for hypothyroidism in order to insure early detection and treatment. In a preliminary report, Dussault and colleagues (1978) have reported that 20 infants detected and treated through their screening program in Quebec have been tested at 12 months of age, and all were found to have normal verbal and motor skills. The report unfortunately does not indicate the time of onset of therapy in this group. Because the histological development of the human brain undergoes rapid changes in the period immediately prior to and after birth (Raaf and Kernohan, 1944; Dobbing and Sands, 1973), it is clear that onset of treatment even at 2–3 months of age, considered early by recent investigators, may in fact represent significant delay.

Eayrs and Taylor (1951) first tested the effect of neonatal hypothyroidism in rats on development of automatic behavior. Whereas in normals the righting reflex was present in 10% of the rats at 15 days and in 90% at 19 days of age, this reflex had not developed in any hypothyroid rats by 15 days and was only present in 20% at 19 days. Similarly, the placing reflex was present in 70% of normals at 19 days but was not observed in any of the experimental animals at that age. Eayrs and Lishman (1955) after demonstrating reduced learning ability in propylthiouracil-treated neonates in a T-maze suggested that they also had reduced sensitivity to their surroundings and were less inhibited by unexpected situations. This conclusion was based on finding an increased ratio for hypothyroid rats of errors made to accumulated time necessary for completion of the test runs.

A series of studies have demonstrated the ability of excess hormone to accelerate the development of indices of neurological development. Schapiro and colleagues have reported that there is earlier maturation of the startle response, as well as acceleration of the sensorimotor cortex evoked potential response to sciatic nerve stimulation (Schapiro and Norman, 1967; Schapiro, 1968). Acceleration of reflex activity was only affected if excess thyroid hormone was administered prior to the fourteenth day of age (Eayrs, 1964). Schapiro *et al.* (1970), as well as Hamburgh and Vicari (1957), also observed earlier development of coordinated swimming behavior in thyroxine-treated newborns. Although these neonates also showed improved performance in active avoidance tests (Schapiro, 1968) they did more poorly in maze testing. This suggestion of improved learning ability in active avoidance tests induced by thyroid hormone is disputed by Davenport and Gonzales (1973) and Stone and Greenough (1975). These authors suggest that the better performance was due, not to improved learning ability, but simply to advanced locomotor function. This would also be consistent with the finding by Schapiro *et al.* (1970) of earlier swimming competence. Sjöden and Lindqvist (1976) and Rastogi and Singhal (1976) have observed the rates of activities of hyperthyroid neonates and report that these animals are hyperactive.. In tests of learning in which rates of activity were not a significant factor, hyperthyroid neonates were shown to perform equally as well as controls. However, Eayrs (1964), Khamsi and Eayrs (1966), and Stone and Greenough (1975) have reported that adult animals that had been treated with excess hormones during the neonatal period show consistent learning deficits. This is obviously of significance in the design of a treatment regimen for newborn hypothyroid children. There are, in fact, several reports in the literature (Daneman and Howard, 1980; Riggs *et al.*, 1972) of children, presenting obvious signs of hyperthyroidism at birth, whose mental development was significantly retarded at later follow-up. A report by Hollingsworth and Mabry (1976) describes four cases of neonatal hyperthyroidism, all of whom had normal I.Q. levels. However, two of these demonstrated residual hyperactivity after 5–8 years of therapy begun soon after birth, suggesting possible residual cerebellar defects.

C. Nucleic Acids and Protein

Consistent with the earlier findings with regard to the effect of thyroid hormones on the morphological development of the brain are the observations of alterations of nucleic acid and protein accumulation in this organ. Neonatal hypothyroidism does not appear to interfere with the accumulation of normal cell numbers in the cerebral cortex, because total DNA content is found to be unchanged (Geel and Timiras, 1967; Pasquini *et al.*, 1967; Balazs *et al.*, 1968). However, whereas the DNA concentration per gram of tissue decreases in normal neonates, reflecting decreased cell density due to development of the neuropil, this process is slowed in thyroidectomized neonates. This results in a 15% greater than normal concentration of DNA per gram, which is detectable by 10–12 days of age. In normal animals, the RNA content per cell (mg/mg DNA) rises sharply during the first weeks of life (Fig. 2A) and the concentration of protein per cell rises more than sixfold during the first 30 days (Fig. 3A). However, as also shown, neonatal hypothyroidism leads to a severe reduction in both these indices. These biochemical alterations are consistent with the observation of deficient morphological differentiation of the cerebrum discussed earlier. With a reduction in RNA and protein content per cell, the basis for the finding of decreased neuronal cell size becomes apparent. And, as observed with other indices of brain maturation, Krawiec *et al.* (1969) have demonstrated that replacement with T_3 prior to the tenth day can restore DNA concentration and RNA/DNA ratios to normal. If treatment was delayed to the fifteenth day, it was only partially effective.

Several reports have indicated that the reduced protein content of the cerebral cortex is likely the result of reduced rates of synthesis (Geel *et al.*, 1967; Jarlstedt and Norstrom, 1972; Szijan *et al.*, 1971). All demonstrated reduced rates of incorporation of radiolabeled amino acids into cortical protein. No information is available as to whether there may be alterations in the rate of protein degradation as well.

The mechanism responsible for a reduction in RNA concentrations is undefined. Data from Balazs *et al.* (1968) suggested that both the rate of conversion of [^{14}C]orotic acid into uridine monophosphate, and its incorporation into RNA are normal in thyroidectomized neonates. Gomez *et al.* (1972) have also reported decreased rates of incorporation of [^{14}C]orotic acid into rapidly labeled RNA during the first 60 minutes after injection. The significance of these findings

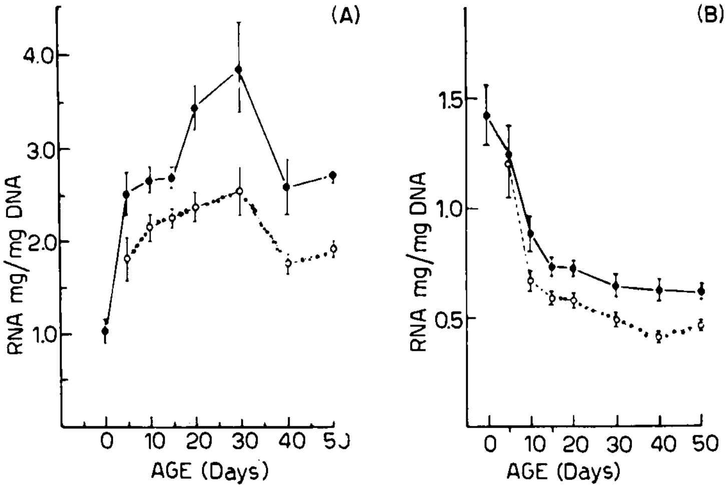

Fig. 2. Changes in RNA content in developing brain of normal and thyroidectomized neonatal rats. (A) Cerebral cortex; (B) cerebellum. Each point represents the mean and SEM for eight rats at each age. (●) normal rats; (○), thyroidectomized rats. Differences in the cortex are significant from day 5 onward; in the cerebellum from day 10 onward. Data taken from Pasquini (1967).

remains unclear because neither of these groups actually measured the mass of the precursor pool in order to take into account possible alterations in the specific activity of the tracer.

In the cerebellum, total DNA content, a reflection of cell number, is transiently reduced by 26% at 14 days of age in neonatally thyroidectomized rats but is essentially normal by 35 days of age (Balazs *et al.*, 1968; Pasquini *et al.*, 1967). This observation is consistent with the finding of Hamburg *et al.* (1971) and Nicholson and Altman (1972a) of an extended period of cell proliferation. However, both RNA and protein content were reduced (Figs. 2B and 3B) indicating that cell size must be reduced. Whether this deficiency is permanent is unknown. Although Geel and Timiras (1967) demonstrated that thyroxine replacement begun on the sixth day restored these parameters to normal in the rat, the reports of Anderson (1971) and others, described earlier, of persistent cerebellar dysfunction in cretinous children suggests that some defects may be permanent despite treatment due to the later stage of development at birth in humans. Zamenhof *et al.* (1971) demonstrated in the rat that hypothyroidism during fetal life induced by thiouracil treatment of the pregnant mother from the fifteenth day of gestation did not cause any abnormality of fetal development. This suggests that the role of thyroid hormones, insofar as brain development is concerned, is confined to the immediate postnatal period in this species. But since the rat is born at a time in development generally regarded to be approximately equivalent to the beginning of the third trimester in human, this period of human pregnancy may be critical for thyroid hormone-dependent development of the central nervous system.

Autopsy studies by Dobbing and Sands (1973) of 139 human brains from 10 weeks of gestation to 7 years of age suggest that cerebral DNA content (cell number) doubles in the period from birth to 2 years with a slow increase continuing as late as 7 years of age (Fig. 4). However, cellular concentration (DNA/g) falls markedly (Fig. 5) by the time of birth with little change thereafter, suggesting that major increases in cell size, dendritic branching, and development of other interneuronal elements occur prior to birth. On the other hand, accumulation of the largest fraction of the eventual total cerebellar DNA (approximately 80%) occurs after birth and is complete by approximately 2 years of age (Fig. 4). These findings confirm earlier histological studies by Raaf and Kernohan (1944) of cerebellar development. Those authors demonstrated little change in width of the external granular layer of the cerebellum during fetal life. From birth to 9 months of age, a sharp drop in width occurs (80–85%) with further progressive loss of this layer until it disappears at 18–20 months of age. This is associated with a sharp increase in granule cell numbers and in the width of the molecular layer which is essentially complete by the end of 1 year.

Of importance, Dainat and Rebiere (1978) have reported that excess hormone administered to neonatal rats causes a transient increase in cerebellar DNA content (cell number) at day 6. However, from days 10 to 35, the content of DNA was below normal. Balazs *et al.* (1971a) also reported a permanent deficit in cerebellar cell proliferation caused by excess hormone. These findings are

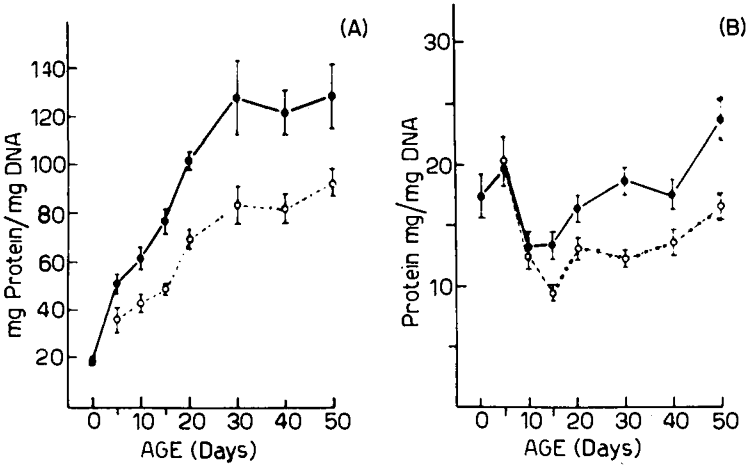

Fig. 3. Changes in protein content in developing brain of normal and thyroidectomized neonatal rats. (A) Cerebral cortex; (B) cerebellum. Each point represents the mean and SEM for eight rats at each age. (●) Normal rats; (○), thyroidectomized rats. Differences are significant from day 5 onward in cortex and from day 15 onward in the cerebellum. Data taken from Pasquini (1967).

obviously relevant to the clinical treatment of hypothyroid newborns and emphasizes the necessity of careful evaluation of replacement doses of thyroid hormones.

Weichsel and co-workers have examined the role of thyroid hormones in control of several enzymes that take part in cerebellar nucleic acid synthesis including aspartate transcarbamylase, thymidine kinase, and thymidilate synthetase. In order to determine which of these might be rate limiting and, therefore, possible points of hormonal control, they studied the temporal relationship of the response of these enzymes to the hormone-induced accumulation of DNA. Thymidine kinase is known to be involved with the so-called "salvage" pathway of DNA synthesis. Thyroid hormone administration to normal neonates raised the level of activity of this enzyme (Weichsel, 1974). However, after cessation of hormone treatment, the activity of thymidine kinase was reduced below con-

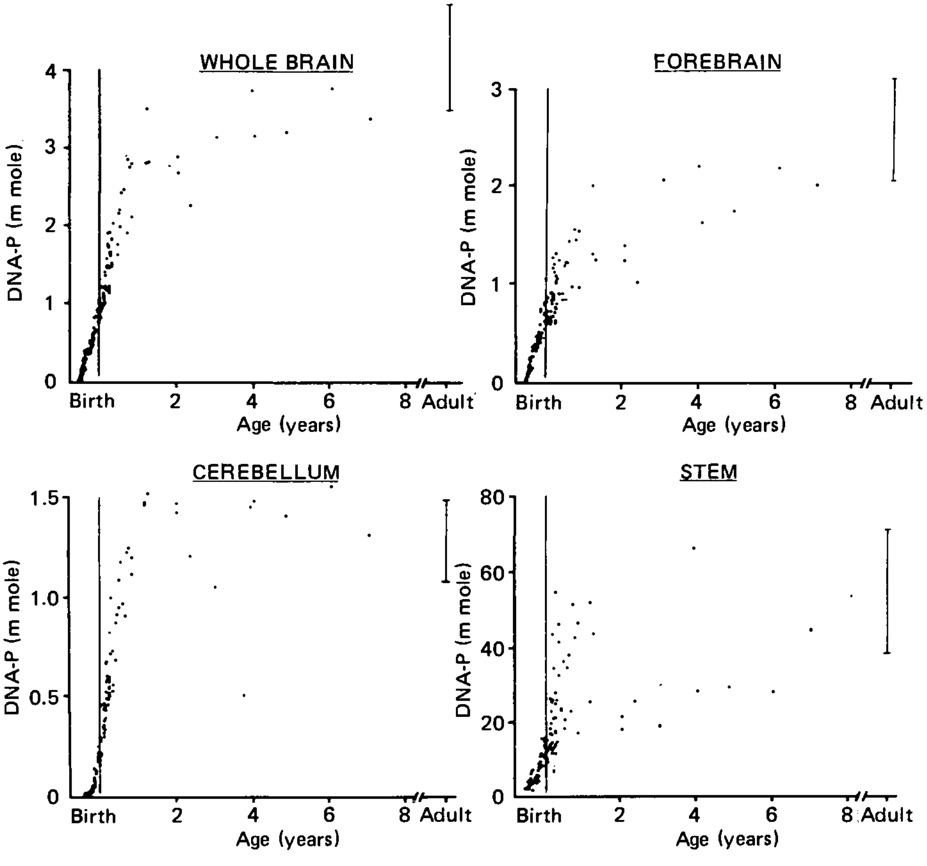

Fig. 4. Total DNA-P content, equivalent to total cell number in whole brain, forebrain, cerebellum, and stem during growth of human brain. Data taken from Dobbing and Sands (1973).

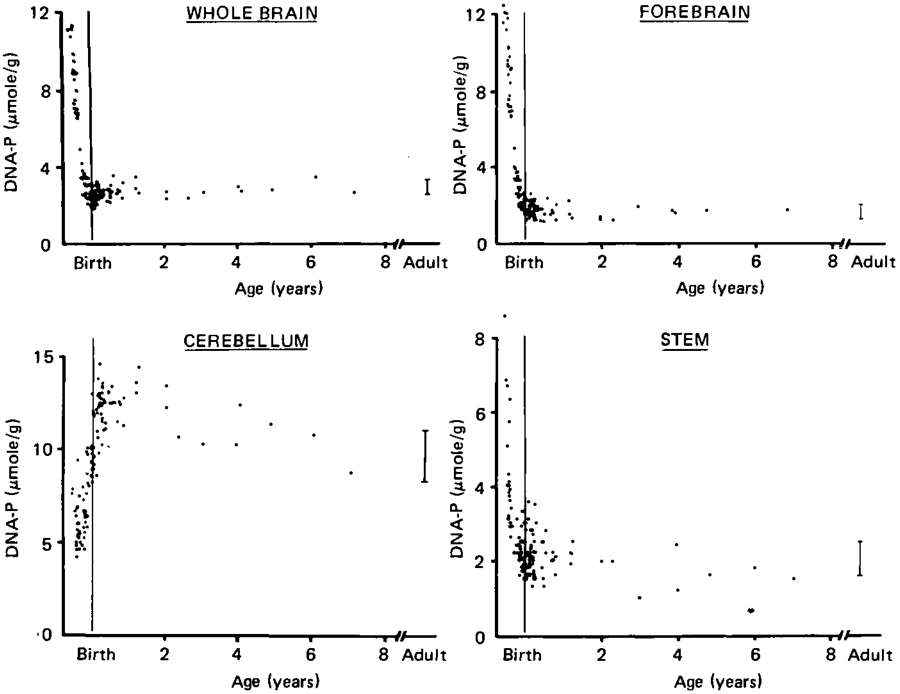

Fig. 5. Concentration of DNA-P per unit of fresh weight of whole brain, forebrain, cerebellum, and stem during growth of human brain. Data taken from Dobbing and Sands (1973).

trol levels significantly in advance of the rate of fall in the rate of DNA accumulation. The activity of aspartate transcarbamylase, an enzyme involved in *de novo* pyrimidine synthesis, is also enhanced by hormone excess and decreased in its absence (Weichsel, 1976). Studies by Clark and Weichsel (1977) and Clark *et al.* (1978) demonstrated that rates of cerebellar DNA accumulation were thyroid hormone dependent. Moreover, the activity of thymidylate synthetase closely paralleled changes in DNA accumulation, falling in hypothyroid neonates and increasing in response to excess thyroid hormone. This enzyme catalyzes the final, perhaps rate-limiting, step in pyrimidine interconversion that transforms UMP, a ribonucleotide to thymidine monophosphate, a deoxyribonucleotide.

D. Myelination

As had earlier been described in clinical autopsies, neonatal hypothyroidism in the rat leads to marked reduction in myelination. Walravens and Chase (1969) and Balazs *et al.* (1969) reported that total brain lipids, but not phospholipids, are reduced by approximately 15%. The content of cholesterol, cerebroside, and sulfatide components of myelin were even more markedly affected. However, the quantitative relationships of the various lipid components of myelin were not altered by hypothyroidism (Balazs *et al.*, 1969; Grippo and Menkes, 1971). The dry weight of myelin was reduced by 30%. Walravens and Chase (1969) reported that daily triiodothyronine administration to hypothyroid neonates normalized the developmental pattern of [^{35}S]sulfate incorporation into myelin. Balazs *et al.* (1971b), however, found no change in [^{35}S]sulfate incorporation after administering excess hormone to normal neonates.

As with myelin lipids, total myelin proteins are significantly reduced (40%) in hypothyroid neonates, but the ratios of the various proteins to each other are not altered (Matthieu *et al.*, 1975). For example, although total 2′,3′-nucleotide 3′-phosphodihydrolase, an enzyme component of myelin, is reduced by 40%, its specific activity is unchanged (Wysocki and Segal, 1972; Matthieu *et al.*, 1975). This suggests that both the lipid and protein composition of myelin are normal despite their reduced content. Two morphological studies suggest that the re-

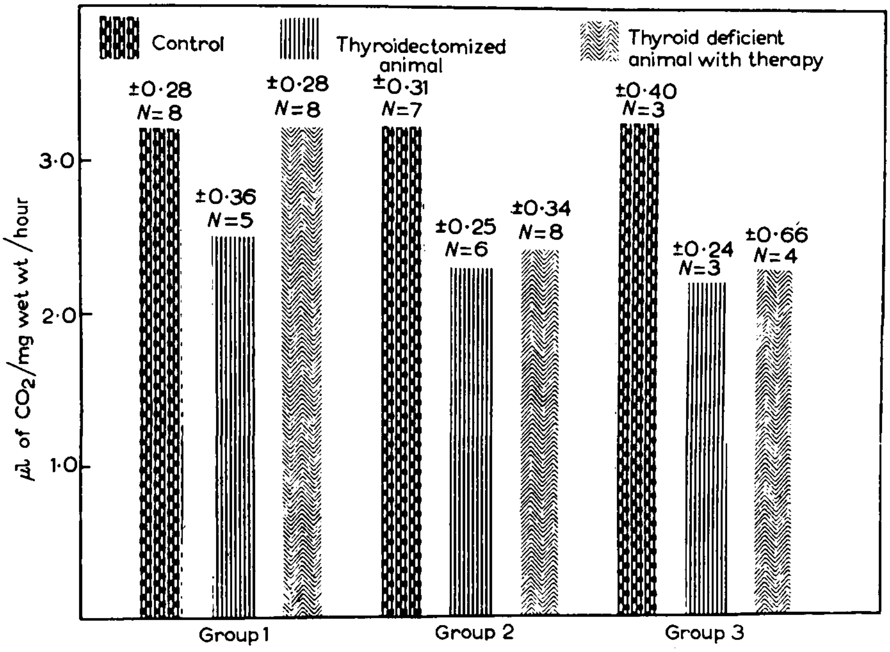

Fig. 6. Effect of replacement therapy on succinic dehydrogenase activity in animals thyroidectomized at birth. (Group 1) Therapy started on day 10 and continued for 15 days; (Group 2) therapy started on day 15 and continued for 15 days in six animals, for 50 days in one animal and for 60 days in one animal; (Group 3) therapy started on the day 20 and continued for 15 days. Over each bar, the height of which represents the mean, is placed a SEM and N = the number of rats in each group. Data taken from Hamburgh and Flexner (1957).

duced myelination may be primarily related to a reduction in available axonal surface (Rosman *et al.*, 1972; Freundl and Van Wynsberghe, 1978). Both groups reported that the numbers of axons per unit area were unaltered, but that the total area of axonal surface per field was decreased.

Studies of several enzymes involved in myelin synthesis suggest that reduced synthesis, not increased degradation, is the cause of the observed decrease in myelin accumulation in the absence of thyroid hormone. Wysocki and Segal (1972) reported that UDPgalactose:sphingosine galactosyltransferase activity is reduced by almost half. This enzyme is involved in the synthesis of psychosine, a cerebroside precursor. Triiodothyronine administered to hypothyroid neonates in the first week of life caused full recovery of activity. Flynn *et al.* (1977) studied the effect of thyroid hormone on a number of enzymes involved in various pathways of diacylglycerol synthesis and degradation and found a 40% reduction in monogalactosyl diacylglycerol concentration in the brains of 20- and 23-day-old rats made hypothyroid at birth. These same animals had a retarded pattern of development of galactosyltransferase activity. In rats made neonatally hyperthyroid, there was a premature rise in monogalactosyl diacylglycerol: galactosyltransferase activity. This enzyme is the first in the pathway committed to galactosyl diacylglycerol synthesis. Other enzymes involved in precursor synthesis, such as α-glycerophosphate dehydrogenase (cytosol and mitochondrial forms) and glycerol-3-phosphate:fatty acyl-CoA acyltransferase were unaffected. β-Galactosidase and galactolipase, enzymes responsible for diacylglycerol degradation, were also not affected by thyroidal state.

E. Intermediate Metabolism

A wide variety of enzymes and metabolic pathways have been studied in relation to the role of thyroid hormones. As with most of the indices already described, the critical period of change in the parameters to be discussed is approximately the tenth day of life in the normal course of development, and it is at this time that the effects of neonatal hypo- or hyperthyroidism become apparent. For example, succinic dehydrogenase, a mitochondrial enzyme, normally begins to rise to adult levels at the tenth day of life. In neonates made hypothyroid at birth, reduced activity is observed between the tenth and fifteenth days (Hamburgh and Flexner, 1957; Garcia-Argiz *et al.*, 1967). Replacement therapy begun at the tenth day leads to normalization of enzyme activity (Fig. 6). If treatment is delayed to the fifteenth or twentieth day, the hormone has little effect (Hamburgh and Flexner, 1957). This critical period of hormone-dependent biochemical maturation extends to a wide variety of enzymes including glutamate dehydrogenase, aspartate aminotransferase, γ-aminobutyric-acid transaminase, and cholinesterase (Krawiec *et al.*, 1969; Garcia-Argiz *et al.*, 1967; Hamburgh and Flexner, 1957; Geel and Timiras, 1967).

Despite the wide range of enzyme activities in brain, which are sensitive to thyroidal status during this early period of life, it is of interest that the mitochondrial enzyme α-glycerophosphate dehydrogenase, which is used so commonly as a hepatic marker of thyroid hormone response, does not respond. Schapiro and Percin (1966) showed that in the liver α-glycerophosphate dehydrogenase responds to administration of thyroid hormone as early as the fetal stage, yet in the brain there is a complete absence of response at any age despite changes of activity of other mitochondrial enzymes in response to the hormone. This represents a primary example of the tissue specificity of response to thyroid hormone, which has recently been discussed by Oppenheimer (1979).

Cocks *et al.* (1970) and Patel and Balazs (1971) demonstrated a role for thyroid hormone in the development of various biochemical pathways in brain as reflected in the capacity for rapid and extensive conversion of glucose into amino acids. This capacity develops during a short period from 10 to 21 days after birth. Cocks *et al.* (1970) administered [^{14}C]glucose to neonates and measured its incorporation into glutamate, glutamine, aspartate, and γ-aminobutyric acid. The rate of glucose incorporation into amino acids was reduced in thyroidectomized neonates after the twelfth day and increased at about the same time in hyperthyroid neonates in comparison to normal controls. However, by the eighteenth day of life, the rate of incorporation in hyperthyroids was equal to that in normal animals of that age. This suggests that excess hormone had accelerated the maturational process. A detailed analysis of the pathway of glucose utilization demonstrated that the total glutamate content of the cerebrum did not vary with thyroidal state. However, the rate at which the glucose carbon passed through this pool was lower in hypothyroids and increased by excess hormone. These metabolic alterations occurred simultaneously with the hormone-dependent maturation of the neuronal dendritic fields and nerve terminals. These authors and others have suggested that the greater part of energy-yielding metabolism in the brain is associated with the dendrites rather than the cell bodies of the neurons.

F. Relationships of Thyroid Hormone and Other Growth-Promoting Factors

It has long been known that thyroidectomy leads to a rapid decline in pituitary growth hormone content to almost undetectable levels (Solomon and Greep, 1959; Contopoulos *et al.*, 1958; Hervas *et al.*, 1975). It was logical, therefore, to suppose that the action of thyroid hormone on the neonatal nervous system may, in fact, be secondary to regulation of the concentration of growth hormone or other growth-promoting factors such as the somatomedins and nerve growth factor. However, as in studies of the interaction of these hormones in general growth, the results appear to suggest a more complex relationship.

A series of papers from Evans Laboratory (Walker *et al.*, 1950, 1952; Asling *et al.*, 1950) demonstrated that despite hypophysectomy at a very early age, 6 days, brain weight was not found to be abnormal at 30 days of age. However, it was found that all these animals died by 75 days of age, suffering with symptoms of cerebellar dysfunction: dyspnea, dysphagia, a hopping gait, or paralysis of the hind limbs. Because brain weight was normal despite retardation of skeletal development, the calvarium of these animals bulged outward, and at autopsy it was found that the medulla had herniated through the foramen magnum. Replacement of these animals with growth hormone from 30 days of age renewed skeletal growth and led to extended survival. Diamond (1968) and Gregory and Diamond (1968) repeated these studies and were able to demonstrate that despite a lack of growth hormone during a critical period of brain maturation, the brains of these animals were normal in dimensions and in several indices of histological maturation, including visual and somesthetic cortical depths and complexity of dendritic branching. These data imply that despite hypophysectomy sufficient thyroid hormone was secreted to maintain normal brain development. However, it is also possible that a non-growth hormone-dependent somatomedin or other factors may be active intermediates in this process, and it may be these factors that are regulated by thyroid hormone. Recent studies by Walker *et al.* (1979) suggested that brain concentrations of nerve growth factor are sensitive to thyroid hormone levels. Another (Burstein *et al.*, 1979) reports that propylthiouracil-induced hypothyroidism in neonates leads to a decrease in serum concentrations of insulin-like growth factor at 10 days of age without any significant change in serum growth hormone concentration. There are also several reports that suggest that serum concentrations of somatomedin C activity are responsive to thyroid hormone levels (Gaspard *et al.*, 1978; Holder and Wallis, 1977), presumably secondary to altered growth hormone concentrations.

Despite the apparent lack of effect of growth hormone deficiency on brain development in neonatally hypophysectomized rats, there have been a number of reports that suggest that this hormone can restore to normal a variety of biochemical indices in brains of thyroidectomized neonates. Krawiec *et al.* (1969) reported that growth hormone given to thyroidectomized rats normalized the brain weight and the DNA and RNA concentrations. This has been disputed by Geel and Timiras (1970), who found growth hormone only slightly affected these parameters. Krawiec *et al.* (1969) also reported that growth hormone could substitute for triiodothyronine in restoring to normal a variety of respiratory enzymes. Gomez *et al.* (1966) reported that growth hormone was equally as effective as thyroxine in correcting the deficit in QO_2, glucose uptake, and lactic acid production measured in brain slices in vitro. However, Eayrs (1961) reported that growth hormone could not correct the abnormal length to width ratio of the brain nor did it stimulate differentiation of neuronal dendritic fields in hypothyroid neonates. Lastly, Drash and colleagues (1968) reported that for a group of 36 idiopathic hypopituitary dwarfs the mean and distribution of scores attained on the Wechsler and Stanford–Binet tests were normal. If, as has been observed in other species (Eayrs, 1971), this result can be related to normal histological and biochemical development, it suggests that GH is not necessary for normal development of the central nervous system.

The sum of these data suggests that, as in the development of the skeleton and other organs, growth hormone and possibly the growth hormone-dependent growth factors may affect processes related to growth and cellular proliferation, but that cellular differentiation and maturation of the central nervous system are thyroid hormone dependent.

V. LUNG

During the past decade there has been growing interest in the role of thyroid hormones in the development of the fetal lung. This interest has been spurred by a number of clinical and laboratory reports that suggest that the respiratory distress syndrome observed in significant numbers of premature infants may be related to inappropriately low plasma thyroxine and triiodothyronine levels. Respiratory distress syndrome appears to be caused by a deficiency of surfactant in the immature lung. The surfactant, composed mainly of phospholipids, reduces the work required for breathing by decreasing the surface tension at the air–liquid interface. The ability of thyroxine to act upon type II pneumonocytes to alter surfactant production was first demonstrated by Redding and co-workers (1972). Measurement of the phospholipid content of lung tissue from hypothyroid and hyperthyroid adult rats demonstrated that in the absence of thyroid hormone there was a marked decrease in surfactant concentrations, whereas hyperthyroidism led to a significant increase. Histological examination showed that only the type II cell was affected by altered thyroid states. These cells in lung tissue from hypothyroid animals were reduced in diameter, contained fewer lamellar bodies, and their nuclear chromatin was clumped, all signs of reduced synthetic activity. Both cell diameter and concentrations of lamellar bodies were increased by excess hormone.

These findings were confirmed in a study by Wu and colleagues (1973) in which development of fetal rabbit lung was accelerated by direct injection of 1 μg of thyroxine into each fetus. Morphological examination demonstrated acceleration of maturation of the lung as a whole but particularly of the type II alveolar epithelium, which contained increased numbers of secretory granules. These histological observations were consistent with chemical measurement of increased surfactant activity. Wu and co-workers (1973) were unable to demonstrate any affect on fetal lung maturation if the thyroid hormone was administered to the pregnant doe. This is consistent with the general finding that virtually no maternal thyroid hormone crosses the placental barrier to affect fetal development.

The increasing concentrations of phospholipids in the surfactant coating the alveolar membranes during maturation apparently alter the absorptive properties of the membranes. This has been demonstrated by measuring the ability of the lung to absorb such lipid-insoluble substances as mannitol and *p*-aminohippuric acid. Normally these drugs are more easily absorbed prior to 12 days of age than at 18 days (Hemberger and Schanker, 1978). When thyroxine was administered to neonatal rats during the first 4 days of life, the developmental process was accelerated so that significantly reduced permeability was already evident at the twelfth day of life.

Hitchcock (1979) carried out a series of histological studies in the rat, that demonstrated that the action of thyroxine on fetal lung is likely not totally dependent on the presence of glucocorticoids, which are known to affect maturation of the lungs, but that the two hormones may act together to affect full development of this organ. Although pretreatment of the fetuses with Metopirone led to some reduction in the effectiveness of thyroxine, the latter clearly increased the maturational process. A sidelight of interest arising from these same studies (Hitchcock, 1979) is the finding that the fetuses in one uterine horn are not unaffected by direct treatment of fetuses in the contralateral horn as has been heretofore accepted in some studies. Saline-injected fetuses in the control horn contained as much as 10% of the [^{131}I]thyroxine measured in the hormone-treated fetuses from the contralateral horn. In addition, their lungs showed clear evidence of some histological maturation when compared to age-matched controls taken from other mothers.

Presumably the demonstration of the ability of thyroid hormones to affect lung tissue implies the presence of receptors for the hormones (Oppenheimer *et al.*, 1974). Three recent studies have been reported demonstrating that this tissue in rat, rabbit, and humans, does, in fact, contain specific nuclear receptors for triiodothyronine. Morishige and Guernsey (178) measured the binding capacity of these receptors in adult rat lung, 0.16 ng T_3/mg DNA, which was relatively low when compared to that in liver, 0.66 ng T_3/mg DNA. The concentration of receptors in the lung appears to be similar to that found in rat kidney (Surks and Oppenheimer, 1977). Lindenberg *et al.* (1978) demonstrated in rabbit lung tissue that there is a fall in nuclear receptor concentration with age. In the fetal lung they measured 2400 nuclear sites per cell (0.44 ng T_3/mg DNA), whereas in the adult the concentration was 1120 sites per cell (0.20 ng T_3/mg DNA) with no alteration in the affinity of the receptor for triiodothyronine. This age-related decrease in nuclear receptor concentration in lung is similar to that reported by Schwartz and Oppenheimer (1978b) for those in rat brain. The physiological significance of the decrease in these tissues is not readily apparent. However, Gonzales and Ballard (1981), in a recent study of nuclear T_3 receptors in human fetal lung, found that receptor concentration rose from 0.17 ng T_3/mg DNA at 12–13 weeks of gestation to 0.27 ng T_3/mg DNA at 16–19 weeks. This same period is one in which thyroid hormones first appear in the fetal circulation (Greenberg *et al.*, 1970) in very low concentrations. It is tempting to speculate that the rise in receptor concentration in fetal lung, and in neonatal rat brain, acts to concentrate scarce hormone at the site of hormonal action at critical periods of development.

The receptor concentration in the adult rabbit appears to be quite similar to that found by Morishiga and Guernsey (1978) in adult rat lung. Schuster and coworkers (1979) have also reported near identity of the receptor concentrations for human and rat liver and kidney. These data suggest the possibility that receptor concentrations may, for any given tissue, be very similar in various mammalian species.

These laboratory findings have prompted several clinical studies of the relationship of insufficiency of thyroid hormone in the fetus and the onset of respiratory distress. Several groups have reported lowered cord blood levels of thyroxine and triiodothyroine in premature infants with the respiratory distress syndrome (Redding and Pereira, 1974; Cuestas *et al.*, 1976; Schoenberger *et al.*, 1979a). Two groups have reported trials of the prophylactic use of thyroxine in premature infants (Schoenberger *et al.*, 1979b) and in cases of high-risk pregnancy (Mashiach *et al.*, 1978) and claim to have reduced the incidence of respiratory distress syndrome. In the latter case, thyroxine was administered intra-amniotically. These studies have provoked considerable discussion; however, several factors must be considered before accepting the conclusions of these reports. First, these studies were poorly controlled with regard to the gestational age at birth of the infants treated in the study by Schoenberger *et al.* (1979b) and for the not insignificant stress effect of amniocentesis. Moreover, not all groups have observed an association of respiratory distress syndrome with decreased thyroid hormone concentrations (Hadeed *et al.*, 1980). Lastly, the respiratory distress syndrome is not common in cases of congenital hypothyrodism. This suggests that thyroid hormones are not primary determinants of the disease. It is true, however, that cretins are generally born at full term and it is possible that during the added weeks of gestation the presence of other factors such as glucocorticoids may result in normal lung maturation. Moreover, since glucocorticoids can effectively accelerate lung maturation when administered to the pregnant mother, use of this hormone would obviate the risks attendant on the need to administer thyroid hormones via amniocentesis.

VI. CONCLUSION

Clearly, growth and differentiation are complex processes that are dependent on genetic, hormonal, nutritional, and other factors. Though we have in the foregoing discussion described the overwhelming evidence for a role for thyroid hormones in the development of the organism we are as yet largely ignorant of the mechanism of its action. Recent evidence suggests that the thyroid hormones act within the cell primarily through interaction with a set of specific nuclear receptors to modulate transcriptional activity (Oppenheimer, 1979).

We have earlier referred to the tissue specificity of response to thyroid hormones, that is, that the profile of metabolic responses observed after exposure to thyroid hormone varies from tissue to tissue. It is also true that tissues enter the differentiative phase at varying times after synthesis and secretion of thyroid hormones have begun. For example, maturation of rat lung is affected by thyroid hormone during fetal stages whereas brain development appears to require thyroid hormone during the postnatal period. This suggests that the temporal sequence of tissue maturation as well as the profile of responses of adult tissues to thyroid hormone is governed by intracellular factors and not by thyroid hormone or other hormone-dependent circulating factors. To date, no information is available as to whether thyroid hormones induce production of a tissue specific protein product(s) that might act as a signal for cellular differentiation. Clearly, if this were the case it is likely that the ''clock'' governing the time of synthesis of such a protein would reside within the cell and that this signal protein would act locally, within the tissue of origin.

The timing of the shift from a proliferative to a differentiative phase of development may alternatively be signaled, not by a specific protein, but rather when a particular set of metabolic pathways reaches a critical level of activity. The role of thyroid hormone in this case might be to stimulate the activity of these enzymes to the critical level. In this case, too, the time of response to hormone would be controlled within the cell. Thyroid hormone action in this model would entail modulation of transcription of genes already expressed rather than causing the expression of previously quiescent genes. At present, the available data do not allow us to choose among the various possibilities. We also lack understanding as to why, apparently in the brain alone, there is a critical period of time within which thyroid hormone must act in order for normal maturation of the central nervous system to proceed. Where and what is the block to its action at later times? Recent reports by Schwartz and Oppenheimer (1978a) suggested that the block to thyroid hormone action in the brain must be beyond the interaction of hormone and nuclear receptors. The receptors in the adult brain appear to be identical with those in the liver and are present in concentrations higher than those in the lung or kidney, tissues that respond to thyroid hormone in the adult animal.

Many questions remain both as to the mechanism of action of the thyroid hormones in general and specifically in relation to their role in maturation of the organism. Hopefully, application of the newer techniques of molecular endocrinology will begin to shed some light on this problem and allow formulation of a reasonable model of hormonal induction of differentiation.

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15

Thyroid Hormone Action in Amphibian Metamorphosis

VALERIE ANNE GALTON

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I. INTRODUCTION

Metamorphosis may be defined as a sequence of postembryonic changes in morphology and function which permit a larva to adapt to an environment that is different from its natal environment (Cohen, 1966). Many anuran amphibia undergo a remarkable metamorphosis during which morphological, biochemical, and behavioral changes occur that transform the aquatic larva into a terrestrial

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adult. These changes include regression of some structures and functions required only in the larval form, transformation of others into forms suitable for use in the adult, and development of new systems not present in the larval form but essential to the adult. Metamorphosis is not merely a process of adaptation; the changes occur in anticipation of, rather than in response to, the new environment.

The importance of the thyroid hormones (TH) in initiating metamorphosis in amphibian species has been recognized ever since Gudernatsch (1912) fed mammalian thyroid tissue to young tadpoles and found that they underwent precocious metamorphosis. Subsequently, it was shown that thyroidectomized tadpoles or tadpoles treated with antithyroid agents failed to undergo metamorphosis, although they continued to grow, unless they were treated with appropriate amounts of thyroid tissue (Allen, 1929; Weber, 1967). In addition to their fundamental importance, these dramatic findings were of practical value to scientists interested in the morphology and biochemistry of growth and differentiation since they made it possible to delay or induce developmental processes in a free living system for the purpose of investigation. Over the past 50 years a vast body of literature dealing with the role of the thyroid in metamorphosis has accumulated. Prior to the 1950 s the reports were concerned primarily with the morphological aspects of amphibian development and the role of the thyroid–pituitary system in this process. These subjects have been covered extensively in several books and reviews (Gorbman, 1964; Etkin, 1964, 1968; Weber, 1967; Dent, 1968; Kaltenbach, 1968). In the last 25 years, however, investigators have been studying the biochemical aspects of amphibian metamorphosis. Not surprisingly, it has been shown that the TH-dependent morphological changes, which occur during metamorphosis, are accompanied by profound TH-dependent biochemical changes in almost every tissue of the organism. Moreover, it is now well established that both TH-induced and spontaneous metamorphic processes almost invariably involve the synthesis of new or additional proteins. This finding is of great importance for two reasons. First, it offers the possibility of a common mechanism for the hormonal control of the diverse structural and functional metamorphic changes (anabolic in liver and limbs; catabolic in tail, gills, and gut). Second, it suggests that there are basic similarities in the action of TH in amphibian and mammalian species (Tata, 1969). Many reviews dealing with the biochemical effects of TH in amphibia are available (Cohen, 1966; Frieden, 1967; Frieden and Just, 1970; Cohen, 1970; Tata, 1971; Dodd and Dodd, 1976). A summary of the biological systems known to be extensively modified during anuran metamorphosis is shown in Table I.

Despite the abundance of information concerning the effects of TH in amphibian tissues, our understanding of the mechanism(s) underlying these effects is far from clear. Although this problem also exists in other species, scientists working with mammalian tissues have made major advances, which have led to the

TABLE I

Some Biochemical Systems Known to Be Modified during Metamorphosis[a]

Origin	Biochemical system
Liver	Increased synthesis of urea cycle enzymes
	Increased synthesis of serum albumin, ceruloplasmin
	Increased activity of liver hydrolases
	Increased synthesis of turnover of some hepatic ribonucleic acids
Tail	Increased synthesis of certain hydrolases, including cathepsins, acid phosphatase, β-glucuronidase, collagenase, DNase, and RNase
	Increased proteolysis
	Decreased rate of total protein synthesis
	Altered rate of synthesis and metabolism of some ribonucleic acids
Limb buds	Protein and nucleic acid synthesis leading to development and growth
Intestine	Decreased synthesis of carbohydrases, increased synthesis of proteases
Blood	Increased concentration of total plasma proteins
	Decreased synthesis of larval hemoglobin, increased synthesis of adult hemoglobin
Eye	Change in retinal pigment from porphyropsin to rhodopsin

[a] For specific references see text and reviews (Frieden, 1967; Dodd and Dodd, 1976).

general concept that the first step in the action of TH is its interaction with a receptor located in the cell nucleus. This results in an increase in transcriptional activity, formation of new mRNA's, and synthesis of specific proteins that are ultimately responsible for the expression of TH action at the cellular level (for reviews, see Oppenheimer and Dillman, 1978; Samuels, 1978; Latham *et al.*, 1978). It is important to recognize, however, that this hypothesis has only been partially tested and it avoids the real issue of mechanism of action, and places the site rather vaguely at the level of transcription. Furthermore, although many investigators feel that most, if not all, of the physiological effects of the hormones may be initiated at the level of the nucleus, the possibility that there may be multiple sites of initiation has not yet been ruled out. Nevertheless, this general concept serves as a working hypothesis for many investigators, and evidence in support of it is steadily accumulating.

It is becoming increasingly apparent that a comparable hypothesis may pertain also to the initiation of TH action in amphibian tissues. As indicated above, formation of new proteins is fundamental to most of the changes induced by TH in this species and several investigators have shown that this is preceded or accompanied by synthesis of RNA (Finamore and Frieden, 1960; Cohen, 1970; Tata, 1971; Weber *et al.*, 1974). These findings are complemented by reports from at least two laboratories indicating that putative TH receptors are present in the nuclei of several tissues of anuran tadpoles (Yoshizato *et al.*, 1975a; Kistler *et al.*, 1975a; Galton, 1979, 1980a; Toth and Tabachnick, 1979).

This chapter focuses on the mechanisms involved in initiating the effects of TH, rather than on the effects themselves, and therefore no attempt has been made to present a comprehensive list of their known effects. For this the reader can turn to the several reviews listed above. Instead, specific effects that offer some insight into mechanism of action will be discussed. Emphasis will be placed on a review of our knowledge concerning hormone receptors in amphibian tissues and the relative importance of thyroxine (T_4) and 3,5,3′-triiodothyronine (T_3) in this class of vertebrates. In order to provide an appropriate background for these discussions, a brief outline of the developmental changes of a typical Ranid anuran will be given with appropriate terminology, followed by a survey of thyroid function and hormone economy at various stages in its life cycle.

II. GENERAL PATTERN OF DEVELOPMENT OF ANURAN AMPHIBIA

The postembryonic development of an anuran amphibian (i.e., *Rana catesbeiana*, *Rana pipiens*) can be divided into three phases (Etkin, 1968): (1) a premetamorphic phase characterized by growth but little morphological change; (2) a prometamorphic phase in which the rate of body growth is reduced and morphological changes proceed at a progressively accelerating pace; and (3) a phase of metamorphic climax in which body growth has ceased and differentiative changes proceed with extreme rapidity. In terms of the widely used staging system of Taylor and Kollros (1946), tadpoles are considered premetamorphic until they reach stage XII. Prometamorphosis lasts until stage XX at which point

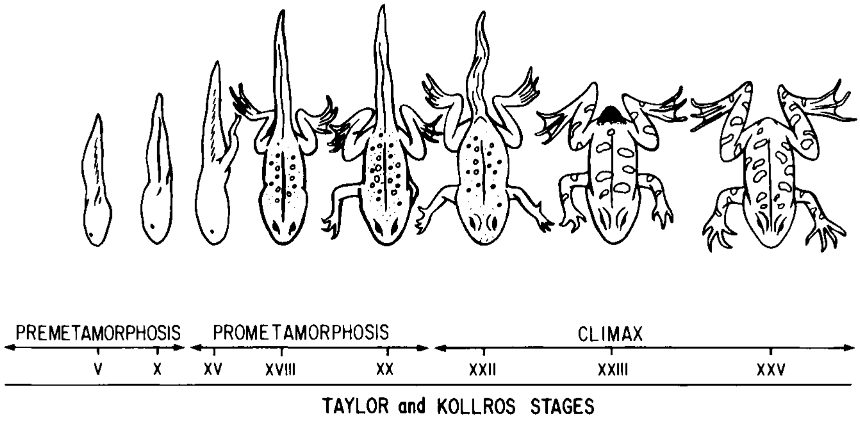

Fig. 1. Pattern of development of the *Rana catesbeiana* tadpole. Tadpoles are staged as described by Taylor and Kollros (1946).

the forelimbs emerge and the animals enter metamorphic climax. Climax ends at stage XXV when tail resorption is complete (Etkin, 1968). The gross anatomical changes observed in the different phases are illustrated in Fig. 1. The duration of the three phases are vastly different. In *Rana catesbeiana* tadpoles, the premetamorphic phase generally lasts for more than a year and prometamorphosis for several weeks. However, the dramatic changes observed in metamorphic climax are usually accomplished in less than 2 weeks.

III. THYROID FUNCTION AND CIRCULATING HORMONE LEVELS IN ANURAN AMPHIBIA

A. During Development

It is generally agreed that most anuran tissues acquire the capacity to respond to TH during late embryonic or early larval life (Etkin, 1950; Tata, 1968). At this time, thyroid tissue can be distinguished, the cells can organify iodide, and, at least in some anuran species, they can synthesize T_4 and T_3 as well as mono- and diiodotyrosine.

The involvement of the pituitary in anuran thyroid function is also evident in early larval life. Kaye (1961) found a difference in thyroid function between normal and hypophysectomized tadpoles as early as stage III. At this stage also, ^{131}I uptake by the thyroid could be depressed by exogenous T_4 or T_3, and administration of the antithyroid compound, thiourea, resulted in a cytological response in the pituitary. The latter finding was taken to indicate that TH is normally present in the circulation by stage III of premetamorphosis, an interpretation that is consistent with the reports that low levels of TH are required for normal development even during early premetamorphosis (Kollros, 1961; Etkin, 1968). However, these levels are too low to be detected by current techniques (Regard *et al.*, 1978).

The ability of young anuran larvae to both synthesize TH and respond with premature metamorphosis to exogenous T_4 and T_3 suggests that the levels of endogenous hormone may be critical to the orderly progression of their life cycle. There is good evidence that this is indeed the case.

First, there is a steady increase in the activity of the thyroid gland during development. Kaye (1961) studied thyroid function, as reflected by the 48 hour uptake of ^{131}I, throughout the embryonic, pre-, and prometamorphic life of *Rana pipiens* tadpoles. She found that ^{131}I uptake was less than 1% of the injected ^{131}I in tadpoles before stage X. Between stages XII and XIV there was a sudden increase in ^{131}I uptake to 12%. This coincided with the first significant sign of metamorphosis (increase in leg growth). ^{131}I uptake continued to increase during prometamorphosis and reached a maximum of 43% at the beginning of metamorphic climax (stage XX). Expressed in terms of tissue weight, a 15-fold increase in uptake occurred between stages XI and XXII. No measurements were made at subsequent stages of climax. Comparable data have been obtained in other anuran species (Dodd and Dodd, 1976). Dodd and Dodd (1976) have also attempted to quantitate the rate of T_4 synthesis during prometamorphosis and metamorphic climax in *Xenopus laevis* tadpoles by measuring the percentage of the total thyroidal ^{131}I that was in the form of $[^{131}I]T_4$ at different times after injection of ^{131}I. After correcting for differences in total uptake it was concluded that the rate of synthesis of T_4 at the onset of metamorphic climax was greater than at any other period during larval life and at least 3 times the rate observed in midclimax, the period of rapid tail resorption. The major iodothyronine in the larval thyroid gland appears to be T_4. Hanaoka *et al.* (1973) have demonstrated that, although at first, more T_3 than T_4 is present in the gland, by the time the premetamorphic stage is reached, T_4 is the predominant iodothyronine observed. Others have reported that T_4, together with trace amounts of T_3, is easily detectable during prometamorphosis and metamorphic climax in *Xenopus laevis* tadpoles. However, by the end of climax, the amount of T_4 was diminished and no T_3 could be found in the gland (Shellabarger and Brown, 1959; Leloup and Fontaine, 1960).

Second, tadpoles exhibit an increased requirement for thyroid hormone during normal development. Kollros (1961) determined the stages reached by *Rana pipiens* tadpoles, which had been hypophysectomized at the tail bud stage and immersed in different concentrations of T_4. The lowest concentration (0.002 μg/liter) was only sufficient for development to stage IX. A 10-fold and 100-fold increase in concentration was required for the tadpoles to progress to stages XV and XIX, respectively.

Third, circulating levels of T_4 and T_3 increase during development. Using sensitive radioimmunoassays (RIA), several investigators have measured T_4 and T_3 levels in anuran tadpoles. Data obtained by Regard *et al.* (1978) in *Rana catesbeiana* tadpoles are shown in Fig. 2. During premetamorphosis, levels of both T_4 and T_3 were below the limits of detection by the RIA procedures employed (< 0.05 μg/100 ml for T_4 and < 5 ng/100 ml for T_3). Significant amounts of T_4 were sometimes but not invariably observed during prometamorphosis; T_3 was rarely detected. Serum levels of T_4 and T_3 increased dramatically between the onset and midpoint of metamorphic climax. Thereafter, they decreased and were undetectable in froglets. Comparable data have been obtained by Miyauchi *et al.* (1977) and by Leloup and Buscaglia (1977) in *Xenopus laevis* tadpoles, although in the latter species, the levels of T_3 observed in midclimax were considerably greater than those shown in Fig. 2. In the case of both T_4 and T_3, approximately 1% of the total hormone present is in the free form (Regard *et al.*, 1978; Galton, 1980a,b).

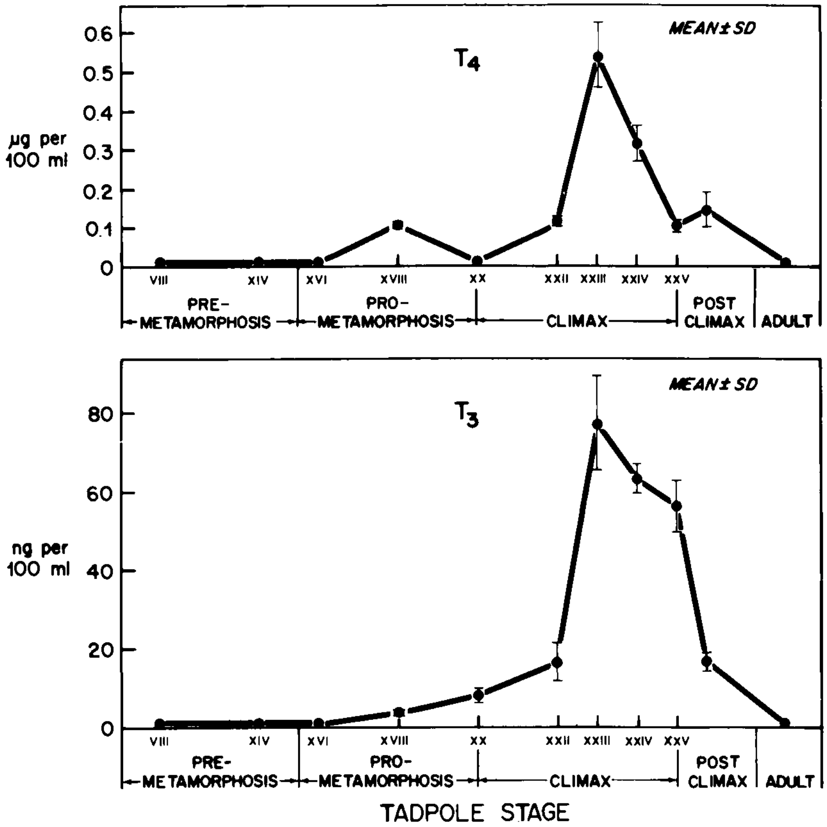

Fig. 2. Plasma T_4 and T_3 in *Rana catesbeiana* tadpoles at various stages of development. From Regard *et al.* (1978).

B. In Adult Anurans

Based on both ^{131}I uptake studies (Leloup and Fontaine, 1960) and histological examination (Coleman *et al.*, 1968), it is evident that thyroid activity is much lower in adult anurans than it is in tadpoles undergoing metamorphic climax. Seasonal variation in activity has also been noted (Rosenkilde, 1972; Ceusters *et al.*, 1978). Despite the apparent low activity, the adult gland can synthesize T_4 and traces of T_3 (Leloup and Fontaine, 1960; Shellabarger and Brown, 1959; Ceusters *et al.*, 1978) and is subject to feedback inhibition by T_4 (Rosenkilde, 1974). Furthermore, a significant decrease in the activity of the adult toad thyroid has been observed after hypophysectomy (Rosenkilde, 1964) and TSH has recently been isolated from bullfrog pituitary glands and found to be about equipotent with mammalian TSH in promoting the release of T_4 from postmetamorphic bullfrogs (MacKenzie *et al.*, 1978).

The first reported values for circulating TH levels in adult amphibia were based on PBI determinations or butanol extraction and chromatography, and values as high or higher than those obtained in mammals were reported (Thornburn, 1967; Van Zyl and Kerrich, 1955; Just, 1972). Values obtained using competitive binding procedures were considerably lower than these; Packard *et al.* (1976) reported a value of 1.7 μg/100 ml for serum T_4 in *Rana pipiens* and Rosenkilde and Jørgensen (1977) obtained values of 1.0–1.3 μg/100 ml in *Bufo viridis* and 0.5 μg/100 ml in *Bufo bufo*. These measurements were made in spring and much lower values were obtained in animals studied in the autumn and winter.

However, recent studies employing sensitive RIA have yielded very different results. Miyauchi *et al.* (1977) were unable to detect either T_4 or T_3 in serum from adult *Rana catesbeiana* frogs, and Regard *et al.* (1978) could not detect either hormone in the adult form of several species of anuran amphibia. Mondou and Kaltenbach (1979) found that plasma T_4 levels were a little higher in the adult form of *Rana catesbeiana* than in the froglet but they were still well below the maximum levels observed during climax and they emphasized that T_4 was not detectable in the serum of all frogs studied. Their measurements were made in plasma from frogs collected in July.

It is not clear why the values obtained by techniques other than RIA are so high, and the possibility cannot be excluded that serum T_4 levels in adults do in fact vary considerably depending on species, season, etc. However, RIA is believed to be more sensitive and specific than the other methods, and thus it seems reasonable to conclude that serum levels of T_4 and T_3 in the adult form of anuran amphibia are below or near the minimal detectable levels throughout much of the year.

In summary, the available data support the concept that TH is essential for normal development at all stages of larval life. During the premetamorphic phase when growth is the major feature, hormone levels are extremely low. As the larva enters the prometamorphic phase and differentiation begins, hormone levels begin to rise, although they still remain near or below the minimum detectable levels until the beginning of climax. Hormone requirement is greatest during climax; the maximum rate of hormone synthesis occurs at the time of *onset* of climax and hormone levels are highest at *mid*climax. Both T_4 and T_3 are present in the serum during climax; in *Rana catesbeiana*, the T_4:T_3 ratio is approximately 5:1. However, once metamorphosis is complete, serum levels of T_4 and T_3 are once again relatively low, suggesting that the requirement for TH is greatly decreased.

Because of the marked changes in the activity of its thyroid system, the anuran amphibian is a unique animal model in which to study the mechanism of action of TH. In such animals, the relationships among the effects of the hormones, their metabolism, and their interactions with specific intracellular binding sites can readily be studied from the time that the animal is unresponsive to T_4 and T_3, through the time when it requires maximum levels for metamorphosis, to postmetamorphic life when, at most, only relatively low levels of hormone are required. Furthermore, in contrast to the situation in mammals, these relationships can be measured not only *in vivo* but in certain isolated systems *in vitro* such as cultured tail tip or tail fin preparations (Weber, 1962) and isolated liver cells (Blatt *et al.*, 1969).

IV. EFFECTS OF THYROID HORMONE IN ANURAN AMPHIBIA

A. Hormone Effects in the Liver

As indicated in Section I, it is well established that both TH-induced and spontaneous metamorphic processes involve the initiation of the synthesis of new or additional proteins. Marked increases occur in the concentration of several serum proteins that are synthesized in the liver (Herner and Frieden, 1961; Inaba and Frieden, 1967) and the activities of many liver enzymes are enhanced (Frieden and Just, 1970). The enzymes that have been explored most extensively are those of the ornithine–urea cycle. The change from ammonotelism to ureotelism, which takes place during either spontaneous or induced metamorphosis, is the result of dramatic increases in the activities of the urea cycle enzymes (Cohen, 1966). The rate-limiting enzyme in this cycle is carbamyl phosphate synthetase (CPS), and there is good evidence that T_4 and T_3 stimulate the *de novo* synthesis of this enzyme. Cohen and his colleagues have isolated CPS from livers of adult *Rana catesbeiana* frogs, raised a specific antibody to it (Marshall and Cohen, 1961), and used this antibody to precipitate newly synthesized CPS labeled with [^{14}C]leucine. They have shown that, following administration of TH either *in vivo* (Metzenberg *et al.*, 1961) or *in vitro* in preparations of cubed liver (Shambaugh *et al.*, 1969), the increase in CPS activity was associated with an increase in amount of immunoprecipitable [^{14}C]CPS.

Although it is evident that the ultimate result of TH action in tadpole liver is the formation of new or additional proteins, the underlying mechanisms are only partly understood. In the last two decades, investigators have approached this question by exploring the molecular events that occur during the lag period that precedes the synthesis of new protein. There is general agreement that T_4 and T_3 influence the pretranslational phase of protein synthesis at some point since enhanced synthesis of RNA is routinely observed during the lag period (Cohen, 1970; Frieden and Just, 1970; Tata, 1971; Weber *et al.*, 1974). However, as will be discussed below, attempts to demonstrate that specific types of RNA, in particular mRNA, are formed as an early response to TH have led to conflicting results, some of which may be due to differences in the techniques and labeled precursors employed in different laboratories. There is also some evidence that TH influence RNA metabolism at the posttranscriptional lévell (Weber *et al.*, 1974) and also the translational phase of protein synthesis (Unsworth and Cohen, 1968; Tonoue *et al.*, 1969).

Finamore and Frieden (1960) were the first to report an effect of TH on hepatic nucleic acid synthesis in tadpoles. These workers found that the rate of synthesis of RNA was increased, as indicated by the rate of incorporation of ^{32}P; 2 days after injection of T_3, no change in base composition of the RNA was observed. In a subsequent study, Eaton and Frieden (1969) observed a significant increase in the specific activity of total RNA within 3 hours of injection of T_3. Although an increase was also observed in the specific activity of the nucleotide pool, the increase in ^{32}P incorporation into RNA appeared to be due to increased RNA synthesis since it was not observed if actinomycin D, a compound that blocks RNA synthesis, was injected prior to the T_3 (Frieden and Just, 1970). It was noted that the base composition of the rapidly formed RNA was different in the T_3-treated and control animals (Eaton and Frieden, 1969).

Tata (1967a, 1971) has studied the time sequence of the changes in RNA and protein synthesis in the liver of *Rana catesbeiana* tadpoles following a single injection of T_3. His findings are presented in schematic form in Fig. 3. After 48 hours, a marked stimulation in the rate of synthesis of rapidly labeled nuclear RNA was observed and this was soon followed by an increase in labeled ribosomal RNA. Since there was little net accumulation of RNA in the cytoplasm, the increased rate of formation of new ribosomal RNA reflected an accelerated turnover of ribosomes. An increase in the fraction of the ribosomes that were present as heavier polysomal aggregates was also noted. This buildup of polyribosomes, which occurred prior to the synthesis of new proteins, is a feature common to regulation of protein synthesis during development (Tata, 1967b). Enhanced protein synthesis, as indicated by increases in the activities of CPS and cytochrome oxidase, the appearance of serum albumin in blood, and an increase in total liver protein, was evident only after several days had elapsed. Unfortunately Tata *et al.* were unable to demonstrate, by base analysis, sucrose density gradient fractionation, and DNA–RNA hybridization studies, that a significant part of the RNA synthesized soon after injection of T_3 was messenger or even DNA-like RNA (Tata, 1971; Wyatt and Tata, 1968). Nevertheless, as Tata originally pointed out (1971), the methods probably were not sufficiently sensitive to detect small changes in a wide spectrum of nuclear RNA molecules.

Ryffel and Weber (1973) have explored the nature of the newly synthesized RNA formed in the liver of control and T_4-treated *Xenopus laevis* tadpoles by subjecting RNA extracts to electrophoresis on exponential polyacrylamide gels

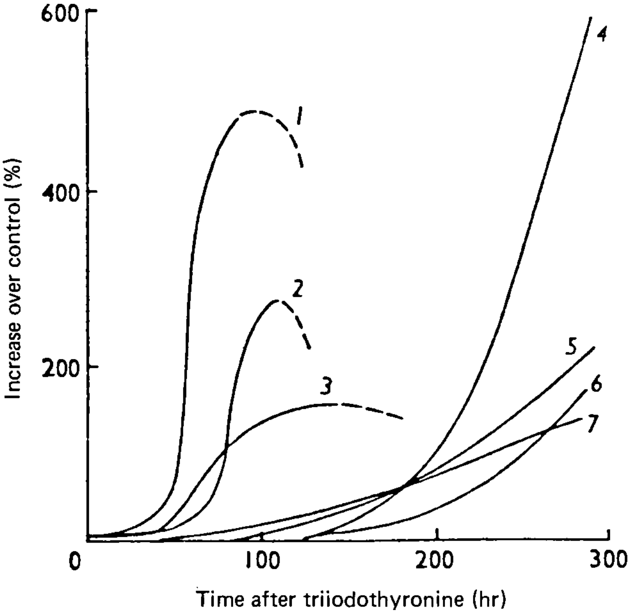

Fig. 3. Schematic representation of sequential stimulation of rates of RNA and phospholipid synthesis in relation to the increases in enzymes and proteins synthesized during the precocious induction of metamorphosis in *Rana catesbeiana* tadpoles with T_3. (Curve 1) Rate of rapidly labeled nuclear RNA synthesis; (curve 2) specific activity of RNA in cytoplasmic ribosomes; (curve 3) rate of microsomal phospholipid synthesis; (curve 4) carbamyl phosphate synthetase activity; (curve 5) cytochrome oxidase activity/mg mitochondrial protein; (curve 6) appearance of serum albumin in the blood; (curve 7) total liver protein/mg wet wt. The values are expressed as percentage increases over those in the noninduced control tadpoles. The broken lines in curves 1, 2, and 3 reflect the dilution of specific radioactivity in precursor molecules due to the onset of regression of tissues such as the tail and intestine. From Tata (1967a).

and analyzing the RNA pattern obtained. Using either [^{3}H]uridine or [^{3}H]guanosine as precursors and a 4 hour labeling period, radioactivity was observed in the RNA's in the 28 S and 18 S (rRNA) fractions and the 4 S (tRNA) region. Label was also present in the areas corresponding to the heavy unstable RNA's that are considered to be precursors of more stable types of RNA. T_4 treatment for 24 hours had no effect on this pattern, but after 4 days of T_4, radioactivity was increased in the areas corresponding to stable RNA's, especially rRNA, and decreased in the unstable fractions. However, no qualitative changes in the RNA pattern were observed. In a subsequent study they found that T_4 had little effect on the size of the precursor pool but markedly stimulated the turnover of ribonucleoside triphosphates (Hagenbuchle *et al.*, 1976). On the basis of their findings these workers concluded that the T_4-induced enhancement of RNA synthesis primarily reflects an increase in transcriptional activity but that T_4 also influences RNA metabolism at the posttranscriptional level by accelerating the maturation of pre-rRNA molecules.

The work of Cohen and his colleagues has been directed primarily toward the elucidation of the mechanism of induction of hepatic CPS. Nakagawa *et al.* (1967) investigated the changes in both RNA metabolism and CPS activity in livers of tadpoles immersed in T_4-containing water for different lengths of time. They found that the rate of synthesis of total hepatic RNA, as indicated by the incorporation of [6-^{14}C]orotic acid during a 2 hour period, was biphasic. There was a pronounced increase after exposure to T_4 for 36 to 48 hours, followed by a decrease and then a gradual increase during the period of days 5–15. Pearson and Paik (1972) have reported similar findings. Nakagawa *et al.* (1967) also examined total RNA turnover in subcellular fractions of tadpole liver. In untreated animals, specific activity was highest in the soluble RNA fraction and little change in turnover was observed after 24 hours of exposure to T_4. After 48 hours of T_4, some increase in turnover of RNA was noted in all fractions, but the most striking increases were in the microsomal and supernatant fractions, suggesting that an early effect of T_4 is a marked increase in the rate of synthesis of ribosomal and transfer RNA. These effects differ to some extent from those of Tata (1967a), who reported that nuclear RNA synthesis was enhanced before any change in cytoplasmic RNA synthesis was evident. At least part of the RNA synthesized was necessary for the induction of CPS: induction of CPS was prevented by actinomycin D if the compound was administered before or with the T_4 (Kim and Cohen, 1968) but not if it was given after the lag period (Nakagawa *et al.*, 1967). Moreover, induction of CPS was not dependent on continuous exposure to T_4. When tadpoles were exposed to T_4-containing water for 2 days and then transferred to hormone-free water, an increase in CPS activity was first evident after 3 days and activity increased slowly but continuously over the next 10 days (Nakagawa *et al.*, 1967). Similarly, when hormone was administered as a single injection, at least 4 days elapsed before any significant increase in CPS activity could be detected, and activity was greater after 7 than after 5 days. Yet, from studies with labeled T_4 it was evident that less than 10% of the hormone detected in liver and serum shortly after injection was still present after 4 days (V. A. Galton, unpublished observations). These findings are consistent with the suggestion that the formation of a relatively stable messenger for CPS synthesis is an early response to TH (Nakagawa *et al.*, 1976).

To obtain further information concerning the types of RNA synthesized after exposure to T_4, Nakagawa and Cohen (1967) fractionated total liver RNA using zonal sucrose density gradient centrifugation. In untreated animals, the distribution of radioactivity 2 hours after injection of precursor ([6-^{14}C]orotic acid) was limited to the area corresponding to RNA fractions of 4–10 S and labeling was greatest in the 4 S (transfer RNA) region. Radioactivity was not found in the 18 and 29 S RNA's until 24 hours. No change in pattern of RNA synthesis was observed after 1 day of exposure to T_4 but after 2 days, when the turnover of bulk RNA was enhanced, most of the rapidly labeled RNA (2–4 hours) was located in the 6 S rather than the 4 S fraction. Furthermore, radioactivity was detected in the 18 S and 29 S RNA fraction by 8 hours. In a comparable study, Pearson and Paik (1972) obtained similar results except that they detected radioactivity in the 6 S region in untreated as well as treated tadpoles.

The studies of Nakagawa and Cohen (1967) differ from those of Ryffel and Weber (1973) in two respects; the latter workers found that significant amounts of radioactivity were present within 4 hours in the ribosomal RNA fractions and the heavier (40 S) fractions. Moreover, they did not observe any new types of RNA following T_4 treatment. They suggest that the rapidly labeled material detected by Nakagawa and Cohen (1967) in the 6 S region may have consisted of degradation products formed from the heavy RNA molecules during the analytical procedures. Nakagawa and Cohen (1967) think this explanation is unlikely for several reasons. First, their procedures were modified to minimize such degradation. Second, radioactivity was present in the 6 S fraction long before any was found in the heavier RNA fractions. Third, the new RNA was found only in the T_4-treated tadpoles; if it consisted only of degradation products formed during analysis, it should have been present in control livers also, unless the activity of RNA degrading enzymes was enhanced by T_4 treatment.

In the studies of Nakagawa and Cohen (1967), the base composition of the 18 S and 29 S RNA fractions isolated from tadpole liver were comparable to that of the bulk RNA, but that of the 6 S fraction was quite different. Furthermore, the base composition of the rapidly labeled RNA detected after treatment with T_4 was similar to that of tadpole DNA. Thus, on the basis of size and composition, this material could contain mRNA. This circumstantial evidence that T_4 stimulates the synthesis of mRNA was recently substantiated by Mori *et al.* (1979) who, using a cell-free translation system, were able to demonstrate increased levels of translatable mRNA for CPS in liver from tadpoles treated with T_4. The increase was observed within 24 hours and did not change substantially during the next 4 days of treatment.

Some information concerning the mechanisms underlying the stimulation of RNA synthesis has been obtained. Kim and Cohen (1966) found that administration of T_4 to tadpoles caused a modification of liver chromatin so that its efficiency as a template for RNA synthesis was increased. T_4 treatment also resulted in an increase in the activities of two DNA-dependent RNA polymerases in tadpole liver nuclei (Griswold and Cohen, 1972). This effect was not due to changes in endogenous template activity since it was observed when solubilized enzyme was assayed on exogenous templates (Griswold and Cohen, 1973). However, it is unlikely that the early effects of T_4 on RNA synthesis can be attributed to increased polymerase activity, since the increases paralleled rather than preceded CPS induction, and the base composition of the RNA produced by the polymerases from control and T_4-treated tadpoles was very similar.

In summary, the available information suggests that thyroid hormone-induced stimulation of protein synthesis in tadpole liver is the result, at least in part, of effects of the hormone on RNA metabolism. The hormones influence RNA synthesis at the level of transcription and possibly also at some point in the post-transcriptional phase. Most of the new RNA formed appears to be of the ribosomal or transfer type, but it may include also mRNA for specific proteins. There is some evidence that the hormones also influence protein synthesis at the level of translation.

B. Hormone Effects in the Tail

The most dramatic event in the metamorphosis of the anuran amphibian is the complete resorption of the tail, and the dependence of this resorption on the local action of TH has been clearly established. Much of the original work on the biochemical basis of tail resorption was carried out in the laboratories of Weber and Frieden (Weber, 1967; Frieden, 1967; Frieden and Just, 1970) and the subject was reviewed in 1976 by Dodd and Dodd. The process of tail resorption is characterized by significant increases in the activities of several hydrolytic enzymes typically associated with tissue destruction, including cathepsins (Weber, 1957; Eeckhout, 1965), acid phosphatase (Weber and Niehus, 1961; Eeckhout, 1965; Robinson, 1970), β-glucuronidase (Kubler and Frieden, 1964; Eeckhout, 1965), collagenase (Lapiere and Gross, 1963; Davis *et al.*, 1975), acid DNase (Coleman, 1962; Eeckhout, 1969), and RNase (Eeckhout, 1969). These changes are observed not only when tail resorption takes place in the intact animal, but also in isolated tail pieces studied *in vitro*. The latter preparation has been widely used for the study of the TH-induced tail regression and it has been shown that the activities of cathepsin (Weber, 1962, 1969; Tata, 1966), collagenase (Davis *et al.*, 1975), β-glucuronidase (Kubler and Frieden, 1964), acid phosphatase (Robinson, 1970), and RNase (Tata, 1966) are all elevated during TH-induced tail resorption. The importance of hydrolytic enzymes in tail resorption is emphasized by the recent work of Seshimo *et al.* (1977) who reported that the T_3-induced regression of tadpole tail fin is inhibited in the presence of the proteolytic enzyme inhibitor, pepstatin. Pepstatin is known to inhibit nearly all acid proteases and is a very strong inhibitor of cathepsin D.

These findings have led to the suggestion that tail resorption is a controlled expression of the activity of intracellular catabolic enzymes and that TH is intimately involved in this control (Frieden and Just, 1970). In the last 20 years, investigators have been studying the mechanism of this control. In 1963, DeDuve proposed the "lysosomal" hypothesis in which controlled tissue resorption would be achieved as a result of the controlled disintegration of intracellular lysosomes. The basis of this hypothesis was the finding that lysosomes contain many acid hydrolases. However, the hypothesis has received little support. As pointed out by Frieden and Just (1970), the distribution of, and changes in, cathepsin, β-glucuronidase, and phosphatases do not correspond to the predictions of the lysosome hypothesis. In fact, the enzymes are distributed almost uniformly throughout the extranuclear fraction of the cell (Eeckhout, 1969).

On the basis of their data, Frieden and Just (1970) concluded that TH initiate *de novo* synthesis of at least some of the hydrolytic enzymes prior to the dissolution of tail tissue. The observations of Weber (1965), Eeckhout (1965), and Tata (1966) strongly support this idea. Eeckhout demonstrated that T_4-induced tail regression was prevented by ethionine, which blocks protein synthesis, and by actinomycin D and puromycin, and Weber (1965) made similar observations with actinomycin D in both intact *Xenopus* larvae and isolated tail pieces. Using isolated tail pieces of *Rana temporaria,* Tata (1966) made a detailed study of RNA and protein biosynthesis during tail resorption. He demonstrated that cycloheximide, actinomycin D, and puromycin prevented not only the T_3-induced tail regression, but also the T_3-induced increase in hydrolase activity. He also found that T_3 increased the rate of total protein and RNA synthesis (as indicated by the increases in the rate of incorporation of [^{14}C]amino acids and [^{3}H]uridine). Furthermore, under conditions in which regression was blocked, actinomycin D blocked the incorporation of [^{3}H]uridine into RNA, and puromycin and cycloheximide that of [^{14}C]amino acids into protein. However, in contrast to his finding in liver tissue (Tata, 1971), the increase in RNA synthesis did not precede the increase in protein synthesis.

Although the findings of Tata are consistent with the view that thyroid hormones stimulate the synthesis *de novo* of enzymes involved in tail resorption, his observation that T_3 enhances the overall rate of protein synthesis in tail tissue has not been confirmed; in fact, subsequent work in the intact tadpole indicates that the rate of protein synthesis is decreased in both induced and spontaneous metamorphosis. Tonoue and Frieden (1970) found that the incorporation of [^{3}H]leucine into tadpole tail proteins was greatly reduced 6–48 hours after injection of T_3, and Kistler *et al.* (1975b), using labeled leucine and glycine, found that protein synthesis was depressed 3 days after T_3 and showed that the decreased synthesis was not merely the result of changes in amino acid transport. Saleem and Atkinson (1978) followed the effect of a large dose of T_3 on both protein synthesis and tail regression *in vivo.* Tail regression was apparent at 4 days and by 8 days 40% of the tail had been lost. A 30% decrease in the rate of protein synthesis in the tail muscle was observed within 24 hours of T_3 administration and by 8 days only 10% of the original activity remained.

Studies of protein turnover in tail tissue during normal metamorphosis indicated that two processes are involved: a decrease in total protein synthesis, which occurs progressively between stages XVII and XX, and a marked increase in the rate of protein degradation, which becomes evident during the actual period of metamorphic climax (Little *et al.*, 1973).

In spite of the fact that hormone-induced tail regression is associated with an overall decrease in protein synthesis, an increase in the rate of RNA synthesis has been demonstrated (Tata, 1966; Ryffel and Weber, 1973), suggesting an effect of TH at the level of transcription. Ryffel and Weber (1973) found that untreated tail muscle contained a high molecular weight RNA fraction, which was not present in tail muscle from T_4-treated tadpoles. This RNA fraction was not seen in other tissues. Because of its location and sedimentation behavior, they think that it might be the 26 S myosin mRNA which Heywood and Nwagwu (1969) have identified in chick embryo muscle. If this can be confirmed, these finding raise the possibility that T_4 acts in this instance by gene repression.

Saleen and Atkinson (1978) have obtained evidence that TH also influence the regulation of protein synthesis in tail tissue at the level of translation. They found that the *in vitro* translational efficiency of polyribosomal complexes from T_3-treated tadpoles was much less than that of a comparable quantity of polyribosomes prepared from untreated tadpoles. Further experiments have revealed that the depressed synthesis of protein was not the result of increased ribonuclease, proteolytic activity, or depletion of elongation factors, but was due to the presence of a factor(s) in the postribosomal supernatant that interacts with the polyribosomes to inhibit their translational efficiency. The inhibitory factor may be a protein; it is thermolabile and sensitive to trypsin (Saleen and Atkinson, 1978, 1979).

In summary, TH-induced or spontaneous tail regression is associated with marked changes in the metabolism of proteins. The synthesis of some proteins, including hydrolytic enzymes is enhanced, whereas the overall synthesis of protein is markedly decreased. The rate of protein degradation is greatly enhanced presumably due to the increased activity of proteolytic enzymes. None of these changes occurs if RNA or protein synthesis is blocked. The available information is consistent with the view that TH controls tail resorption, at least in part, by stimulating the synthesis of specific proteins, such as hydrolytic enzymes and regulation factors. As postulated for the liver, these effects appear to result from actions of TH at the level of transcription.

C. Hormone Effects in the Adult Anuran

The role of the thyroid hormones in adult amphibia is poorly understood. As discussed in Section III,B, although the adult thyroid gland is less active than that of the metamorphosing tadpole, it can synthesize iodothyronines, primarily T_4, and some investigators have detected T_4 in the serum of adult anurans. Many workers concerned with the role of TH in adult amphibia have utilized the metabolic response as an index of TH action. This type of response can be readily obtained both in mammalian species (Barker, 1964) and in tadpoles (Lewis and Frieden, 1959). However, a metabolic response to TH has not been invariably obtained in adult frogs and toads. On one hand, Warren (1940) found that TH administration resulted in a rise in oxygen consumption of leopard frogs (*Rana pipiens*) and Calhoun (1955) demonstrated that propylthiouracil caused a fall in oxygen consumption in the same species. Furthermore, Jankowski (1960) found that metabolic rate of *Rana temporaria* was reduced by surgical thyroidectomy and that the operated animals were unable to effect the short-term compensations for temperature changes observed in control animals. On the other hand, several investigators were unable to demonstrate an increase in oxygen consumption following administration of thyromimetic substances (Henschel and Steuber, 1931; Drexler and von Issekutz, 1935; Galton and Ingbar, 1962). Galton and Ingbar (1962) were also unable to demonstrate any effect of T_4 in frogs on several other systems often used as indices of TH action in mammalian species. These included hepatic glucose-6-phosphatase and ATPase activities and also histological changes in frog pituitary.

Information obtained in the last 15 years has provided a plausible explanation for these seemingly conflicting findings. It appears that some frogs and toads can only respond metabolically to T_4 if they are acclimated to relatively warm temperatures. When several species of frog and toad (*Rana pipiens, Bufo woodhousi, Scaphiopus bombifrons*), acclimated to warm temperatures (above 20°C), were given injections of T_4 for several days, an increase in oxygen consumption was observed both in the intact animal (Maher, 1967; McNabb, 1969; May and Packer, 1976) and in isolated liver slices (Packard and Packard, 1973, 1975). In contrast, an increase was seen in only one species (*Bufo woodhousi* (Maher, 1967) when the animals were acclimated to temperatures below 18°C. T_4 had no effect in *Rana pipiens* (Maher, 1967; McNabb, 1969; Packard and Packard, 1975) and caused a decrease in respiration in tissues from the toad, *Scaphiopus bombifrons* (Packard and Packard, 1973). A temperature-dependent effect of T_4 on mobilization of hepatic glycogen reserves has also been observed (Packard and Randall, 1975).

Another index of response that has been studied in adult frogs is the mitogenic action of T_4 and T_3. This action of TH is readily demonstrable in tissues of larval amphibia (Lim, 1920; Atkinson *et al.*, 1972; Wright, 1977) and the effect has been studied in detail in tissues of the larval eye (Kaltenbach and Hobbs, 1972; Beach and Jacobson, 1979). Similar effects have been observed in the adult frog. In 1972, Weinsieder *et al.* reported that T_4 and T_3 enhanced mitotic activity in ocular tissues. Others have observed the same effect in many tissues of the adult frog and have shown that enhancement of mitogensis is accompanied by a marked stimulation in the rate of synthesis of DNA (Rothstein and Worgul, 1973; Rothstein *et al.*, 1973). The doses of T_4 and T_3 used in these latter experiments were large (0.5 μg/g). However, a significant increase in the mitotic activity of the lens was observed following injection of TSH (Rothstein *et al.*, 1973). Recently, Weinsieder and Roberts (1980) have shown that mitotic activity in the lens epithelium of adult frogs is greatly diminished 3–4 weeks after hypophysectomy. If T_3 or TSH is administered shortly after surgery, this change can be prevented and either hormone will restore proliferative activity to the lens even if treatment is deferred until well after all mitotic activity has ceased (Weinsieder and Roberts, 1980).

These findings provide strong evidence that the adult anuran amphibian can respond to exogenous TH and at least one of the responses can be obtained with amounts of TH that are either present or formed in the thyroid gland during the study. Furthermore, although relatively large amounts of exogenous hormone were employed in most of the studies and normal serum levels of endogenous T_4 and T_3 are low in comparison with levels in mammalian species, the observations made in hypophysectomized and thyroidectomized animals provide good evidence that TH does play some role in the adult anuran.

V. THYROID HORMONE RECEPTORS IN AMPHIBIAN TISSUES

A. Nuclear Binding Sites

1. Evidence for the Presence of High Affinity Binding Sites for Thyroid Hormone

The initial studies concerning TH receptors in amphibian tissues were published before the first reports describing the presence of putative nuclear receptors in mammalian tissues appeared (Oppenheimer *et al.*, 1972; Samuels and Tsai, 1973; DeGroot and Strausser, 1974). In 1970, Tata reported that acquisition of metamorphic competence of developing *Xenopus laevis* was accompanied by the appearance of TH "receptors" that could be distinguished by their temperature sensitivity from hormone binding components present at earlier developmental stages. These phenomena were observed 40–60 hours after hatching. The term *receptor* was hardly justified in this study since binding was assessed in whole embryos and the distinction between "specific" and "nonspecific" binding sites was made solely on the basis of temperature sensitivity. Indeed, it is probable that most of the sites were of the nonsaturable variety since the values given for maximum binding capacity (MBC) for T_3 were several orders of magnitude higher than those subsequently reported for the saturable nuclear binding sites in premetamorphic *Rana catesbeiana* tadpoles (Kistler *et al.*, 1975a; Yoshizato *et al.*, 1975a; Galton, 1980a). However, it was shown that the majority of the binding components were located in the structural elements of the cell that sedimented at 105,000 *g* and were not merely plasma or soluble intracellular binding proteins. Thus, nuclear binding components would have contributed to the total binding activity observed. Furthermore the relative binding affinities of the larvae for different T_4 analogs corresponded quite well with the relative biological activities of the compounds.

In 1972, Griswold and Cohen reported data concerning the intracellular distribution of $[^{14}C]T_4$ injected into premetamorphic *Rana catesbeiana* tadpoles. After 24 hours, more than 50% of the T_4 in the liver was located in the nuclear fraction and was found to be tightly bound to chromatin; nuclear binding of T_4 was not observed if the tadpoles were held at 5°C. In this study a substantial amount of hormone was injected (138 nmol/tadpole) and the amount of T_4 present in the liver nuclei after 24 hours exceeded 30 nmol/g liver or approximately 15 nmoles/mg liver DNA [the DNA content of liver of premetamorphic tadpoles is approximately 2 mg/g liver (Galton, 1980c)]. The MBC of saturable TH binding sites in rat liver has been estimated to be about 1.0 pmol T_3/mg DNA (Oppenheimer and Dillman, 1978). Values obtained for premetamorphic tadpole liver are even lower than this (Galton, 1980a). Thus, the values obtained in the experiments with $[^{14}C]T_4$ must have reflected primarily binding of hormone to the nonsaturable binding sites.

The first evidence that tadpole tissues contain TH binding sites that possess high affinity, low capacity characteristics similar to those of the putative TH receptors detected in mammalian nuclei came from the laboratory of E. Frieden. In two companion studies, he and his colleagues demonstrated the presence of saturable binding sites for both T_4 and T_3 in tissues of early prometamorphic *Rana catesbeiana* tadpoles. One study was performed in isolated tail fin bricks (Yoshizato *et al.*, 1975a) and the other in isolated liver cells (Kistler *et al.*, 1975a). In both studies, uptake of T_3 and T_4 was examined in both the nuclear and extranuclear fractions of the tissue. In the tail fin study, maximum uptake in the nuclear and extranuclear fractions, respectively, was 0.5% and 15% of the added $[^{125}I]T_3$ and 0.2% and 0.7% of the $[^{125}I]T_4$ and uptake was complete after 3 hours. Nuclear uptake was retarded but not prevented during incubation at 4°C. Binding sites, which were saturated at *free* hormone concentrations in the medium of approximately 10^{-9} *M*, were demonstrated in preparations of purified nuclei, but not in the extranuclear fraction. From their Scatchard analyses of the data, Yoshizato *et al.* (1975a) determined that the dissociation constants (K_d) for both T_3 and T_4 were approximately 1×10^{-10} *M*. Based on the estimated number of cells in the tissue used in each incubation and allowing for a DNA content of 13 pg/cell, they calculated that each tail nucleus can bind a mean of approximately 1500 molecules of T_3 and 800 molecules of T_4. On the basis of these findings, the authors suggest that there are two sets of high affinity TH binding sites in tadpole tail tissue; one set that binds both T_3 and T_4 with equal affinities and a second set that binds only T_3. From the characteristics of their plot they conclude that both sets of sites have the same affinity for T_3.

Comparable findings were obtained in the isolated liver cell preparation (Kistler *et al.*, 1975a). In this tissue, uptake of both T_3 and T_4 was complete within 45 minutes at 25°C and by 90 minutes at 4°C. As much as 90 and 85% of the T_3 and T_4, respectively, were found in the extranuclear fraction while only 0.9 and 0.2% were recovered in the nuclear fraction. Again binding sites that were saturated at free hormone concentrations of approximately 10^{-9} M were detected in the nuclear but not the extranuclear fraction. From their Scatchard analyses of the nuclear binding data, the authors estimated an apparent K_d of 6.8×10^{-10} M for T_3 and 4.6×10^{-10} M for T_4. The number of sites per nucleus was estimated to be 12,300 for T_3 and 2300 for T_4. On the basis of these estimations together with data from competitive binding studies, the authors concluded that, as in tail nuclei, there are two sets of TH binding sites, one that binds only T_3 and a second set that binds both T_3 and T_4.

The data from these elegant studies are extremely important and provide the first convincing evidence of the presence of TH receptors in amphibian tissues. However, there is a problem with the authors' analysis of the nonsaturable binding data. Although this does not detract from the major conclusions drawn, it does affect the values derived for the number of nuclear binding sites. As indicated in their text (Kistler *et al.*, 1975a; Yoshizato *et al.*, 1975a), both saturable and nonsaturable data were included in the Scatchard plots. It appears from the plots presented in both papers that the MBC's of the high affinity sites were determined from the straight line drawn through the points representing the data obtained at nonsaturating concentrations of hormone. The slope and intercepts of this line were obtained by linear regression. The intercept on the axis representing moles of hormone bound was taken as the MBC, and the value thus obtained was then corrected for nonsaturable binding (the percentage of labeled hormone not displaced with 1000-fold excess of unlabeled hormone). It was stated that correction for nonsaturable binding did not affect the K_d. Scatchard analysis of data consisting of saturable and nonsaturable sites has been discussed in detail by Munck (1976). In Figure 3 of his paper Munck has illustrated the pertinent points very clearly. First, it is evident that if the data are plotted without first being corrected for the nonsaturable fraction, the points do not fall on a straight line. Second, correction of the points on the graph for nonsaturable binding certainly does result in a change of the slope (K_d) relative to the "best fit" straight line through uncorrected points. In studies where the nonsaturable binding is only a very small fraction of the total binding, the method used by Kistler and Yoshizato would give values for MBC and K_d that are close to the correct values. However, when this fraction is large, a major discrepancy would ensue. It seems likely that significant discrepancies do exist in the liver cell study; in the case of T_4, nonsaturable binding was reported to be roughly 40% of the total binding.

For T_3, the value given was 16–20% of total binding, although the plot was not expanded to include the data obtained at this concentration. However, correction must be made also for the second binding component that is evident on this plot. This presumably includes the fraction the authors consider to be the nonsaturable fraction. Such a correction would result in a fairly substantial change in both the K_d and MBC. This correction may be important since Kistler *et al.* (1975a) have estimated that there are 12,300 T_3-binding sites per liver nucleus and this value is considerably higher than values reported by other investigators for this tissue (Toth and Tabachnick, 1979; Galton, 1980a). A rough correction of the data of Kistler *et al.* (1975a) for nonsaturable binding performed as described by Munck (1976) yields an MBC of 10 fmoles/10^6 cells (6000 sites/nucleus) and a K_d of 3.4×10^{-10} *M*.

High affinity, low capacity binding sites for T_4 and T_3 have also been detected in hepatic nuclei of premetamorphic *Rana catesbeiana* tadpoles using *in vivo* techniques (Galton, 1979, 1980a; Toth and Tabachnick, 1979). In the studies of Galton (1979, 1980a), premetamorphic tadpoles were injected with $[^{125}I]T_3$ or $[^{125}I]T_4$ (0.001–10 nmol/tadpole). Twenty hours later, when the ratios of nuclear-bound hormone to serum hormone were maximal and relatively constant, the amounts of hormone bound to purified nuclei and present in serum were determined. Liver nuclei were found to possess binding sites for T_3 and T_4 that were saturated after administration of approximately 33 ng T_3 or 80 ng T_4/tadpole. Chromatographic analysis of nuclei and serum revealed that the injected hormone was the only labeled organic compound present in detectable amounts in nuclei or serum. Typical Scatchard plots of data obtained with T_3 and T_4 are shown in Fig. 4. In these plots, the value for total nuclear-bound hormone was corrected for the nonsaturable binding fraction prior to construction of the plot (9 and 11% of the total T_3 and T_4 binding, respectively). From such plots, the mean K_d for T_3 was 1.6×10^{-12} *M* and for T_4 was 3.9×10^{-12} *M*. Corresponding mean values for MBC were 0.15 and 0.5 pmol/mg DNA. On the basis of these values it appears that the affinity of the nuclear sites for T_3 is almost twice that for T_4 but that there are only ¼ the number of sites. Although these observations raise the possibility that the nuclei contain separate sets of binding sites for T_4 and T_3, the finding that T_3 and T_4 compete with each other for at least some of the sites shows that this is not the complete answer. An alternative possibility is that the nuclei contain two classes of binding sites, one in which the binding capacity for the two hormones is identical and the K_d values are different and a second set that binds only T_4. Since Scatchard analysis of T_4 binding did not reveal a two-component line, it must be assumed that this second set differs from the first in capacity but not in affinity for T_4. In this respect, the present data differ from those of Kistler *et al.* (1975a), who found that the nuclei contained more binding sites for T_3 than for T_4.

Toth and Tabachnick's experiments (1979) involved comparable amounts of

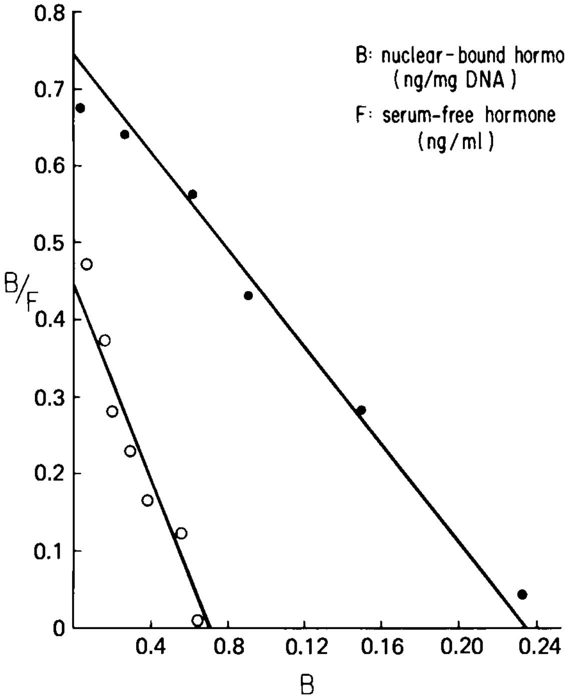

Fig. 4. Scatchard plots of T_3 and T_4 binding *in vivo* by liver cell nuclei in tadpoles (stage X). Each point represents the mean of values observed in six or more tadpoles. Values were corrected for nonsaturable binding. For T_3: $K_d = 2.4 \times 10^{-12} M$; MBC = 0.07 ng/mg DNA. For T_4: $K_d = 4.1 \times 10^{-12} M$; MBC = 0.23 ng/mg DNA.

T_3 but tadpoles were studied 2 hours after injection of hormone and, since hormone levels in serum were not determined, Scatchard analysis of the data was not performed and no values for K_d were presented. However, a value for MBC was obtained by subtracting the nonsaturable fraction determined following injection of 2000 pmol T_3/g body weight from the values obtained for total nuclear T_3 at each dose level and then calculating the absolute amount of hormone bound to saturable sites. MBC, determined in this manner, was approximately 0.350 pmol/mg DNA or 2700 molecules/nucleus. No data for the binding of T_4 were reported from this laboratory, but nuclear binding of T_4 has been studied in a comparable manner by Ergezen and Gorbman (1977), who used *Rana pipiens* tadpoles. Saturable binding was demonstrated in the nuclear fraction of brain, liver and tail tissue 6 hours after injection of T_4; no other information concerning the properties of these sites was presented.

Thus, at least four groups of investigators have demonstrated that saturable binding sites for T_3 and/or T_4 are present in nuclei of tadpole liver. However, on the basis of the available data it cannot immediately be considered that the same set(s) of sites is involved in each study. It does seem probable that Galton (1980a) and Toth and Tabachnick (1979) were studying the same set of T_3-binding sites. Although the sites cannot be compared on the basis of the apparent K_d, the values reported for MBC in the two studies were 0.150 and 0.350 pmol/mg DNA. As indicated above, these values were obtained by different methods and it is quite possible that more nonsaturable binding is included in latter than in former value. On the other hand, the differences in the values for K_d and MBC obtained by *in vivo* techniques (Galton, 1980a) and in the liver cell system (Kistler *et al.*, 1975a) are substantial, and this raises the question of whether these differences can be accounted for on a technical basis or whether, in fact, the two studies involved different sets of binding sites. At this time, the bulk of the published data and additional personal unpublished observations favor technical differences as the explanation. For example, in mammalian systems, values for apparent K_d of nuclear T_3-binding sites are generally lower when determined by *in vivo* techniques than when the measurements are made *in vitro*. This apparent discrepancy has been attributed to lack of an optimal incubation environment in the *in vitro* studies (Oppenheimer and Surks, 1975). The same reasoning may be used to explain the comparable discrepancy in the binding data obtained *in vivo* and *in vitro* in tadpole liver. However, another possibility is that, in the *in vivo* studies, hormone levels decrease more rapidly in plasma than in the nuclei (i.e., the system is not in true equilibrium) resulting in spuriously low values for the K_d. This is a plausible explanation in tadpole liver in view of the relatively slow rate of dissociation of T_3 from the saturable nuclear binding sites (Galton 1980c).

The differences in the estimated number of sites for T_3 and T_4 in the two studies are a significant problem. Kistler *et al.* (1975a) cite 12,000 and 2300 for T_3 and T_4, respectively. Corresponding values obtained by *in vivo* techniques are 1100 and 5000. As discussed above, the values reported for T_3 by Kistler were corrected for nonsaturable binding in a manner that would yield erroneously high figures. However, the difference in the values for number of T_3 sites/nucleus is likely to remain at approximately 6:1 even after correction and no satisfactory explanation for the remaining difference has been put forward as yet. A comparable reanalysis of the T_4 data would lead to an increase in the discrepancy in the number of binding sites, although the increase is small (Kistler *et al.*, 1975a, Fig. 5). The validity, not only of the observed differences in values obtained in the two laboratories but also of the finding that there are more sites for one hormone than the other, needs to be clearly established since the two sets of data lead to dissimilar conclusions. Kistler *et al.* (1975a) suggest that two sets of sites are present in hepatic nuclei, one set that binds both T_4 and T_3 and a second that binds only T_3. Galton (1980a), on the other hand, has suggested that there are two sets of sites for T_4, only one of which can bind T_3.

2. *Are These Nuclear Binding Sites Hormone Receptors?*

The ultimate proof that a hormone binding site is a hormone receptor rests in demonstrating that the bound hormone complex is essential to the action of the hormone. Since such proof is not at hand for these amphibian sites, their importance in the action of TH must be considered in the light of the few available pieces of circumstantial evidence.

a. AFFINITY AND BINDING CAPACITY. The two most frequently discussed properties of hormone receptors are their affinity and capacity for the hormone in question. Although it should not be essential, it is, in fact, generally the case that putative receptors, in contrast to other types of binding sites, exhibit a very high affinity and a low capacity for the corresponding hormone. This is certainly true of the nuclear sites postulated to be TH receptors in mammalian tissues (Oppenheimer and Dillman, 1978; Samuels, 1978; Latham *et al.*, 1978), and as discussed above, it is also true of the sites detected in tadpole liver nuclei and tail tissue. In fact, the affinities of tadpole liver and rat liver nuclei for T_3 are remarkably similar (Oppenheimer and Dillman, 1978; Galton, 1980a). However, the K_d of the tadpole sites is not so low that the sites are fully occupied at endogenous levels of hormone. If it were then it would be difficult to assign them a role in hormone action. It can, in fact, be estimated that very few sites are saturated in the premetamorphic tadpole (Galton, 1980a). The K_d for T_3 is $1.6 \times 10^{-12} M$, i.e., the concentration of T_3 for half saturation of the sites is approximately 0.1 ng free T_3/100 ml. In premetamorphic tadpoles the total concentration of endogenous T_3 in plasma is below the detectable limit of the available RIA (< 5 ng/100 ml, Regard *et al.*, 1978). However, even if the concentration of endogenous hormone was only just below the detectable limit, i.e., 4 ng T_3/100 ml serum, the free T_3 concentration (1% of total T_3, Galton, 1980a,b) would be only 0.04 ng/100 ml and at this concentration only a small proportion of sites would be occupied by hormone. Theoretically, therefore, the effects that result from the administration of T_3 to premetamorphic tadpoles could follow the association of T_3 with the unoccupied binding sites. It is not certain, however, if the same statement can be made for tadpoles undergoing metamorphic climax. Plasma T_3 levels rise sharply during this phase and can reach concentrations of at least 1.0 ng free T_3/100 ml. Unless there is a marked change in the affinity or capacity of the nuclear sites during this phase, they would be fully saturated at this level of T_3. If this is the case then hormone effects should not occur following injection of additional T_4 and T_3. M. P. Shannon and V. A. Galton (unpublished data) have found this to be true with respect to hepatic CPS activity. The activity of this enzyme system was invariably increased in pre- and prometamorphic tadpoles, 6 days after injection of 5.0 nmoles of T_3, but not in animals at the peak of metamorphic climax (Fig. 5). Although other possible

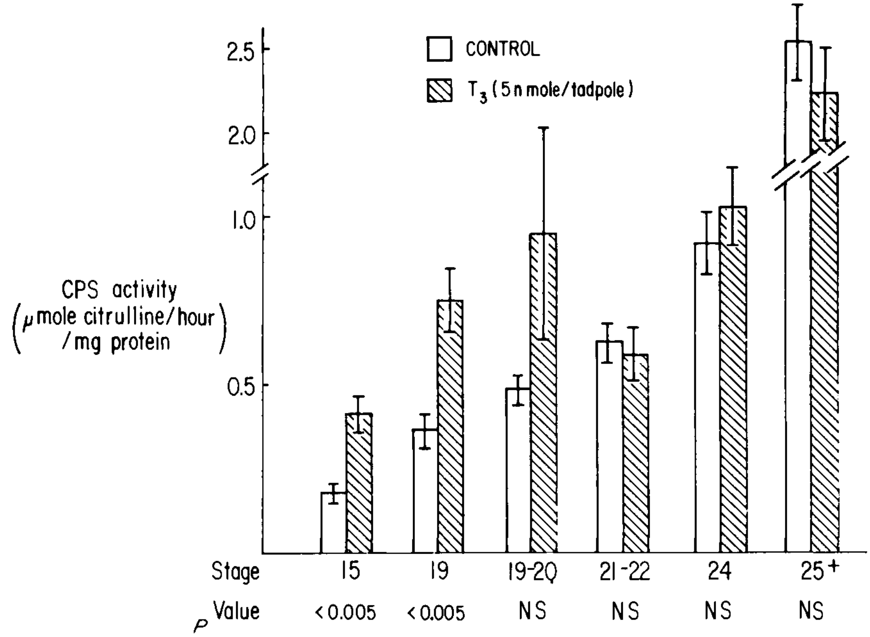

Fig. 5. Effect of T_3 on carbamyl phosphate synthetase activity (CPS) during prometamorphosis and metamorphic climax. Tadpoles received a single injection of T_3 (5.0 nmol in 0.1 ml 1% human serum albumin) or vehicle, and hepatic CPS activity was measured 6 days later as described by Galton and Cohen (1980). Bars indicate mean ± SE of values obtained of at least 10 animals.

explanations for this finding can be envisaged, it is consistent with saturation of the receptors by endogenous hormone.

b. RELATIVE BINDING AFFINITY FOR T_4 ANALOGS. A second line of evidence often used in support of the biological relevance of a set of binding sites is the correlation between the binding affinity of the sites for various hormone analogs and the biological potency of the analogs. This is one of the major pieces of evidence supporting the concept that the mammalian nuclear binding sites are hormone receptors (Oppenheimer and Dillman, 1978). In amphibia, the relative potency of an analog depends to some extent on the nature of the response chosen and the mode of administration of the compound. Nevertheless, it has been demonstrated in several studies that T_3 has a greater physiological effect than T_4 in tadpoles, and in general terms it can be stated that triiodinated compounds are more active than the corresponding tetraiodinated compounds, D-isomers are less active than their corresponding L-isomers, and diiodinated derivatives have relatively little activity (Frieden, 1967).

The limited data presented in Table II suggest that a similar pattern exists for the relative abilities of the different compounds to compete with $[^{125}I]T_3$ for the

TABLE II

Competition for T_3-Binding Sites in Tadpole Liver Nuclei by Thyroid Hormone Analogs[a]

Analog	No. of experiments	Dose range[b] for 50% inhibition of $[^{125}I]T_3$[c] binding (ng/tadpole)
T_3	>20	3.6–13
T_4	4	7.8–32
TA_3	5	6.3–13
TA_4	4	150–225
TP_3	4	6–12
D-T_4	4	78–320
T_2	4	560–5600
rT_3	4	65–650

[a] From Galton (1980a). T_3, 3,5,3′-triiodothyronine; T_4, thyroxine; TA_3, triac (acetic acid derivative of T_3); TA_4, tetrac (acetic acid derivative of T_4); TP_3, propionic acid derivative of T_3; D-T_4, dextrothyroxine; T_2, 3,5-diiodotyrosine; rT_3, "reverse" T_3.

[b] Dose for 50% inhibition of $[^{125}I]T_3$ binding to saturable sites varied slightly from experiment to experiment; the range given indicates the maximum variation observed for the analog. Data obtained in tadpoles given 6500 ng $[^{125}I]T_3$ were used to correct for nonsaturable binding sites.

[c] 0.65 ng $[^{125}I]T_3$/tadpole.

nuclear sites. The greater potency of T_3 compared to T_4, however, is seemingly at variance with the *in vivo* data in hepatic nuclei showing more binding sites for T_4 to T_3 (Galton, 1980a; Galton and Cohen, 1980). However, results obtained in *in vivo* studies in which a single injection of hormone is employed must be carefully interpreted. The biological effectiveness of a given compound administered in a single injection depends not only on the total amount of compound that becomes associated with the nuclear binding sites and the "intrinsic activity" of "efficacy" of the compound, but also on the duration of binding of the compound by the sites (Oppenheimer and Dillman, 1978). Both the total amount bound and the duration of binding are influenced by the biological half-life of the compound. However, the latter factor depends also on the rate of dissociation of the hormone–receptor complex, and there is reason to believe that the rates of dissociation of T_4 and T_3 from tadpole liver nuclei are very different. The data shown in Fig. 6 indicate that the time required for 50% dissociation of hormone from the bound complex in liver nuclei is more than 19 hours for T_3 and less than 3 hours for T_4. It is possible, therefore, that the reason that T_3 is more effective than T_4 when injected *in vivo* is due in part to differences in the duration of occupancy of the nuclear sites by the two hormones. Clearly, much more information in this important area is needed (in particular, quantitative data comparing the biological effect of compounds with the extent of binding to, and duration of occupancy of, the nuclear sites).

C. TEMPERATURE SENSITIVITY. The effect of temperature on TH action and binding in tadpoles has proved to be less helpful with respect to establishing physiological relevance of the binding sites than was originally hoped. There is no doubt that both spontaneous and T_4-induced metamorphosis is greatly retarded or even prevented if tadpoles are kept in the cold (Frieden, 1967). However, attempts to confirm the findings of Griswold *et al.* (1972) that cold completely inhibits the uptake of T_4 by hepatic nuclei *in vivo* have failed. Kistler *et al.* (1975a), using the liver cell system, found that the total nuclear binding of T_4 and T_3 was slower at 4°C than at 25°C but by 90 minutes the same proportion of hormone was bound at both temperatures. In addition, it has recently been clearly demonstrated that nuclear binding of T_3 *in vivo* does take place in premetamorphic tadpoles maintained at 4°C (Galton, 1980a; Toth and Tabachnick, 1980). In the studies of Kistler *et al.* (1975a) and Toth and Tabachnick (1980), no distinction was made between saturable and unsaturable nuclear binding in the cold. This distinction was made in the experiment shown in Fig. 7 (Galton,

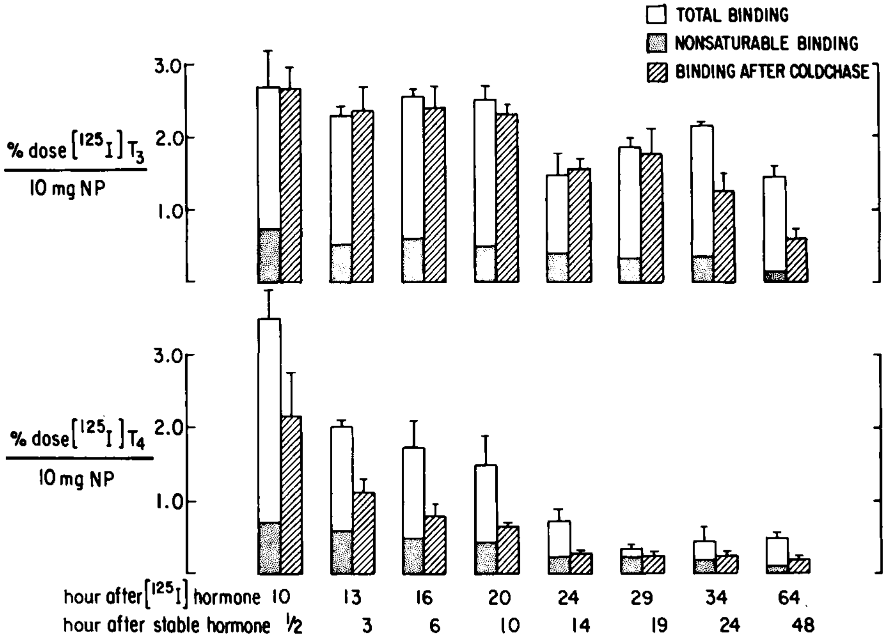

Fig. 6. Effect of a chase dose of stable hormone on the binding of $[^{125}I]T_3$ or $[^{125}I]T_4$ by hepatic nuclei of premetamorphic tadpoles. (Hatched bars) Results (mean ± SE) obtained in animals given ^{125}I-labeled hormone (0.65 ng T_3 or 0.78 ng T_4), followed 10 hours later by an injection of stable hormone (6500 or 7800 ng/tadpole). (Clear bars) Values obtained in animals given vehicle instead of stable hormone. (Stippled bars) Values for nonsaturable binding, obtained in tadpoles given 6500 ng $[^{125}I]T_3$ or 7800 ng $[^{125}I]T_4$. From Galton (1980c).

1980a). Total binding was measured in tadpoles given 0.001 nmol [^{125}I]T_3 and the nonsaturable binding fraction was determined in tadpoles given 1 nmol [^{125}I]T_3. It is evident that the rate of association of T_3 with the saturable binding sites was greatly retarded but not prevented in the cold. Not until 72 hours after injection did the amount of T_3 bound to saturable sites approach the values obtained in warm animals. This was not due to a decrease in the amount of T_3 present in either the whole liver or the hepatic nuclei per se, since the concentration of T_3 in liver and serum of the cold animals greatly exceeded that in warm animals at all time intervals studied. By 6 hours the concentrations of hormone in the nuclei were comparable in both groups. In warm animals, maximum values

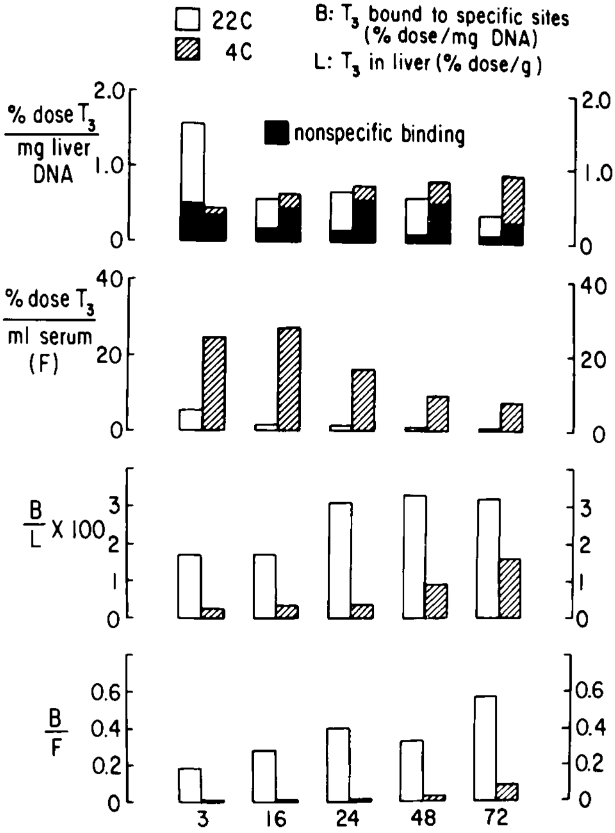

Fig. 7. Saturable and unsaturable binding of [^{125}I]T_3 at various times after injection into tadpoles maintained at 22° or 4°C. Bars indicate the mean of values obtained in at least four animals. By 36 hours, serum [^{125}I]T_3 levels in warm animals were generally low and variable. Thus, B:F ratios had a wide SD. There was no statistical difference in the B:F values determined at 24 and 72 hours. From Galton (1980a).

for nonsaturable binding were observed when hepatic T_3 concentrations were the highest 3 hours after injection. Comparable values for nonsaturable binding were observed in cold animals from 3 to 48 hours after injection. During this period, hepatic T_3 concentrations in the cold animals were similar to the high values seem in warm animals at 3 hours. These findings suggest that binding to nonsaturable sites is relatively unaffected by cold.

These data indicate that the saturable and nonsaturable binding components of tadpole liver nuclei can be distinguished on the basis of the temperature sensitivity of their binding interactions. However, it is evident that the failure of tadpoles to respond to T_4 at 4°C cannot be attributed to the failure of the hormone to bind to the saturable nuclear binding sites.

d. DEVELOPMENT OF NUCLEAR RECEPTORS. In the course of its life the anuran amphibian exhibits wide variations in its requirement for TH as discussed in Section III. One possible explanation for the initial unresponsiveness is an absence of hormone receptors. It is also possible that the marked increase in TH synthesis and secretion which occurs at metamorphic climax is accompanied also by an increase in the number of the hormone receptors. Clearly, strong evidence for the physiological relevance of the saturable nuclear binding sites would be provided by the finding that appropriate changes in either number or affinity occur during the life cycle of the frog. In this context, the observations of Tata (1970) that metamorphic competence of *Xenopus laevis* larvae is accompanied by the appearance of temperature sensitive "receptors" may be significant.

As indicated above, it is likely that very few of the high affinity nuclear sites present in the premetamorphic tadpole are occupied with endogenous hormone. This situation is consistent with minimal differentiation under normal conditions, and it allows for the metamorphic response which occurs when exogenous TH is administered. Both the affinity and capacity of the hepatic nuclear sites were found to be the same in pre- and prometamorphic *Rana catesbeiana* tadpoles (Galton, 1980c). Endogenous hormone levels increase during this phase (Section III), but although this must increase the proportion of occupied sites, saturation would still not be achieved. However, as discussed above, serum T_4 and T_3 levels during climax are more than sufficient to saturate the sites present during pre- and prometamorphosis, and thus an increase in number of receptor sites would be necessary if full advantage is to be taken of the high hormone levels experienced during climax. Some evidence that this may indeed be the case has been obtained by Yoshizato and Frieden (1975) who demonstrated an increase in the number of T_3 binding sites per nucleus in tadpole tail fin cells from 1300 at stage X to 2800 by the end of prometamorphosis. The estimated number had fallen somewhat by stage XX, but the authors suggest that this may be due to an increase in the proportion of sites that were occupied with endogenous hormone. No significant change in the affinity of the sites for T_3 was observed. These data were apparently obtained from Scatchard plots which included data from nonsaturable binding sites. However, the difference between MBC at stages X and XIX was substantial and is likely to be real. No data were presented for tadpoles at subsequent stages of climax.

Attempts to demonstrate a change in number or affinity of the hepatic nuclear sites during climax has so far met with little success. When nuclear binding of [^{125}I]T_3 or T_4 *in vivo* was studied in tadpoles at stages XX to XXV using methods similar to those employed in premetamorphic tadpoles (Galton, 1980a), the fraction of the injected [^{125}I]hormone bound to saturable binding sites was notably depressed by stage XX and only minimal binding was evident by stage XXIII (Galton, 1980c). The possibility that this was due to the absence of binding sites is unrealistic. It is difficult to accept that the binding sites, if they are hormone receptors, disappear or are blocked during metamorphic climax, when the animal's requirement for TH is maximal, particularly since saturable binding sites have been detected in the adult form (Galton, 1980c). It is more likely that the decreased binding of [^{125}I]hormone to the nuclear sites is related to the increase in the concentration of endogenous hormone that occurs at climax. This would result in decreases in both the specific activity of the [^{125}I]hormone achieved *in vivo* and the number of unoccupied nuclear sites. In any event, some other approach will have to be taken to determine the number and affinity of the binding sites during metamorphic climax.

B. Extranuclear Binding Sites

Although most of the available information concerning mammalian tissues favors the concept that TH action is initiated at the nuclear level, the possibility that extranuclear initiation sites exist has not been ruled out. In fact, Sterling and Milch (1975) reported that isolated mammalian mitochondria contain high affinity, low capacity binding sites for T_3. Unfortunately, this work has not been confirmed by others, and it has been impossible to verify the existence of these sites using *in vivo* techniques (Oppenheimer and Dillman, 1978).

Several investigators have examined the binding of TH in extranuclear fractions of amphibian tissues. Yoshizato *et al.* (1975b) have reported the presence of temperature-sensitive, saturable binding sites for T_3 in preparations of cytosol of tail fin, tail muscle, liver, and kidney from premetamorphic *Rana catesbeiana* tadpoles (stages X–XIII). Cytosol was defined as the supernatant obtained from tissue homogenates after high speed centrifugation (140,000 *g*). The values for the K_d of the tail fin and liver cytosol sites were 1.4×10^{-7} and $1.5 \times 10^{-8}\,M$, respectively, which were considerably higher than values obtained for the corresponding nuclear sites (Kistler *et al.*, 1975a; Yoshizato *et al.*, 1975a). Many more sites were present in tail fin than in liver cytosol (MBC's were 10.4 and 0.04 pmol/mg protein). In contrast, no T_4 binding sites with comparable K_d were detected in tail tissue cytosol (Yoshizato *et al.*, 1976), and Kistler *et al.* (1977) have shown that the concentration of T_4 required for 50% inhibition of $[^{125}I]T_3$ binding in tail tissue cytosol was 250 times the concentration of T_3 needed for the same effect. Jaffe and Gold (1977) have also detected saturable sites for T_3 in tadpole tail fin and liver tissue and they too found that the affinity of these sites for T_4 was relatively low. However, although their values for K_d approximated those reported by Yoshizato *et al.* (1975b), they obtained a value for MBC for the liver cytosol sites that was much higher than that reported by Yoshizato *et al.* (1975b). Binding constants and capacities were similar in tadpoles at stages V and XX (Jaffe and Gold, 1977).

It is quite possible that the same sets of binding sites were involved in these two studies. Galton (1980b) has shown that the fraction of unbound or free T_4 and T_3, when measured directly using an equilibrium dialysis method, is less than 1% of the total hormone present in cytosol. Moreover, the free fraction is independent of hormone concentration between 10^{-10} and 10^{-6} M. However, values for the free fraction determined by other methods (in particular, adsorption on dextran-coated charcoal) were much higher than those determined directly and it was evident that the values included hormone that had dissociated rapidly from the bound form in the course of making the measurement. Both Yoshizato *et al.* (1975b) and Jaffe and Gold (1977) mentioned this problem and Jaffe and Gold, unlike Yoshizato, elected not to use charcoal but to separate the bound complexes from the free hormone by precipitating them with protamine sulfate. This may explain why their values for K_d were lower than those obtained by Yoshizato *et al.* (1975b) and may also account in part for the difference in the binding capacities reported for liver cytosol.

In contrast to findings discussed above, Durban and Paik (1976) have detected saturable binding sites for T_4 in tadpole tail and liver cytosol with an affinity that was higher than the values reported by the others for T_3 ($K_d = 9 \times 10^{-10}$ M). These investigators also found that the affinity of the sites was increased fourfold by treatment with T_4.

All these studies were performed in the soluble fraction of the cell from which organelles such as mitochondria and microsomes had been removed. Results obtained in comparable studies performed in the extranuclear fraction of liver homogenate (800 *g* supernatant) indicate that cytosol contains several sets of sites that bind TH, and these sites are distinguishable by differences in the rates of dissociation of the protein–hormone complexes. Some of the sites could be saturated with T_4 or T_3 but, compared with the nuclear sites in the same tissue, the affinity of the sites for either hormone was relatively low. In competitive binding studies it was shown that T_4 was more effective than T_3 in displacing $[^{125}I]T_4$ from the sites and equally as effective as T_3 in displacing bound $[^{125}I]T_3$ (Galton, 1980b).

There is no compelling reason to believe that any of the saturable TH binding sites detected in tadpole tissue cytosol are hormone receptors. In fact, the available data indicate otherwise. First, clearly, none of the reported sets of sites has an affinity for either hormone that approaches the apparent affinity of the nuclear sites for T_3 and T_4 (Galton, 1980a). Second, correlation between binding affinity and biological activity of several T_4 analogs (in particular, T_4 and T_3) is poor. In tadpole tissues, T_4 has ⅓ to ⅒ of the biological activity of T_3, yet Kistler *et al.* (1977) have found that the affinity of the cytosolic sites for T_3 is 250 times that for T_4 and an even greater difference was reported by Jaffe and Gold (1977). Correlation was also poor in the crude cytosol preparation since T_4 competed as well or better than T_3 for the saturable binding sites (Galton, 1980b). Jaffe and Gold (1977) have also shown that D-T_4 competes as well as L-T_3 for the sites and Kistler *et al.* (1977), in a study of eight compounds, found few instances of good correlation. Third, the binding characteristics of T_4 and T_3 in the extranuclear fraction of tadpole liver homogenate are similar, from both the quantitative and qualitative standpoint, to those observed in serum, which is not considered to be a target organ of the hormones (Galton, 1980b).

Thus, it seems unlikely that these binding sites are involved in the initiation of TH action. Nevertheless, in view of the complexity and extensive nature of extranuclear binding, it is premature to rule out completely the possibility that extranuclear receptors are present in amphibian tissues.

VI. RELATIVE IMPORTANCE OF T_4 AND T_3 IN AMPHIBIA

A. In Premetamorphic Tadpoles

It is generally accepted that T_3 is responsible for most of the physiological actions of the TH in mammals (Oppenheimer *et al.*, 1979). Although T_4 is normally more abundant than T_3 and can elicit substantial thyromimetic response it is bound only weakly to mammalian nuclear receptors and it has been shown that it derives most, though not necessarily all, of its physiological activity through its conversion to T_3 in peripheral tissues (Surks *et al.*, 1973; Surks and Oppenheimer, 1977).

There is good reason to believe that a different situation may exist in premetamorphic tadpoles. First, tadpoles in this phase appear unable to convert T_4 to T_3 *in vivo*. Following injection of $[^{125}I]T_4$, the only labeled iodothyronine present in detectable amounts in liver nuclei, liver homogenate, and serum was T_4; $[^{125}I]T_3$ was not observed (Galton and Cohen, 1980). Since the generation of $[^{125}I]$iodide from $[^{125}I]T_3$ is slower than from $[^{125}I]T_4$ in these tadpoles, it should have been quite evident if any $[^{125}I]T_3$ had been formed from $[^{125}I]T_4$. These findings indicate that the biological action of T_4 in premetamorphic tadpoles is effected without prior conversion to T_3. Second, in contrast to the situation in mammals (Oppenheimer and Dillman, 1978), the tadpole hepatic nuclear binding sites have an affinity for T_4 that approximates that for T_3 (Kistler *et al.*, 1975a; Galton, 1980a). Third, it has been shown that more T_4 than T_3 is bound to the hepatic nuclear sites during the 24 hours after injection of a solution containing equimolar amounts of T_4 and T_3 (Galton and Cohen, 1980). Although this would be an unlikely finding in mammals, it was not altogether unexpected in these tadpoles. The amount of hormone bound to a set of binding sites is a function of the number of binding sites, the affinity of the sites for the hormone, and the concentration of free hormone in the system. In premetamorphic tadpoles, the affinity of the nuclear binding sites for T_4 determined by *in vivo* techniques is approximately half that for T_3, and it is likely that more binding sites are available for T_4 than for T_3 (Galton, 1980a). When T_4 and T_3 were administered simultaneously, the concentrations of T_4 in the liver was approximately three times that of T_3 during the first 24 hours. Since the same fractions of total T_4 and T_3 in cytosol are present in the free form, when both hormones are present the free T_4:T_3 ratio must be equal to the total T_4:T_3 ratio. Thus, when the number of binding sites, affinity of binding sites, and concentration of free hormone are all taken into consideration, one would in fact predict that more T_4 than T_3 would be bound to the nuclear sites under the conditions of these experiments.

Although at the present time it is not possible to make any definitive statement regarding the relative contribution of each hormone to growth and development during this phase since it is not known in what proportion the two hormones are released from the thyroid, some speculations can be made. Except in the embryonic stages, the predominant iodothyronine in the thyroid gland of both the developing tadpole and the adult frog is T_4 (Section III). The same is true for the serum at least during metamorphic climax when levels can be measured. At this time the T_4:T_3 is at least 3:1 (Regard *et al.*, 1978; Miyauchi *et al.*, 1977). More T_4 than T_3 is probably also present in serum during prometamorphosis as shown by Regard *et al.* (1978). Although Miyauchi *et al.* (1977) were able to detect only T_3 in serum during this phase their T_3 assay was 50 times more sensitive than their T_4 assay and thus no conclusion can be drawn from their data regarding the ratio of T_4:T_3. However, when Regard *et al.* (1978) were able to detect T_4 during prometamorphosis, the T_4 to T_3 ratio was invariable greater than one. In view of these observations in thyroid and serum it is probable that serum of premetamorphic tadpoles also contains more T_4 than T_3. Given then that T_4 is likely to be the predominent circulating TH and that premetamorphic tadpoles cannot convert T_4 to T_3, a major fraction of total TH action during this phase is probably derived from T_4.

B. In Metamorphosing Tadpoles

Once metamorphic climax is reached there is no doubt that T_4 is present in greater amounts than T_3 in the circulation. Furthermore as indicated above, the

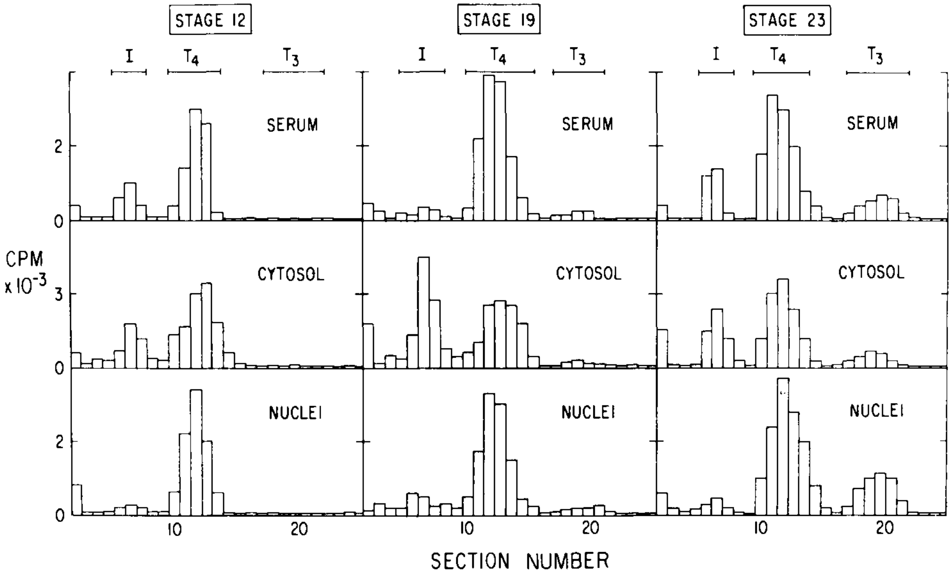

Fig. 8. ^{125}I-Labeled compounds in serum and liver of tadpoles at different stages of development, 10 hours after injection of [^{125}I]T_4 (10 pmol). Samples of serum or liver preparations were applied directly to Whatman No. 1 chromatography strips and developed in *tert*-amyl alcohol–2 *N* NH_4OH (1:1) solvent system. Stable carriers (I^-, T_4, and T_3) were cochromatographed on some strips for identification purposes. The locations of the visualized carriers are shown. The strips were cut up into 0.7 cm sections, the ^{125}I on each section determined in a γ counter, and the counts per minute on each section plotted as indicated. From Galton and Munck (1981).

free and total T_4:T_3 ratios are comparable. However, in contrast to premetamorphic tadpoles, metamorphosing animals can 5′-monodeiodinate T_4 to T_3 (Fig. 8). Since this converting system can be induced in premetamorphic tadpoles by pretreatment with T_4 or T_3 (Galton and Munck, 1981), it may not be a coincidence that normal development of the system occurs in late prometamorphosis at the time when endogenous TH levels in serum are rising rapidly. In view of the importance of the T_4 5′-monodeiodination system in overall TH economy in mammals, it is tempting to attribute some significance to the acquisition of a comparable system by tadpoles at the time when their requirement for TH is at its height. Unfortunately, as discussed in Section V,A,2, it has not been possible as yet to determine whether metamorphic climax is also associated with an increase in the number or affinity of the nuclear T_3 receptors. However, indirect evidence of a change has been observed. When tadpoles undergoing metamorphic climax were injected with a mixture of radioactive T_4 and T_3 in equimolar amounts, the ratio of T_4 to T_3 on the nucleus at 3 or 24 hours was invariably less than 1:1 (V. A. Galton and M. P. Shannon, unpublished observations), in marked contrast to the value of 2:1 obtained in premetamorphic tadpoles (Galton and Cohen, 1980). This change in ratio could be due either to a change in the affinity or number of hepatic nuclear receptors in favor of T_3, or to extensive peripheral conversion of T_4 to T_3. Although these data must be interpreted with caution since endogenous T_4 and T_3 were not taken into account, they are consistent with the view that the T_3 gains importance during metamorphic climax.

VII. CONCLUDING COMMENTS

The data presented herein support the concept that the biological actions of TH in amphibia are initiated at the level of the cell nucleus. Although the detailed mechanism remains to be defined, the first step is the interaction of TH with specific nuclear binding sites. This results in increased DNA transcription followed by synthesis of certain proteins that are responsible for the expression of TH action in the cell. Although the possibility that there are extranuclear sites of initiation of TH action in the amphibian cell cannot be excluded, so far attempts to identify such sites have been unsuccessful.

Premetamorphic tadpoles cannot convert T_4 to T_3 and it appears that T_4 rather than T_3 is the hormone primarily responsible for development through mid-prometamorphosis. However, by the time they reach metamorphic climax, tadpoles can generate significant amounts of T_3 from T_4. These findings together with reports from other workers that T_3 generation from T_4 is minimal or absent in developing forms of other species (Wu *et al.*, 1978; Harris *et al.*, 1978; Segall-Blank, 1978; Borges *et al.*, 1979) suggest that acquisition of a T_4 5′-monodeiodinating system is a general phenomenon of development. Thus information concerning the relative importance of T_3 and T_4 in the tadpole during metamorphic climax and in the mammalian fetus could be very significant.

It is evident that many aspects of TH action in amphibia are similar to those proposed for mammalian species and it is tempting to speculate that there is a basic mechanism of action for TH that is common to all species. The limited information available for amphibian species provides a solid background for future studies which must include an analysis of the nuclear events that lead to enhanced DNA transcription and an analysis of the importance of T_3 during metamorphic climax. Investigations along these lines will contribute much to a full understanding of the mechanism of action of TH at the cellular level.

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