Biochemistry of Human Cancer

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To
Barbara Biber Bodansky
and
Margery B. Franklin

Preface

Biochemistry emerged as a well-defined discipline during the last quarter of the nineteenth century. Its application to the study of human cancer was begun almost simultaneously. In 1885 Freund published his studies on hyperglycemia and in 1889 Müller described his investigations on nitrogen balance. The pace of biochemical investigations in human cancer has accelerated rapidly since then, and in the past twenty-five years particularly a most intensive effort has been made in this direction.

The purpose of this book is to describe and evaluate the present status of these biochemical studies in human cancer and the knowledge we have gained. Biochemical studies of cancer at molecular, cellular, and animal levels have been considered in a number of monographs. Reference to these investigations in this book are brief, and are presented as the background for human studies. Early investigations in various fields of human cancer are frequently noted, not only as an acknowledgment to those who initiated the work but also as a guide to those who may wish to engage in similar studies. In this, as in other areas, one cannot fail to be aware of Santayana's statement that "he who does not remember the past is condemned to repeat it."

It was felt that this book would be most useful if the material

were arranged generally according to the organ site of the neoplasms rather than under general biochemical categories. However, to avoid repetition, certain features of human cancer such as general metabolic characteristics, enzymic aspects, and immunochemical considerations have been described in the first five chapters. Although separate chapters on pulmonary and prostatic neoplasms are not presented, the important biochemical aspects of these neoplasms such as those characterizing carcinoid and ectopic pulmonary neoplasms and the serum acid and alkaline phosphatase activities of prostatic carcinoma have been described in other chapters. A monograph on the "Biochemistry of Brain Tumors" has recently been written by M. Wolleman (University Park Press, Baltimore, Maryland, 1974). The normal human biochemistry of the various organs in which the major types of neoplasms occur has usually been presented, to the extent deemed relevant, as introductory portions to the chapters or occasionally has been interwoven with the discussion of the various types of neoplasms resident in the organ. Where it was considered useful, brief case reports from the literature have been presented to illustrate correlations between biochemical and clinical findings.

We have attempted to indicate the clinical importance of various types of neoplasms by noting the incidence and mortality rates at the beginning of each chapter dealing with a particular group of neoplasms. It has not always been possible to apportion space according to such clinical importance, for many of the common tumors have received relatively little biochemical or, for that matter, other basic science study, whereas some groups of rare and esoteric neoplasms have been the subject of much successful biochemical investigation.

The author has avoided an encyclopedic presentation of his material for obviously such an approach would have increased the size of this volume substantially. Accordingly, references to original work are illustrative rather than comprehensive, and the author feels an apology is due to the investigators both in this country and abroad whose studies it was not possible to cite because of limitation of space.

In reproducing various illustrations and tabular data, the author has indicated the source of the material in the appropriate places throughout the book. He wishes to take this opportunity to express his gratitude to authors and publishers of various journals and monographs for granting permission to reproduce such material.

It is also a great pleasure to acknowledge the assistance of Susan London who, throughout a period of three years, has industriously and faithfully typed the drafts of the manuscript and helped to verify references.

The author is indebted to his many clinical and research colleagues and

friends at Memorial Hospital and Sloan–Kettering Institute who, in formal presentations as well as in informal luncheon discussions and corridor conversations during a period of over twenty-five years, helped to supply a background for the contents of this book. In particular, he wishes to thank Dr. Martin Lipkin, Dr. W. P. Laird Myers, and Dr. Robert D. Leeper, who have been kind enough to read Chapters 8, 10, and 13, respectively, of the final manuscript and to offer helpful comments. The author will at all times welcome suggestions from the readers of this book.

Oscar Bodansky

1

General Metabolic Characteristics in Cancer

I. Introduction

For many years, the concept has recurred that human cancer, regardless of its particular site or pathological nature, is characterized by certain general metabolic features. These have included the existence of anorexia and cachexia, a greater than normal intake of protein to maintain nitrogen equilibrium, the depletion of body fat, disturbances in carbohydrate metabolism, and changes in the protein and lipid patterns of blood serum. At various times, and as an ancillary to these concepts, the idea has also arisen that it is possible to devise a general diagnostic blood test for human cancer, again regardless of its particular type or site. The present chapter will review and assess the evidence for these general beliefs.

II. Protein Metabolism

A. Introduction

The various aspects of normal protein metabolism such as the digestion of protein, absorption of amino acids, the biosynthesis of amino acids and peptides, and the biosynthesis of protein constitute a vast and impor-

tant area which is discussed in detail in various biochemistry texts and to which the reader of this volume is referred. Questions naturally arise concerning the extent to which these mechanisms apply in tumor cells or in tumor-bearing animals, and whether there are any discernible differences between the modes of protein biosynthesis in normal and tumor-bearing animals.

Protein biosynthesis has been studied in such tumor cell systems as mouse ascites tumor cells (Littlefield and Keller, 1957), L-1210 mouse ascites leukemia cells (Ochoa and Weinstein, 1964), and the Novikoff rat ascites tumor (Griffin *et al.*, 1965). Protein biosynthesis in tumor has been compared with that in microbial and normal mammalian systems, particularly with respect to (a) specificity of aminoacyl transfer ribonucleic acid and transfer enzymes, (b) aminoacyl transfer ribonucleic acid ribosomal system, (c) the ribosomal polysomal complex, and (d) the coding characteristics. In a review of such studies, Griffin (1967) noted that there appeared to be no evidence for any differences in the mechanism of protein biosynthesis between normal and cancer cells. However, there may be some tumors as, for example, multiple myeloma, in which specific γ-globulins are synthesized to an excessive extent (Chapter 5).

B. Overall Protein and Nitrogen Metabolism

1. Introduction

The food nitrogen which is ingested by the human organism consists mostly of protein, but to some extent also some of the metabolic products such as purines or amino acids which are found in the particular plant or animal substance that is eaten. The pepsin of the stomach, derived from its zymogen precursor, pepsinogen, and the trypsin and chymotrypsin convertible from their precursors in the pancreatic secretions break down the large protein molecules to polypeptides and free amino acids. Complete digestion to amino acids is accomplished by the action of carboxypeptidase from the pancreas and the amino-, tri-, and di-peptidases of the intestinal secretions. Normally, about 95% of food protein is digested, so that the feces contain only about 5% protein, chiefly keratin, which remains undigested.

2. Fate of Amino Acids

The amino acids and other small nitrogen-containing molecules which thus arise by digestion of the food are absorbed into the bloodstream

and pass into the various tissues. There, they mix with the corresponding molecules to form the various metabolic pools which are in ceaseless, dynamic equilibrium with each other. Whether derived from the diet or from endogenous sources, amino acids may be channeled into one or more of the following metabolic pathways: (a) incorporation into peptides and proteins, (b) utilization of the nitrogen and/or carbon for the synthesis of different amino acids, (c) utilization for synthesis of nitrogenous compounds which are not amino acids, and (d) removal of the α-amino group by transamination or oxidation with the subsequent formation of ammonia which may itself follow any of several paths.

Some of it recycles into the metabolic stream by entering into the synthesis of glutamic acid, glutamine, and carbamyl phosphate. Part of it is excreted as such, constituting about 5–6% of the total nitrogen in the urine of the normal person but changing in response to alterations in the acid-base balance of the individual. However, most of the ammonia is converted to urea, constituting about 90% of the total nitrogen excreted by man in the urine. The formation of urea occurs by the entrance of NH_3 into a cycle of reactions. Under the influence of carbamyl phosphate synthetase in the liver, NH_3 interacts with CO_2 and ATP to form carbamyl phosphate. Ornithine transcarbamylase then catalyzes the interaction of carbamyl phosphate with ornithine to form citrulline and inorganic phosphate. In the next reaction, catalyzed by arginosuccinic acid synthetase in the presence of ATP and Mg^{2+}, citrulline interacts with aspartic acid to form arginosuccinic acid, AMP, and pyrophosphate. Arginosuccinase cleaves arginosuccinic to form arginine and fumaric acid. Finally, arginase catalyzes the hydrolysis of arginine to form urea and ornithine, and the latter compound is then recycled again.

3. Relationship between Nitrogen Intake and Excretion

Much of our information on the intermediary metabolism of protein has been gained from studies of lower organisms such as bacteria and yeasts and of smaller animals such as mice and rats. Obviously, intermediary metabolism, either in normal man or in the patient with cancer, is much less accessible to study, and much of our available knowledge concerns the overall metabolism of proteins. San Pietro and Rittenberg (1953) formulated the kinetic interrelationships in human protein metabolism. Using equations developed from assumptions for the nitrogen content of the body, the urea pool, the intake and excretion of nitrogen, and analyzing the urine for total nitrogen and ^{15}N concentrations of ammonia, urea and total nitrogen following the intravenous injection

of ^{15}N-labeled glycine, they obtained values for protein synthesis of 0.58 gm/kg body weight per day for one individual and 0.86 and 1.28 per kg body weight per day for a second individual on two different occasions.

In 1920, Sherman summarized the results of 109 nitrogen balance studies that had been conducted on humans since the early studies of Hirschfeld (1887). In many of these studies and, indeed, in Hirschfeld's own study, only the urinary nitrogen was determined and assumed to represent the total excretion of nitrogen. The protein requirement or the point at which the nitrogen intake in protein was equivalent to the nitrogen excretion was, on the average, 0.64 gm protein or 102 mg nitrogen per kg of body weight. Sherman's own balance study (1920) on one individual included the fecal nitrogen, and yielded a requirement of 74 mg nitrogen per kg of body weight.

Since these early reports, more elaborate studies have determined balances at varying protein intakes. Regression equations have been developed which connect (a) nitrogen balances as ordinates versus nitrogen intake as abscissae, both expressed as gm per day per m^2 of body surface (Hegsted *et al.*, 1946); (b) nitrogen intake as ordinates versus nitrogen balance as abscissae, both in mg per day per basal calorie (Bricker *et al.*, 1945); (c) nitrogen balance as ordinates versus nitrogen as intake, both in mg per day per kg of body weight (Beattie *et al.*, 1948); and (d) nitrogen excretion as ordinates versus nitrogen intake as abscissae, both as mg per day per kg of body weight (Schwartz *et al.*, 1956). Obviously, where the nitrogen balances are ordinates, the value of the abscissae at $y = 0$ represents the intake, x, necessary to keep the subject in balance. Where the values for nitrogen excretion are on the ordinates, and those for nitrogen intake are the abscissae, the point at which x is equal to y also yields the intake necessary for balance. Calculations from equations based on studies of well-fed persons have yielded values of 67–74 mg/kg/day as the amount of nitrogen necessary for balance (Bricker *et al.*, 1945; Hegsted *et al.*, 1946). Calculations from the equations formulated from the data of von Hoesslin (1919) and of Beattie *et al.* (1948) on subjects recovering from severe malnutrition yielded a value of 112 mg nitrogen per kg per day (Schwartz *et al.*, 1956).

The daily intakes necessary to obtain zero balance vary with the type of protein in the diet. Thus, from the data of Bricker *et al.* (1945), it may be calculated that, on a milk diet, a protein intake of 58 mg nitrogen per kg per day would suffice, whereas, on a white flour intake, 103 mg nitrogen per kg per day would be necessary. These differences depend to some degree on the digestibility of the protein and, hence,

on the extent to which it finds its way into the fecal residue, but they probably depend to a much greater degree on the biological value of the protein, that is, on its content of essential amino acids. Employing mixtures of relatively purified amino acids, Rose (1949) found that lysine, tryptophan, phenylalanine, leucine, isoleucine, threonine, and valine were necessary to maintain a positive nitrogen balance in the adult. In addition, histidine was essential in the growing infant (Holt and Snyderman, 1965).

Nitrogen excretion in feces has occasionally been neglected in balance studies. Yet it may be appreciable, even in normal individuals (Hegsted *et al.*, 1946; Forsyth *et al.*, 1954). The latter study resulted in the following equation:

$$Y = 0.5 + 0.067X$$

where Y was the daily fecal nitrogen and X was the daily dietary nitrogen, both expressed in grams.

4. Response in the Normal Individual to Decrease of Protein Intake

The cancer patient very often has anorexia, and his protein intake is, therefore, low. Chemotherapy or radiotherapy may also affect his desire for food. Again, the performance of surgical operations frequently necessitates the supply of caloric requirements through the intravenous administration of nonprotein nutrients, chiefly glucose and fructose. In order, therefore, to evaluate nitrogen metabolism in the cancer patients, it is important to know the effects in normal persons of inanition or of deprivation of protein.

In his classic review of studies on overall protein metabolism, the physiologist, Graham Lusk (1921), observed that the urinary nitrogen excretion in previously well-nourished men is remarkably constant during the first week of fasting. As fasting continues, the excretion of urinary nitrogen falls, until usually, though not invariably, it reaches levels of about 3–4 gm/day during the third or fourth week of starvation. For example, the professional faster, Succi, excreted 17.0 gm of urinary nitrogen on the first day of his fast. The excretion then dropped to a level fluctuating between 9 and 11 gm/day for the next 8 days, and began to decline slowly until it was 3.3 gm on the twentieth day of starvation and 2.8 gm on the twenty-first.

The decrease in urinary nitrogen excretion is much more sudden if the diet remains calorically adequate, with the dietary protein being replaced by fat and carbohydrate. Many such studies have been carried out since the initial investigation of Folin (1905). For example, Smith

(1926) studied a healthy and active medical student who, during a preliminary period of about a week, had ingested about 75 gm protein daily and had excreted about 9–14 gm urinary nitrogen per day. When the student was placed on a palatable diet containing only 0.3–1.0 gm nitrogen per day, the urinary nitrogen dropped precipitously to 7.3 gm urinary nitrogen on the second day, 3.9 gm on the fourth day, 2.0 gm on the twenty-second day, and 1.58 on the twenty-fourth day, the lowest levels recorded for man until then. The urea nitrogen was 0.35 gm on that day. The nitrogen intake in his food was 0.33 gm on the last 2 days. During the entire period, the subject lost 80.1 gm nitrogen, but his weight remained constant, and there was no objective evidence of deterioration of strength.

The greater depression of urinary nitrogen excretion in the presence of a calorically adequate, protein-poor diet than occurs in starvation illustrates, of course, the sparing influence of carbohydrate oxidation upon protein metabolism. This phenomenon was well appreciated by students of metabolism in the early part of the century.

5. Response in the Normal Individual to Increase of Nitrogen Intake

When an individual has lost considerable protein, either as a result of starvation or as a result of a calorically adequate but protein-deficient diet, the subsequent intake of large amounts of protein results, at the beginning, in relatively low excretions of nitrogen and a consequent replenishment of the body's protein stores. In one of the early significant experiments in nutrition, Thomas (1910) placed himself on a high carbohydrate, protein-free diet for 15 days. The negative balances during this period can be calculated from his data as summing up to a total loss of 51.7 gm nitrogen. He then placed himself on a small protein intake of 3.2–3.7 gm nitrogen per day for 5 days. During this period, the nitrogen excretions were slightly in excess of the intakes, and another 0.56 gm nitrogen was lost. An extremely high protein intake, ranging from 71–87 gm nitrogen per day, was given for the next 4 days. Both fecal and urinary nitrogen contents were determined. The balances were substantially positive, namely, 43, 25, and 8 gm nitrogen per day for the first 3 days, before becoming negative on the fourth day. Thus, a total of 76 gm nitrogen were retained during the first 3 days.

6. Overall Protein in Metabolism in Cancer

Early as well as more recent metabolic data on patients with cancer have indicated that the protein intake which is adequate for nitrogen

balance in normal persons is insufficient in patients with cancer (Mueller, 1889; Wallerstein, 1914; Bolker, 1953). A possible explanation for this is the "nitrogen trap" theory of Mider (1951). He found that the nitrogen content of Walker carcinoma 256 in rats exceeded the nitrogen stored by the host during the period of tumor growth. The normal tissues in the residual carcasses lost nitrogen during the period of tumor growth and contained much less nitrogen than the carcasses of pair-fed controls. These results led to the concept that there was a shift of nitrogen from normal to neoplastic tissue, with the normal tissues providing nitrogenous building blocks for the tumor. In other words, tumor tissue was a "trap" for nitrogen (Mider, 1951).

The extent to which this concept applies in human cancer may now be examined. Waterhouse *et al.* (1951) studied 8 patients with cancer during successive 6-day balance periods. On diets well balanced with regard to carbohydrate and fat and containing 11–12 gm protein nitrogen per day, the nitrogen balance was almost always positive and amounted to about 1 gm/day. In a few instances, the nitrogen intake was 16–18 gm, and the positive nitrogen balance amounted to about 2–3 gm/day. Yet the caloric expenditures, calculated according to the method of Newburgh *et al.* (1937; see also Newburgh, 1942), were frequently greater than the caloric intakes, and the patients lost weight.

These results indicated that patients in whom a malignant neoplasm is growing rapidly may store nitrogen readily while the body is wasting. Presumably, it is the tumor that is storing protein, and the question arises whether the ingestion of large amounts of protein can satisfy the demands of the tumor and, in addition, prevent weight loss of the patient.

Patients who had been on control diets containing 6.1–10.8 gm nitrogen per day were force-fed diets containing about 1000–1500 more calories and 4–7 gm protein nitrogen more per day (Terepka and Waterhouse, 1956). This feeding resulted in daily increases in the weight of the patient by about 240 gm and, in the daily nitrogen balance, by about 2–4 gm. It may be seen that this retention of nitrogen is similar to that which we have previously described as occurring in presumably normal individuals who had been starved and then fed high levels of protein. Terepka and Waterhouse (1956) have, however, maintained that there is a difference. The cancer patient initially exhibits an increasingly positive nitrogen balance during forced feeding but, as the supplementation continues, the positive balance approaches an equilibrium value, and the cancer patients do not retain nitrogen as strongly as do individuals with "simple" starvation. They proposed that, in the presence of an abundant supply of nitrogen and calories, the repletion of host tissues in the cancer patient

cannot fully occur "because of some restraining influence exerted by the growing tumor on the host." In support of this, they suggested that gains in weight appeared to be largely the result of accumulation of intracellular water, and weight loss was rapid when the forced feeding was discontinued.

The question arises whether the apparently higher nitrogen and caloric intakes that are necessary to keep the cancer patient in balance are specific for cancer or hold for any grave disease. Pareira *et al.* (1955) compared a group of cancer patients who were cachectic and had lost about 20% of their previous average weight with a group of cachectic noncancer patients. Both groups were tube-fed a calorically adequate diet that contained 33.6 gm of protein nitrogen per day. The range of weight gains and positive nitrogen balances as well as the clinical improvement were essentially the same in both groups. The only difference observed was that the serum protein levels were restored toward normal values less effectively in the cancer patients than in the noncancer patients. According to Pareira *et al.* (1955), there appeared to be no fundamental specific impairment in protein metabolism in patients with widespread cancer.

7. Nitrogen Metabolism in Totally Gastrectomized Patients

The overall metabolism of nitrogen is also of interest in patients with cancer who have been subjected to total gastrectomy. Buerger and Konjetzny (1923) first noted that the fecal excretion of nitrogen appeared to be higher in such patients, and this observation has been confirmed by others (Everson, 1952; Kelley *et al.*, 1954).

The relationship between nitrogen intake and total nitrogen excretion after total gastrectomy has been evaluated more quantitatively (Schwartz *et al.*, 1956). Five patients with cancer and two with benign ulcer who had undergone gastrectomies 4 months to 5 years previously were studied for a total of 15 3-day metabolic periods. The relationship between the nitrogen intake, X, and the fecal nitrogen excretion, Y, both expressed as grams per day, could be summarized by the equation for the regression line

$$Y = 1.44 + 0.066X$$

The correlation coefficient was $r = 0.68$ ($p < 0.01$). This relationship in normal persons is

$$Y = 0.5 + 0.067X$$

with a correlation coefficient of $r = 0.66$ ($p < 0.01$) (Forsyth *et al.*, 1954). It may be seen that, for any given intake, the fecal excretion is substantially greater in the gastrectomized patients. For example, at an intake, X, of 8 gm nitrogen, the mean fecal excretion would be 1.03 gm, or about 12% of the intake, in normal persons and approximately 1.97 gm, or about 25% of the intake, in the gastrectomized patients.

The relationship between the nitrogen intake and the nitrogen excretion for the gastrectomized patient is shown in Fig. 1-1. The point at which the excretion is equal to the intake, or the point of zero balance, is 234 mg/kg/day. This is much higher than the values of about 70 mg nitrogen per kg per day for normal persons in a good nutritional state (Sherman, 1920; Bricker *et al.*, 1945; Hegsted *et al.*, 1946) or higher than the value of 112 mg/kg/day for malnourished persons who were being repleted (von Hoesslin, 1919; Beattie *et al.*, 1948).

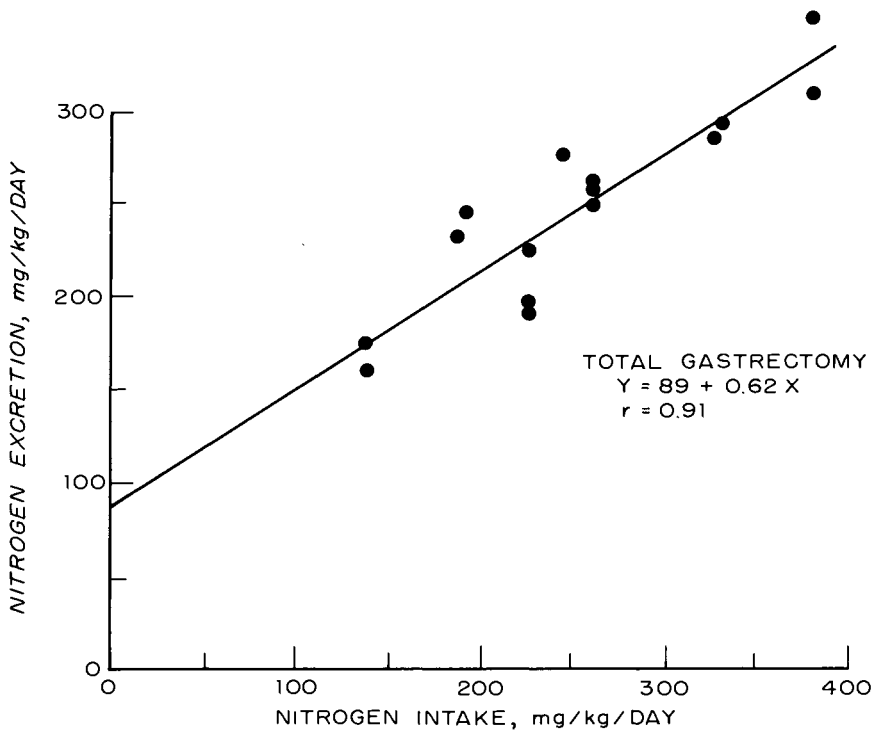


Fig. 1-1 Relationship between nitrogen intake and nitrogen excretion in totally gastrectomized patients. From Schwartz *et al.* (1956). Reproduced by permission of the American Society for Clinical Nutrition, Inc.

C. Serum Proteins

1. Introduction

Changes in the concentration of various protein components in the serum or plasma of patients with cancer have been studied for over 50 years. Methods of determining these individual components have, of course, been refined during this period. Toennies' review of data available in 1947 showed that, in general, neoplastic disease was characterized by a decreased concentration of total serum protein and increased concentrations of blood fibrinogen and serum globulin.

2. Electrophoretic Pattern of Serum Proteins in Cancer

More detailed analysis of the changes in plasma proteins was supplied by electrophoretic studies (Seibert *et al.*, 1947; Petermann *et al.*, 1948; Mider *et al.*, 1950; Ogryzlo *et al.*, 1960), some of which were reviewed by Bodansky (1956). Practically all of the earlier studies agreed with respect to the existence of significant decreases in the albumin component and significant rises in the α-globulins, but there were differences concerning the presence of statistically significant increases in the concentration of β- and γ-globulins.

Consideration of these and other reports indicated that the extent of the changes were dependent upon the progress and extent of the cancer. This is well illustrated by the study of Mider *et al.* (1950) as shown in Table 1-1. The concentrations of α_1-globulin, expressed either in absolute terms or as a fraction of the total protein, increased with the severity of the disease, but did not vary according to the type of cancer. The differences between the mean value for normal adults and those for the groups with localized and late cancer were statistically significant. A similar situation obtained for α_2-globulin. The increases in β- and γ-globulins were less, but were significant in late cancer.

However, this kind of change does not appear to be specific for any particular kind of cancer or, indeed, for cancer as a whole. Seibert and her associates (1947) found that advanced tuberculosis was also characterized by a decrease in the concentration of serum albumin and increases in the concentrations of α-, β-, and γ-globulins. In 1960, Ogryzlo *et al.*, employing paper electrophoresis, summarized the results of more than 3000 such determinations and found that hyperglobulinemia with hypergammaglobulinemia may be found in a wide variety of diseases such as rheumatoid arthritis, chronic liver disease, chronic ulcerative

TABLE 1-1

Electrophoretic Components in the Plasma of Normal Individuals and Cancer Patients[a]

Group	No. of cases	Total protein	Concentration as gm per 100 ml					
			Albumin	α_1-globulin	α_2-globulin	β-globulin	Fibrinogen	γ-globulin
Normal adults	—	6.83	4.04	0.38	0.66	0.76	0.31	0.66
Cancer, all stages[c]	222	6.60[b]	2.94[b]	0.53[b]	0.90[b]	0.89[b]	0.58[b]	0.75
Late cancer	35	6.55	2.38[b]	0.65[b]	1.05[b]	0.99[b]	0.82[b]	0.82

[a] From Bodansky (1956). Reproduced by permission of W. B. Saunders Company.

[b] The significance of the difference between the mean of each group and that of the normal adults has been calculated from the values of the standard deviations given by Mider *et al.* (1950). Designates significance at a 5% level or less ($p < 0.05$).

[c] This group included the 35 patients with late cancer.

colitis, sarcoidosis, certain infectious states, and various types of thrombocytopenic and nonthrombocytopenic purpuras and lymphomas.

These findings are not to be interpreted as indicating that there is no specific serum protein pattern for various types of cancer. In a subsequent chapter (Chapter 5), we shall discuss the subject of immunoglobulins and their presence in the serum and urine of patients with various neoplastic diseases.

3. Cancer Tests and Serum Protein Alterations

As may be seen from the preceding considerations, it has long been recognized that the nature of the serum proteins is altered in patients with cancer. Many attempts have been made to utilize such alterations in devising a diagnostic test for the detection of early cancer. Homburger (1950) reviewed more than 60 such tests that had been proposed during the preceding 20 years. Many of these had been based on the differential precipitability of the serum proteins in cancer patients and in normal persons by such compounds as copper acetate, tannic acid and carbol fuchsin, sodium vanadate, nitric acid, ricinoleic acid or sulfosalicylic acid. The decline and disappearance of these procedures attest to their uselessness.

One of the more prominent of these tests was based on the inhibition by iodoacetate of thermal coagulation of the serum proteins, and it was found that smaller quantities of iodoacetate were required to inhibit the thermal coagulation of cancer serum than normal serum (Huggins *et al.*, 1949). It was also found that no normal persons, but 100% of patients with clinically active cancer and 17% of patients with nonmalignant disease, fell below a critical value of the "iodoacetate index." Detailed study by Bodansky and McInnes (1950) indicated that positive results in this cancer test could occur in grave diseases other than cancer, in the depletion of tissue proteins as a result of noxious stimuli to the organism or in the derangement of the normal synthesis of new plasma protein. Conversely, cancer patients in a good nutritional state gave negative tests.

Bodansky (1951, 1956) has pointed out that the establishment of a general blood biochemical test requires the satisfaction of certain criteria. First, such a test requires that material from the tumor or the results of the reaction of the tumor on its surrounding tissue enter the circulation. Second, it must be recognized that the test may be dependent on the clinical status and, therefore, have a phaselike character. It may be positive when the tumor is growing and negative when growth is slow or has temporarily stopped. Alterations in the concentrations of various plasma proteins such as we have discussed in the preceding

section may amount to about 100–200 mg per 100 ml, or to a total of 3–6 gm in the circulation. It is obvious that neoplasms which had just begun to grow, and for which a cancer test would be desirable, would not be capable of effecting such large changes in concentration.

On the other hand, cancer tests based on the presence of enzymes, antibodies, or antigens in the serum or plasma would require only very small amounts of these substances. It has been calculated that the additional presence of 2–3 mg of an enzyme protein in the entire circulation would cause substantial increases in serum enzyme activity (Bodansky, 1951). Positive immunological tests may be obtained with much smaller concentrations of antibody or antigen protein (Eagle, 1935; Lo Gerfo *et al.*, 1971) and, with current labeling techniques, with amounts of the order of nanograms. We shall discuss the alterations of serum enzyme activities in cancer in Chapters 2–4. In later chapters, we shall consider some purportedly specific tests of current or recent interest such as the utilization of the determinations of α-fetoglobulin in hepatic cancer and of carcinoembryonic antigen in cancer of the colon. These procedures have been listed among others in a recent presentation of the programs and plans of the National Cancer Institute for the application of research methods in the diagnosis of cancer (Berlin, 1974).

The establishment of a reliable general diagnostic test for the presence of cancer must overcome several conceptual and practical obstacles. Such a test demands considerable, detailed, and lengthy correlations between the results of the test and the clinical course of the patient: first, in those patients in whom the neoplasm is evident, large, or operable; second, in patients with small or superficial tumors; and, lastly, in those cases with no clinical evidence of neoplasia, but who yield a positive test and must, therefore, be followed for protracted periods of time to determine the significance of such a positive test. It is self-defeating to label such a test as "false-positive," for it is precisely for those patients in whom there is no obvious clinical evidence of cancer that a test for early cancer is being sought. A positive finding would, of course, furnish no information concerning the site or character of the neoplasm but, in our present state of knowledge, would have to be the start of a roentgenographic and surgical search. These considerations emphasize the desirability of turning our attention to specific tests.

III. Carbohydrate Metabolism

A. Introduction

The dietary carbohydrate in man consists chiefly of the disaccharides: sucrose, lactose, and maltose and the polysaccharides: starch, amylose,

amylopectin, glycogen, and pentosans. In addition to the action of gastric acidity, a series of enzymes consisting of the salivary and pancreatic amylases and the various oligosaccharases of the intestinal tract hydrolyze these to monosaccharides, chiefly glucose, which are then transported across the intestinal tract into the blood circulation. Cellulose and pentosans do not contain linkages that are susceptible to hydrolysis by the enzymes present in the intestinal tract and, except for some slight bacterial action in the large intestine, remain undigested and form part of the fecal residue.

After its absorption, glucose becomes involved in a vast network of metabolic reactions, which are beyond the scope of this volume to describe in any detail. But it may be briefly noted that glucose is converted to glucose 6-phosphate by the action of hexokinase, both in the liver and in the extrahepatic tissues. In turn, this compound can enter any of four metabolic pathways: (a) hydrolysis by a phosphatase to glucose and phosphate in the cells of the liver and kidney, and the entry of glucose into the extracellular fluid and circulation; (b) conversion by the enzyme, phosphoglucomutase, to glucose 1-phosphate, and the entrance of this compound into the synthesis of glycogen, various nucleoside diphosphate esters, heteropolysaccharides, etc.; (c) oxidation at C-1 to yield 6-phosphogluconic acid, pentoses, etc.; and (d) its conversion by phosphoglucoisomerase to fructose 6-phosphate, the initial step in the pathways of glycolysis and, subsequently, oxidation.

In man, the most apparent biochemical manifestations of altered carbohydrate metabolism are the level of glucose in the blood and its excretion in the urine. The normal concentration of this component, approximately 8–12 hours after a meal, is about 70–90 mg per 100 ml, and is the dynamic resultant of several immediate processes. Those tending to increase the level of glucose are (a) absorption of glucose from the intestinal tract, either when such glucose is present directly in the diet or arises from breakdown of dietary polysaccharides, and (b) the hydrolysis of glucose 6-phosphate by hepatic phosphatase and the release of glucose into the blood. On the other hand, processes tending to decrease the level are (a) the urinary excretion, ordinarily negligible in the normal individual, and (b) the conversion of glucose to glucose 6-phosphate and, as we have already noted, the passage of this compound into the metabolic mill in liver and in extrahepatic tissues.

The manifestations of altered carbohydrate metabolism that may occur in certain groups of patients with cancer are (a) hypoglycemia, (b) lowered glucose tolerance and hyperglycemia, (c) lowered spinal fluid concentration (hypoglycorrachia), and (d) the occurrence of sialic acid proteins or mucopolysaccharides in the serum. With the exception of

hypoglycemia, these will be discussed generally in the present chapter. On a more basic, though perhaps not yet applicable, level is the consideration of tissue enzyme activities that are involved in carbohydrate metabolism and have been shown to be altered in human neoplastic tissue. These will be discussed in Chapter 2. We shall also postpone to that point the consideration of the glycolytic enzymes that are elevated in the serum in patients with cancer.

B. Decreased Glucose Tolerance and Hyperglycemia in Cancer

That the carbohydrate metabolism in patients with cancer may be generally altered was first pointed out by Freund (1885) and has been reported repeatedly since then. This alteration is usually demonstrated by a decreased glucose tolerance and even by elevated concentrations of fasting blood glucose.

In 1920, Friedenwald and Grove reviewed the literature available up to that time and, in addition, employing the methods then current, they found that in 31 of 32 cases of cancer of the gastrointestinal tract the fasting blood sugar ranged from 120 to 206 mg per 100 ml, as contrasted with a normal range of 90–130 mg per 100 ml. After the ingestion of 100 gm of dextrose, the blood sugar in the cancer patients rose to levels ranging from 187 to 364 mg per 100 ml at 45 minutes, and to levels ranging from 153 to 332 mg per 100 ml at 120 minutes. The corresponding ranges in normal persons were 153–180 mg per 100 ml at 45 minutes, and 90–130 mg per 100 ml at 120 minutes.

This type of phenomenon has continued to be reported, and some of the more recent studies may be cited. Thus, decreased glucose tolerance or "diabetic" curves have been noted in 37% of one series of 628 patients with all types of cancer (Glicksman and Rawson, 1956), in 62% of 31 hospitalized cancer patients (Weisenfeld *et al.*, 1962), and in 56% of 75 patients with endometrial carcinoma (Benjamin and Romney, 1964). When intravenous glucose tolerance tests are performed in patients with cancer, the rate of disappearance of glucose is slower than in normal individuals (Weisenfeld *et al.*, 1962; Marks and Bishop, 1957).

The basis for the decreased glucose tolerance in patients with cancer has received some study. Levine and Haft (1970) have reveiwed the carbohydrate homeostatic mechanisms normally operative. Secretion of insulin is increased in response to the presence of glucose in the duodenum or to the occurrence of an elevated blood sugar. The stimulus to the presence of duodenal glucose appears to be mediated through the evocation of a local "hormone" in the duodenum which, in turn,

possibly stimulates release of insulin from the pancreas. The elevated blood glucose acts more directly as a stimulus. The insulin may be in two pools—a compartment of the beta cell which releases the insulin rapidly, and a second pool in which slower, but continuous release, is coupled to synthesis.

Decreased glucose tolerance may reflect either diminished secretion of, or a diminished responsiveness to, endogenous insulin. Such endogenous responses may be determined by the measurement of insulin in the serum, but sensitive techniques for such measurement have been available only since 1960 (Yalow and Berson, 1960), and no studies with this technique appear to have been performed in patients with cancer who were tested with glucose orally or intravenously. However, earlier studies (Bishop and Marks, 1959) with exogenous insulin intravenously administered at a dose of 0.1 unit/kg body weight 75 minutes after intravenous glucose administration showed that the rate of decline in blood glucose concentration was slower in patients with neoplastic disease than in normal individuals. In other words, the patients exhibited a decreased sensitivity to insulin, as compared with normal individuals.

If the high glycolysis displayed by neoplasms *in vitro* were characteristic of their behavior *in vivo*, 400–500 gm of neoplastic tissue would use up about 4 gm of glucose per hour, an amount equivalent to the use by all the tissues of the normal body with the exception of the nervous system. The measurement of rates of decline of blood glucose–specific activity after a single injection of labeled [^{14}C]glucose into 7 patients with cancer gave a value of 192 ± 66 (SD) mg/kg/hour for the blood replacement, not significantly different from a value of 161 ± 37 (SD) mg/kg/hour in 4 normal persons (Reichard *et al.*, 1964).

It may be seen, therefore, that no substantial explanation has been submitted for the occasional decreased glucose tolerance and hyperglycemia that occur in the patients with cancer. The question also arises whether these effects are specific for the cancer patient or may not also be present in patients with other grave diseases. In 1921, McBrayer found a high incidence of hyperglycemia in patients with tuberculosis, namely, 40% of 54 cases. In a more recent study in which 62% of 31 hospitalized patients with widespread cancer had shown decreased glucose tolerance, or "diabetic" curves, 78% of 27 patients hospitalized with chronic vascular or neurological disease also showed the same type of curve (Weisenfeld *et al.*, 1962). It is well recognized that the glycemic response to ingestion of glucose depends on several adventitial factors: fasting, exercise, type of diet, and emotional stress. Previous fasting and carbohydrate deprivation causes an exaggerated hyperglycemic response (Shope, 1927; Goldblatt and Ellis, 1932). It is possible that inani-

tion, which occurs in any grave disease, may have been the basis for the hyperglycemia and the hyperglycemic response reported in early studies (Freund, 1885; Friedenwald and Grove, 1920; McBrayer, 1921), but in more recent studies (Benjamin and Romney, 1964), the patients received an adequate caloric diet, including carbohydrates, daily for at least a week before any glucose tolerance tests were performed.

C. Glucose in the Cerebrospinal Fluid

Although modern techniques such as arteriography, ventriculography and radioactive scans are now being used chiefly as aids in the diagnosis of intracranial tumors and metastases, the examination of the spinal fluid for protein, glucose, and cellular content still retains value. Here, we shall consider chiefly the matter of glucose in the cerebrospinal fluid, but the other components deserve brief mention. The roofs of the third and fourth ventricles and the chorioidal tissues of the lateral ventricles have clusters and tufts or plexuses of blood vessels invested by *pia mater*, which is the innermost of the three membranes covering the brain and spinal cord, and by *ependyma*, the lining membrane of the ventricles and the canal of the spinal cord. These plexuses serve as the chief site for the filtration of the blood. Secretion may also be involved.

The circulation of the cerebrospinal fluid takes place from the lateral ventricles, through the foramen of Monroe and, thence, through the aqueduct of Sylvius to the third ventricle and down to the fourth ventricle. From here, it passes through laterally placed foramens. Some fluid passes downward in the spinal subarchnoid space, but most of it flows upward and outward over the brain stem and surfaces of the cerebral hemispheres. The subarachnoid space in which the cerebrospinal fluid circulates is a cavity, variable in size, which exists between the visceral and parietal layers of the piarachnoid membrane. The composition of the spinal fluid depends on several factors, among which is the collective permeability of the blood vessels and membranes that we have just discussed. This permeability has been designated most often as the "blood–brain barrier."

The concentration of protein in the cerebrospinal fluid is usually increased in brain tumors. In a series of 182 cases, Merritt (1935) found that the protein content was normal or less than 45 mg per 100 ml in 31% of the cases, between 46 and 100 mg per 100 ml in 37%, and above 100 mg per 100 ml in the remainder.

Rindfleisch in 1904 appears to have been the first to observe that the cerebrospinal fluid contained very little glucose in the presence of diffuse neoplastic involvement of the meninges. Since then, many reports

have confirmed and amplified this observation, a few of which may be listed (Madow and Alpers, 1951; Dodge *et al.*, 1952; McCormack *et al.*, 1953; McMenemey and Cumings, 1959; Berg, 1953; Kim and Resnick, 1965). Berg in 1953 reviewed the 46 cases reported in the literature until then. These cases included metastatic carcinomatosis, gliomatosis, sarcomatosis, leukemia and lymphoma, melanoma, one thymoma, one pinealoma, and two unclassified tumors. The cerebrospinal fluid glucose was below 40 mg per 100 ml, the lower limit of normal, in 33 cases, or 72%. In some instances, the concentrations of glucose were much lower, down to about 10 mg per 100 ml, and occasionally even to undetectable levels.

Decreased concentrations of spinal fluid glucose (hypoglycorrachia) may also occur in chronic bacterial and fungus infections of the meninges, and is common in acute bacterial meningitis. This situation may become a problem in differential diagnosis for in patients not suspected of having a primary extraneural malignancy there may be vague and poorly localized neurological symptoms, and a low cerebrospinal fluid glucose may be the first clue that there is carcinomatous, gliomatous, or sarcomatous invasion (Glaser and Smith, 1954; Kim and Resnick, 1965).

Several explanations have been submitted for the low glucose concentration in the cerebrospinal fluid of patients with meningeal carcinomatosis. These are (a) utilization of the glucose by leukocytes in the spinal fluid, (b) utilization of glucose by the cells of the neoplastic tissue involving the meninges, and (c) alteration in the blood–brain barrier. The evidence concerning each of these may be briefly considered.

When sterile specimens of dog cerebrospinal fluid were incubated with leukocytes at 37°C for 24 hours, decreases in the glucose did not occur at concentrations below 450 cells/ml, but became more pronounced with higher concentrations until it was 87% at 13,000 cells/ml. However, *in vivo*, when acute aseptic meningitis was caused by various irritants, no decrease in the cerebrospinal fluid glucose occurred at counts of leukocytes ranging from 100 to 13,000 per ml (Baltch and Osborne, 1957). This would indicate that there is some inhibitor in *in vivo* glycolysis or that glucose diffuses or filters from the blood into the cerebrospinal fluid at a rate sufficient to replace the glycolyzed glucose. There do not appear to be any studies on the presence of glycolytic intermediates during *in vitro* and possibly *in vivo* glycolysis which might help resolve this problem.

The second possibility involves glycolysis by tumor cells circulating freely in the cerebrospinal fluid (McCormack *et al.*, 1953). Although tumor cells have two- to threefold the glycolytic rate of normal cells,

it is not possible to quantify the relationship between the number of cells in the fluid or the sheet of tumor that is actively participating in glycolysis and the resultant decrease in spinal fluid glucose.

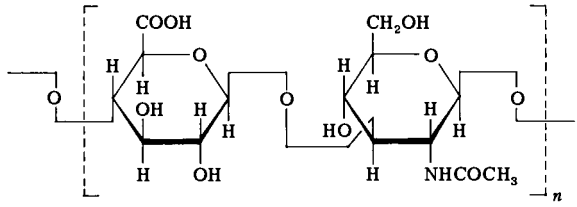
The possibility that the "blood–brain barrier," as we have defined it earlier, may play a role in hypoglycorrachia is subject to experimental approach. Employing dogs, Fishman (1963, 1965) found that the equilibrium between the blood and the spinal fluid possesses the characteristics of saturation, stereospecificity, competitive inhibition, reversibility, and counter transport. Indeed, the term "blood–brain barrer" did not represent a membrane in a simple diffusion process but could be redefined more specifically as a "carrier transport system."

That the transport of glucose in this system may be damaged in neoplastic involvement of the meninges is indicated by Fishmans's (1963) study of 2 patients. Where the intravenous injection of large doses of glucose in dogs raises the blood glucose to levels of 300–450 mg per 100 ml and the spinal fluid glucose also to high saturation levels of 210–240 mg per 100 ml, a similar intravenous injection into a patient with meningeal carcinomatosis caused a marked hyperglycemia but succeeded in raising the spinal fluid glucose from 31 to only 50 mg per 100 ml. Similarly, after intravenous injection of large doses of glucose into a patient with melanomatosis and involvement of the meninges, the cerebrospinal fluid glucose rose from 15 mg per 100 ml to 38 mg per 100 ml.

D. Mucopolysaccharides

1. Introduction

The basis for directing our attention to this subject is the recurrence of reports for over 40 years that the concentration of plasma glycoproteins is elevated in patients with cancer. Mucopolysaccharides are polysaccharides which contain an *N*-acetylated hexosamine in a characteristic repeating disaccharide unit. Thus, the unit of hyaluronic acid consists of D-glucuronic acid attached through its C-1 position to the C-3 position of *N*-acetyl-D-glucosamine, and the latter is attached through its C-1 position to the C-4 position of the following D-glucuronic acid:



Hyaluronic acid unit

Examples of other mucopolysaccharides and their repeating dissacharide units are chondroitin, D-glucuronic acid attached to *N*-acetyl-D-glactosamine; keratosulfate, D-galactose attached to *N*-acetyl-D-galactosamine-6-sulfate. Mucopolysaccharides fall into two categories—the neutral type which contains hexosamine and neutral monosaccharides and the acid type which contains hexosamine combined with uronic acid and/or sulfuric acid.

Only in the case of the hyaluronic acid of the vitreous body and synovial fluids is the polysaccharide free in solution. All other mucopolysaccharides are found in combination with proteins and are designated as "mucoids" or "mucoproteins." The carbohydrate moiety of the mucopolysaccharide is probably linked with the protein through a glycosidic bond to hydroxyl groups of serine residues or to α-carboxyl groups of glutamic acid residues (White *et al.*, 1968). The content of carbohydrate is usually more than 4%, measured as hexosamine. Those mucoproteins which contain less than this amount are often termed "glycoproteins." The carbohydrate moiety may also contain sialic acids which are a group of *N*- and *O*-acetyl derivatives of a 9-carbon, 3-deoxy-5 amino sugar acid called "neuraminic acid." Sialic acids are ubiquitously distributed in nature and are also found as constituents of lipids and of plasma and enzyme proteins.

2. Plasma Glycoproteins

Ordinary electrophoresis at pH 8.6 of human plasma shows that carbohydrate is present in each protein component, but is highest in concentration in the α_1- and α_2-globulin fractions. For example, in the α_1-globulin moiety, the fraction, as grams of carbohydrate per 100 gm protein, was hexose, 7.5; hexosamine, 6.3; sialic acid, 4.1; and fucose, 0.55 (Winzler, 1960). In 1966, Schultze and Heremans listed and surveyed the molecular parameters of the 93 human plasma proteins that up to then had been characterized by physiochemical and immunochemical methods. This tabulation reveals the multiplicity of mucoproteins present in human plasma. Among those with high total carbohydrate content are α_1-acid glycoprotein (orosomucoid), 41.4%; α_{1X}-glycoprotein, 22.7%; haptoglobin, 19.3%; Zn_{α_2}-glycoprotein, 18.2%; and β_2-glycoprotein, 17.1%.

3. Plasma Glycoproteins in Cancer

Beginning with Lustig and Langer in 1931, a number of investigators have noted that the level of polysaccharide associated with serum proteins rose in patients with cancer (Shetlar *et al.*, 1949). But it was

also observed that the concentration of serum polysaccharide was elevated in several other conditions, including hepatic cirrhosis, nephrosis, tuberculosis, and pneumonia. In 1948, Winzler and Smyth reported the following values for plasma mucoproteins, expressed as milligrams tyrosine per 100 ml plasma: 3.33 ± 0.27 for 10 normal persons and 8.53 ± 0.7 for 10 patients with cancer. Table 1-2 shows the results obtained by Shetlar *et al.* (1949) in a more extensive study. It may be seen that the serum mucoproteins, expressed as polysaccharide levels, were elevated significantly in patients with other types of severe disease as well as those with cancer. However, Greenspan (1954) pointed out that serum mucoprotein levels are decreased in diseases with hepatic or endocrine dysfunction and in certain other diseases like the nephrotic syndrome, sarcoidosis, and in one type of neoplastic disease, namely, multiple myeloma.

It would be redundant to describe in detail the many similar reports that have appeared in the intervening years, with the serum levels being measured on serum mucoprotein as such, as protein-bound hexose or as sialic acid (Macbeth and Bekesi, 1962; Rosato and Ravdin, 1967; Singh *et al.*, 1967). In general, diseases characterized by inflammatory, neoplastic, degenerative, thrombotic, or traumatic tissue changes have been associated with a significant incidence of increased serum mucoprotein levels (Greenspan, 1954). The extent of the disease is also an important factor. For example, the levels of serum mucoprotein were substantially elevated in metastatic breast cancer, but were within the normal range if the breast cancer were clinically localized (Macbeth and Bekesi, 1962). At this time, it would appear that the changes in serum mucoprotein levels are nonspecific, although they may have value in following the course of the disease (Harshman *et al.*, 1967). It is possible that, with increasing knowledge of the factors influencing the synthesis of the individual mucoproteins, more specific information about levels of these in the serum of patients with various diseases will become available.

IV. Lipid Metabolism

A. Body Fat Storage in Cancer

It has been frequently observed at operation or autopsy of patients with cancer that the subcutaneous, mesenteric, and retroperitoneal fat stores are greatly depleted or have even completely vanished (Mays, 1969). Quantitative studies in both animal and man support these observations. Mider and his associates (1949) found that the carcasses of

TABLE 1-2

Serum Polysaccharide Levels in Patients with Cancer and Other Pathological Conditions[a]

Group	No. of cases	Nonglucosamine polysaccharide			Glucosamine polysaccharide		
		Average (mg/100 ml)	Range (mg/100 ml)	Standard deviation[b]	Average (mg/100 ml)	Range (mg/100 ml)	Standard deviation[b]
Normal adults	43	111	93–127	9.32	69	61–82	5.18
Malignant neoplasms	105	171	106–308	32.5	95	65–177	17.6
Benign neoplasms	31	123	98–150	14.4	76	65–192	6.38
Nonneoplastic disease	70	149	74–237	29.1	89	50–126	18.0

[a] From Shetlar *et al.* (1949). Reproduced by permission of Cancer Research, Inc.

[b] Standard deviation has been calculated from coefficients of variation listed by Shetlar *et al.* (1949).

rats bearing Walker carcinoma 256 had lipid losses ranging from 19 to 91% of the lipid content of the carcasses of 25 pair-fed control animals. The greater the ratio of the tumor weight to the body weight, the larger the loss of fat from the carcass. These results were confirmed by Haven *et al.* (1949) who, in addition, found that the average percent of total steroid in the adrenal gland decreased to about one-third of the normal value in rats with large tumors, and that the adrenal steroids were replaced to some extent by fat.

Metabolic studies on patients with cancer permit the calculation of caloric balance and the amount of body fat gained or lost (Waterhouse *et al.*, 1951). A specific description of one of the patients on such a study illustrates the principles involved. A 23-year-old female patient with rapidly progressing Hodgkin's disease and previous therapy was placed, for an initial 6-day control period, on a diet containing a total of 96.78 gm of nitrogen and 13,000 calories as determined by calculation from the composition of the diet. Her total output of calories for the 6-day period, as calculated from the determination of insensible water loss by Newburgh's method (1942), was 16,940 calories, with a negative balance of 3,940 calories. The fat loss, estimated by the method of Reifenstein *et al.* (1945), was 423 gm during the 6-day period. Similar calculations showed fat losses for subsequent periods of the study on this patient and for other cancer patients in this study.

B. Lipid Composition of Tumors

1. Introduction

The study of the lipid constitutents of tumors, like that of other constituents, is of importance in order to determine whether any characteristic changes occur in the neoplastic state. Such studies are, of course, hampered by the admixture of connective tissue and areas of necrosis, but we shall presently review the available data on human tumors.

It may be noted, first, that lipids are important components of the cell, for they are involved in various membraneous structures, such as the endoplasmic reticulum, the membrane in the mitochondria that separates the intracristal space from the matrix space, and of the exterior cellular membrane. In cells that can be easily separated and fractionated into various subcellular components, the content and nature of the lipid can be studied in some detail. Such analyses have been carried out on animal tumor cells as, for example, Ehrlich ascites carcinoma cells (Wallach *et al.*, 1960) and the Landschutz ascites carcinoma cells and BP8/3CH ascites sarcoma cells (Gray, 1963).

2. Lipid Content of Human Tumors

There is a substantial but not well-integrated body of information concerning the lipid content of human tumors. Gerstl *et al.* (1965) studied the content of various lipids in 5 pulmonary carcinomas and in 3 normal lungs. The average values, calculated from their results and expressed as millimoles per 100 gm wet weight, were as follows for the normal lungs: lipid phosphorus, 0.81; plasmalogens, 0.16; fatty acids, 3.72; and glycerides, 0.81. The corresponding values were higher for the carcinomas, namely, 1.54, 0.22, 4.77, and 1.60. However, the variability and the small number of cases within each group do not permit any conclusions concerning the significance of the differences. The total polyunsaturated fatty acids averaged 1.13 and 1.56 μM per 100 gm wet weight for the normal lungs and the carcinomas, respectively. The present author has treated these latter data statistically and found the difference to be significant at the 5% level, thus confirming the impression of Gerstl *et al.* (1965).

A more complete study concerning lipid content in breast tumors was submitted more recently by Hilf *et al.* (1970). As may be seen from Table 1-3, there were no significant differences in content of various lipids between the 2 types of control tissue, namely, from normal breast and from breast with fibrocystic disease. In contrast, the contents of cholesterol, cholesterol esters, and free fatty acids showed statistically significant increases in carcinoma, and the content of triglycerides showed a marked and significant decrease.

TABLE 1-3

Lipid Content of Carcinoma of the Breast[a]

Lipid component	Concentration of lipids as mg per gm tissue in		
	Normal breast (20 specimens)	Fibrocystic disease of breast (16 specimens)	Infiltrating ductal carcinoma (48 specimens)
Cholesterol	1.04 ± 0.16	0.98 ± 0.10	2.08 ± 0.18[b,c]
Cholesterol esters	0.31 ± 0.05	0.41 ± 0.05	1.12 ± 0.26[d]
Triglycerides	187 ± 40	136 ± 32	50.4 ± 11[d]
Free fatty acids	1.03 ± 0.10	1.07 ± 0.13	2.51 ± 0.24[b,c]

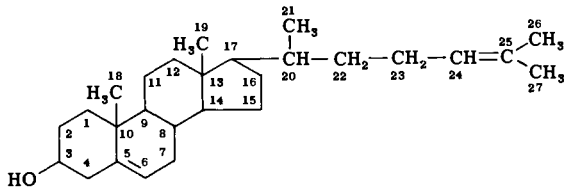
[a] Based on data of Hilf *et al.* (1970)

[b] Significantly different ($p < 0.001$) compared to normal breast tissue.

[c] Significantly different ($p < 0.001$) compared to breast in fibrocystic disease.

[d] Significantly different ($p < 0.001$) compared to normal breast tissue.

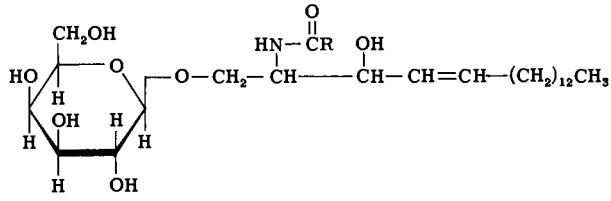
Of compelling interest is the occurrence in neoplastic tissue of lipids that are normally absent from normal tissue. Fumagalli *et al.* (1964) observed that desmosterol (24-dehydrocholesterol) is present in fetal brain, absent in adult brain, and appears again in glioblastomas. The structural formula of desmosterol is shown below.



Desmosterol

This compound constitutes about 7.5% of all brain sterols in the 10-week-old fetus; its concentration decreases to about 2.7% in the 32-week-old fetus, to 1.6% at birth, and, as noted above, is not present in the adult brain. Of 13 glioblastomas studied by Fumagalli *et al.* (1964), 7 contained desmosterol in concentrations ranging from detectable traces to amounts ranging up to 4% of the total brain sterols. It was not found in any of 6 astrocytomas, but was present in an oligodendroglioma. Fumagalli *et al.* (1964) suggested that the presence of desmosterol might result from any of several mechanisms—the rapid proliferation of glial cells, alteration in blood–brain barrier, or higher rate of sterol synthesis. It may be noted, however, that the reappearance of this fetal steroid in brain tumors resembles the reappearance of α-fetoproteins in hepatomas (Chapter 8; Section V,B,3) and of placental alkaline phosphatase in neoplasma (Chapter 3; Section III,B,3), and may all be instances of the phenomenon of derepression.

A number of glycosphingolipids have also been isolated from human neoplastic tissues. These compounds are combinations of a hexose or oligosaccharide with a long chain aliphatic base and are classifiable into three groups—the cerebrosides, the gangliosides, and the ceramide oligosaccharides. For example, a typical cerebroside may be pictured as:



A cerebroside

The monosaccharide is present in a β-glycosidic linkage with the ceramide which is an *N*-acyl fatty acid derivative of the base sphinogosine.

Gangliosides consist of ceramide linked to a hexose and, in addition, contain several moles of carbohydrate such as *N*-acetylgalactosamine or *N*-acetylglucosamine and at least one mole of *N*-acetylneuraminic acid. Ceramide oligosaccharides have a number of moles of carbohydrate joined in glycosidic linkage and attached to ceramide. Some of the glycosphingolipids, particularly the ceramide oligosaccharides, possess antigenic activity (Graf and Rapport, 1960).

Rapport and his associates (1959) isolated ceramide galactosylglucose (ceramide lactoside; cytolipid H) from 3 kg of human epithelial carcinoma grown in rats. A glycosphingolipid with the sequence β-galactosyl-(2-acetamido-2-deoxygluosyl)-galactosyl-glucose-ceramide with a fucosyl residue, probably attached at the terminal galactosyl or at the penultimate 2-amino-2-deoxyglucosyl residue, was obtainable by Hakomori *et al.* (1964, 1967) from a human gastric carcinoma and from a bronchogenic adenocarcinoma, but not from normal gastric mucosa. A ganglioside consisting of fatty acid–sphingosine–glucose–galactose–acetylneuraminic acid in the molecular ratios of 1:1:1:1:1 was found to be present in meningiomas in substantial amounts but absent from normal brain (Seifert, 1966).

3. Serum Lipids and Lipoproteins in Cancer

Changes in the concentration of various lipid components in the serum or plasma of patients with cancer have been reported for many years (Bloor, 1916; Mattick and Buchwald, 1929). The latter investigators obtained values for the following plasma constituents in 10 normal persons and in 21 patients with cancer: total fatty acids, "lecithin," and total cholesterol. They concluded that the plasma total fatty acids and cholesterol were higher in cancer than in normal individuals, although no statistical evaluation was submitted. By current standards, this study failed to take into account a number of factors, such as clinical status, age, and sex of the patient, which might influence the results.

A more rigorous study on one group of plasma lipid components, the plasma unesterified fatty acids, was carried out by Mueller and Watkin (1961). In 30 normal subjects, the mean value for the concentration of this component was 0.35 ± 0.12 (SD) mEq/liter as compared with a value of 0.58 ± 0.30 (SD) mEq/liter for a group of 41 patients with cancer in various stages of the disease. The severity of the disease paralleled the concentration of unesterified fatty acids, and sequential studies indicated that the concentration increased during periods of neoplastic disease activity and decreased when the disease responded to therapy. The mean value for the plasma unesterified fatty acids for

the cancer patients was also significantly higher than that, 0.36 mEq/liter, for a series of 18 patients with chronic diseases other than cancer. Mueller and Watkin (1961) considered various factors such as poor glucose utilization, epinephrine liberation, extreme excitement, prolonged fasting, prolonged inadequate dietary intake, and growth hormone production that might conceivably account for increases of plasma fatty acid levels. They suggested that either impairment of glucose utilization or the evocation of a fat-mobilizing substance might play such a role in rapid tumor growth. However, the significance of these findings has recently been challenged by Mays (1969) who found that only 2 of 15 patients with far advanced cancers showed plasma fatty acid levels that were above the normal range of values obtained in a group of patients of comparable age with debilitating and wasting diseases other than cancer.

There is some evidence, therefore, that disturbances in lipid metabolism, particularly as manifested by the occurrence of abnormal lipids in tumors, are present in human cancer (Hilf *et al.*, 1970; Fumagalli *et al.*, 1964) regardless of their particular nature and site. In later chapters, we shall consider those disturbances in lipid metabolism that are associated with specific neoplasms.

V. Cachexia

A. Introduction

Cachexia may be described as consisting of a marked loss of weight, breakdown of tissue proteins and lipids, negative nitrogen balance, dysfunction of important physiological systems, including muscular weakness, and anorexia. Cachexia, originally considered to be characteristic of cancer, has been deemed to hold for grave disease in general (Pareira *et al.*, 1955). Yet more recent investigations have reawakened interest in this subject, and a symposium was held in 1970 to summarize current views (De Wys, 1970).

B. Biochemical Aspects of Cachexia

Several investigators have again stressed the superior ability of the cancer cell to capture from the internal environment and to concentrate intracellulary the free amino acids necessary for its protein synthesis (Wiseman and Ghadially, 1958; Shapot, 1972). In the summary of a "Working Conference on Anorexia and Cachexia of Neoplastic Disease," De Wys (1970) quoted Cahill as having found alanine in

the blood to be the result of breakdown of muscle protein. Normally, alanine goes to the liver where it is converted to glucose which then passes into the circulation and supplies the glucose requirement of the central nervous system. According to De Wys, Cahill (1970) stated that this excessive catabolic loss of amino acid from muscle may be responsible for the weakness and cachexia of advanced cancer. In contrast, Shapot (1972) has considered that the growing tumor's demand for glucose renders it, in effect, a "glucose trap." This, in turn, leads to a hypoglycemic effect and causes a disturbance of fat and protein metabolism with an increased rate of gluconeogenesis or formation of glucose from amino acids.

Another biochemical approach to the problem of cachexia has been explored by Scherstén and his associates (1969, 1971; Scherstén and Lundholm, 1972). Ghadially and Parry (1965) have reported that a marked increase in the number of lysosomes occurs in the liver cells of rats with induced subcutaneous sarcoma. In view of the intracellular digestive role generally attributed to the hydrolytic enzymes of the lysosome, Scherstén *et al.* (1969) studied several of these enzymes in biopsy specimens of livers from patients with renal carcinoma. It was observed that the free and total activities of aryl sulfatase, of cathepsin, of β-glucuronidase, and of acid phosphatase were higher than the corresponding activities in liver biopsy specimens of a noncancer group. These differences were statistically significant, except for total and free acid phosphatase. The reader will recall that the free activity of a lysosomal enzyme is that present in the tissue homogenate, whereas the total activity is that obtained after treatment with the detergent, Triton X-100.

Further studies showed that the lysosomal enzyme activities in normal renal tissue were of the same order of activity as those in normal liver tissue, but that the activities in both central and peripheral parts of the renal tumor were statistically significantly lower than the corresponding activities in the liver and the kidney tissue of these patients. This suggested that lysosomal enzymes are released from tumor tissue and may then be taken up by other tissues in which they manifest their activity (Scherstén *et al.*, 1971).

Muscle specimens taken from the abdominal wall of patients with various types of carcinoma, chiefly esophageal, gastric, pancreatic, colon, and rectal, were found to have significantly higher total acid phosphatase and cathepsin D activities than specimens from the abdominal wall of control, noncancer patients operated on for uncomplicated gallbladder disease or peptic ulcer (Table 1-4; Scherstén and Lundholm, 1972). These results as well as the others we have noted imply that increased activity of the lysosomal hydrolyzing enzymes may play an important

TABLE 1-4

Total Activity of Cathepsin D and Acid Phosphatase in Muscle Tissue from Patients with Malignant Neoplasms[a]

Group	Cathepsin D		Acid phosphatase	
	No. of patients	Activity as nmole tyrosine/mg protein/min	No. of patients	Activity as nmole P_i liberated/mg protein/min
Patients with malignant neoplasms	19	1.19 ± 0.11[b]	21	0.63 ± 0.07[b]
Control patients with ulcer or gallbladder disease	31	0.68 ± 0.06[b]	31	0.48 ± 0.04[b]
Statistical significance value of p	—	<0.0005	—	<0.05

[a] From Scherstén and Lundholm (1972). Reproduced by permission of J. B. Lippincott Company.

[b] Standard error of the mean.

role in the development of cachexia in patients with malignant neoplasms.

That the serum may also reflect the increased activity of lysosomal enzymes is indicated by the recent finding of Gamklou and Scherstén (1973) that α-1,4-glucosidase is significantly and substantially elevated above normal in patients with various types of cancer.

A number of other mechanisms, which have a lesser degree of experimental background, particularly in man, have also been invoked as exerting a role in the production of cachexia. These include alterations in lipid metabolism, release of hormones or hormonelike substances by the tumor, production of nutritional deficiencies, and psychophysiological factors (De Wys, 1970). Shapot (1972) has suggested distant effects by the tumor on various enzyme systems in the body, and reduction of template activity in tissues distant from the growing tumor. This investigator has stated: "Cancer cachexia seems to be an extremely complex process including both nonspecific and specific features for malignancy."

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2

General Aspects of Enzymes in Cancer: The Glycolytic Sequence

I. Introduction

One of the most notable developments in biochemistry during the past 50 years has been the recognition and description of the individual enzymes that mediate the various steps of intermediary metabolism in tissues. Paralleling this development have been attempts to apply this growing body of knowledge to elucidate possible metabolic derangements in cancer and to utilize the presence of enzymes in blood in the diagnosis and management of various types of cancer.

Enzymes may be classified in one of two major ways, namely, by the metabolic sequence in which they are involved as, for example, the enzymes in glycolysis or by the type of reactions they catalyze. In 1961, the Commission on Enzymes of the International Union of Biochemistry (IUB) drafted specific rules for the classifications and nomenclature of enzymes that were based on the nature of the reaction catalyzed and the type of bond formed or severed. Six main classes (*oxidoreductases, transferases, hydrolases, lyases, isomerases,* and *ligases*) were established and were further divided into subclasses. These were amended in 1972. The IUB designated an Enzyme Commission (EC) number to classify each enzyme, provided a systematic name for the enzyme, recommended a trivial name, but offered no abbreviations. In the discussion that follows, we shall chiefly employ the recom-

mended trivial name but, for convenience in reference to the work of other investigators, may employ the trivial names used by them. The IUB recommendations of 1961, 1964, and 1972 were published in 1962, 1965, and 1973, respectively.

II. The Glycolytic Sequence

A. Respiration and Glycolysis in Cancer Tissue: The Warburg Formulation

The classic studies of Warburg and his colleagues (1930) resulted in several major observations. When slices of normal tissue were incubated with glucose and the proper salt-buffer solution in the presence of oxygen, the formation of lactic acid was negligible or very low. In contrast, in the case of tumor tissue, substantial amounts of lactic acid were formed. In the absence of oxygen, both normal and tumor tissue led to the formation of lactic acid, but that by tumor tissue was considerably higher. Table 2-1 illustrates these points for normal nongrowing tissues, growing tissues, and malignant tissues.

Warburg's data (1930) included human neoplasms as well as animal tumors. Although the human neoplastic tissues used in those studies contained a substantial admixture of nontumor tissue, they showed, in general, high anaerobic and high aerobic glycolytic rates. These observations on human neoplastic tissue have been amply and more precisely

TABLE 2-1

Quantitative Studies of Glycolysis in Tumors[a]

Parameter	Normal nongrowing tissues	Growing tissues	Malignant neoplastic tissues
Respiration, Q_{O_2}[b]	9.3 (3–21)	9.7 (4–14)	5.7 (2.0–10)
Aerobic glycolysis, $Q_{CO_2}^{O_2}$[b]	3.1 (0–10)	7 (6–18)	15.7 (10.1–24)
Anaerobic glycolysis, $Q_{CO_2}^{N_2}$[b]	7.2 (2–19)	20 (13–28)	18.4 (14.0–34.8)

[a] These average values and ranges (in parentheses) are based upon Burk's (1939) summary of Warburg's studies (1930). The values for normal nongrowing tissues were based on 14 different tissues of various animals, including man; those for growing tissues on 7 tissues of 3 different tissue types; and those for malignant tissues on 17 different tumors, most of which were human and were mixed with nontumor tissues.

[b] The term Q_{O_2} designates the microliters of oxygen consumed per milligram dry weight of tissue per hour. $Q_{CO_2}^{O_2}$ and $Q_{CO_2}^{N_2}$ are similar terms for the carbon dioxide liberated that would correspond to the amount of lactate produced; these may also be designated as $Q_L^{O_2}$ or $Q_L^{N_2}$.

confirmed by subsequent investigators. In a large series of determinations, Macbeth and Bekesi (1962a) found that the oxygen consumption of human carcinoma of the breast, stomach, and large bowel, but not of kidney, was higher than that of comparable normal tissue from the same organ. The mean values for anaerobic glycolysis for carcinoma of the rectum, sigmoid colon, and stomach were distinctly higher than the average values for the corresponding normal tissues.

However, there are many exceptions to Warburg's generalization that glycolysis is greater in neoplastic than in normal tissue. His own data (Warburg 1930) show instances of tumor tissue in which the anaerobic and aerobic rates are less than values obtained for some normal tissues such as the exceedingly high values obtained for rat retina: Q_{O_2}, 31; $Q_L{}^{O_2}$, 45; and $Q_L{}^{N_2}$, 88. Macbeth and Bekesi (1962a) found that in two hypernephromas the rates of anerobic glycolysis were less than those of the normal renal cortex. Similar exceptions to Warburg's generalization have been observed by other investigators (Dickens and Weil-Malherbe, 1936, 1941).

In spite of the discrepancies that we have just described, the question naturally arises whether the general tenor of Warburg's *in vitro* findings apply *in vivo*. Even preceding the *in vitro* findings, Cori and Cori (1925a) noted that oral, intraperitoneal, or subcutaneous administration of glucose to rats with Jensen sarcoma or to mice with spontaneous mammary carcinoma markedly increased the lactic acid concentration of the tumors. Venous blood draining tumor-containing organs have considerably more lactic acid and less glucose than the corresponding vein of comparable organs without tumor (Cori and Cori, 1925b). The arteriovenous differences for glucose and lactate are greater for tumor-containing tissues than for corresponding normal tissues (Warburg *et al.*, 1926). Kahler and Robertson (1943) found that the pH levels of hepatomas in mice and rats were 7.0, as compared with a pH of 7.4 in normal liver, and decreased markedly, in some instances to as low as pH 6.4, as more lactic acid was formed following the administration of glucose to the tumor-bearing animals.

As was noted in Chapter 1 (Section III,B), the studies of Reichard *et al.* (1964) showed that rates of replacement and recycling of blood glucose were higher in patients with cancer than in normal individuals, but the differences were not as marked as might have been expected from the generally high glycolytic activity of tumors *in vitro*. These investigators also observed that a survey of the literature showed little, if any, elevation of lactate in the blood of cancer patients. However, a review of more recent literature indicates the occurrence of occasional elevations (Waterhouse, 1974). Employing labeling techniques, Water-

house (1974) also observed that an increased fraction of pyruvate and lactate was recycled to glucose in patients with cancer, indeed, about 2–3 times normal, as compared with an average rise of 50% in the patients studied by Reichard *et al.* (1964). The increased recycling of lactate to glucose was associated with a high rate of entry of glucose into cells. The availability of lactate could be explained by its increased production by the cancer cells. Obviously, determination of arteriovenous differences across the tumor is necessary to substantiate this suggestion (Waterhouse, 1974).

B. Glycolytic Enzyme Pattern in Human Cancer Tissue

1. Introduction

The patterns of glycolytic enzymes in tumor tissue have been studied by Racker and his associates (Wu and Racker, 1959; Racker and Alpers, 1960) on mouse brain HeLa cells and Ehrlich ascites cells. Of greater relevance here are studies which have been carried out on human material such as normal and leukemic leukocytes (Beck, 1958) and carcinomas of the rectum and colon (Shonk *et al.*, 1964, 1965).

The enzyme reactions involved in normal glycolysis are shown in Fig. 2-1, and the enzymes which mediate them are shown in Table 2-2. Some of the enzymes catalyze the reaction in one direction as, for example,

TABLE 2-2

Enzymes of Glycolysis

Reactions in Fig. 2-1	Trivial name recommended by IUB (1965)	EC No.
1	Glucokinase	2.7.1.2
2	Glucose 6-phosphatase	3.1.3.9
3	Glucosephosphate isomerase	5.3.1.9
4	Phosphofructokinase	2.7.1.11
5	Hexosediphosphatase	3.1.3.11
6	Fructosediphosphate aldolase	4.1.2.13
7	Triosephosphate isomerase	5.3.1.1
8	Glyceraldehydephosphate dehydrogenase	1.2.1.12; 1.2.1.13
9	Phosphoglycerate kinase	2.7.2.3
10	Glycerate phosphomutase	2.7.5.4
11	Phosphopyruvate hydratase	4.2.1.11
12	Pyruvate kinase	2.7.1.40
13	Lactate dehydrogenase	1.1.1.27

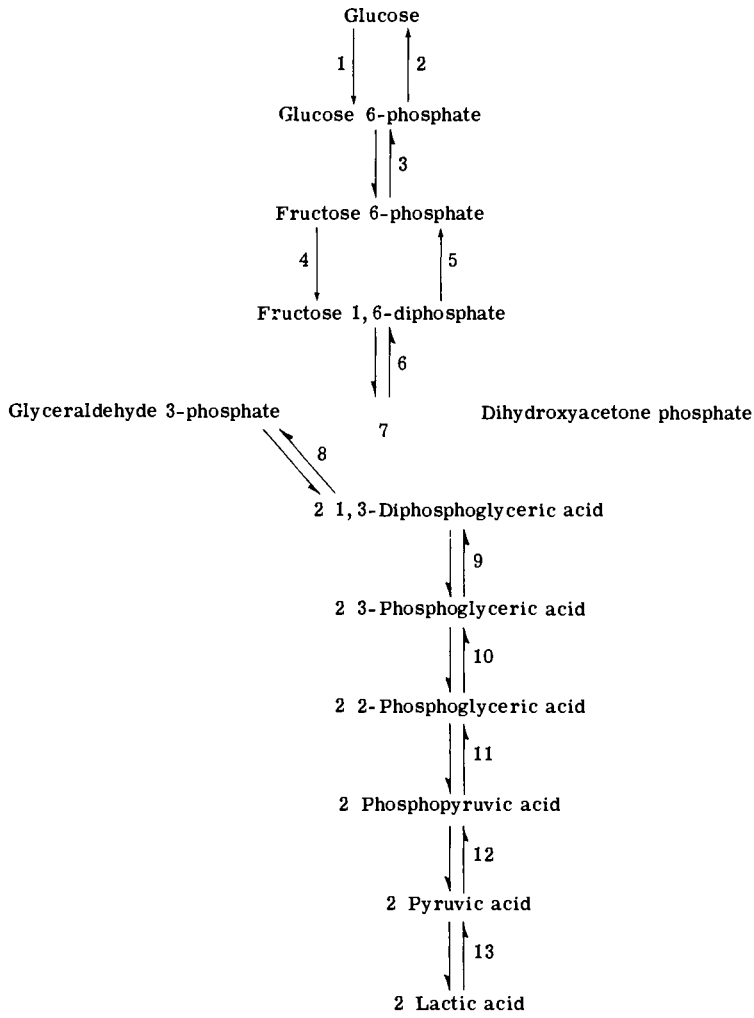

Fig. 2-1 The reactions of glycolysis. Numbers adjacent to the arrows refer to the participating enzymes as listed in Table 2-2.

glucokinase mediating the interaction of glucose with ATP to form glucose 6-phosphate, whereas the reverse reaction is mediated by glucose-6-phosphatase. Most of the other enzymes catalyze reversible reactions, and an equilibrium is attained no matter from which direction the reaction is started. For example, the equilibrium constant for glucose 6-phosphate/fructose 6-phosphate in the reaction catalyzed by glucosephosphate isomerase is 77/23 or 3.3 at 30°C and pH 8.0.

2. Patterns of Glycolytic Enzymes in Normal Human Tissues and Their Neoplastic Counterparts

To obtain human tissues, other than blood, for determination of reliable enzyme activities requires the closest cooperation between the surgeon, the pathologist, and the biochemist. Logistic problems must be surmounted so that representative portions of neoplastic and normal tissue specimens are kept on Dry Ice until enzyme analysis is initiated (Bodansky and Schwartz, 1967). The extent to which autopsy tissue may validly be used for enzyme analysis has been considered by several investigators (Bodansky and Schwartz, 1967; Shonk *et al.*, 1966). The heterogeneity of human neoplastic tissue also poses a problem; the presence of strands of connective tissue and areas of hemorrhage and autolysis raise a question concerning the manner in which the specific activity of the enzyme should be expressed.

Table 2-3 shows the glycolytic enzyme patterns for human normal

TABLE 2-3

Activities of Glycolytic Enzymes in Normal Human Leukocytes and in Leukocytes of Human Leukemia[a]

Enzyme and numbered place in glycolytic sequence[b]	Enzyme activity expressed as micromoles of substrate converted per 10^{10} leukocytes at 37°C in		
	Normals	Lymphocytic leukemia	Myelocytic leukemia
1. Hexokinase	8.9	3.3	3.8
3. Glucose-6-phosphate isomerase	440.0	138.0	469.0
4. Phosphofructokinase	10.2	4.2	4.6
6. Fructosediphosphate aldolase	13.8	11.8	15.8
7. Triosephosphate isomerase	26.0	20.6	29.4
8. Glyceraldehydephosphate dehydrogenase	46.5	21.3	30.3
12. Pyruvate kinase	14.4	4.4	5.7
13. Lactate dehydrogenase	53.4	40.0	34.0

[a] Based on data of Beck (1958) and on review by Knox (1967). Beck (1958) states that data were obtained on the leukocytes of 65 normal subjects, 49 patients with chronic lymphocytic leukemia, and 59 patients with chronic myelocytic leukemia. It would appear that each value represents the mean of separate determinations on the leukocytes of 4–18 individuals, with all but three of the values representing the averages of ten or more determinations.

[b] The designations are those employed by Knox (1967) and are the same as the recommended trivial names.

and leukemic leukocytes, as measured in homogenates of these cells. It may be seen first, that the activity of *hexokinase* is less than that of any other glycolytic enzyme in either the normal or leukemic leukocyte homogenates. Comparison of the kinetic characteristics of the individual glycolytic enzymes indicated that hexokinase is the chief rate-limiting enzyme of glycolysis in both normal and leukemic leukocyte homogenates. The activities of the various glycolytic enzymes in the leukemic leukocytes were, in most instances, substantially less than the activities of the corresponding enzymes in the normal leukocytes (Beck, 1958).

Careful and detailed studies of the glycolytic enzymes in normal and neoplastic human tissues were carried out by Shonk and his associates (1964, 1965, 1966). Table 2-4 shows the activities of the glycolytic enzymes in carcinomas of the rectum and colon, and in the corresponding nonmalignant counterparts. Only those specimens of carcinoma of the colon that contained 30% or more of malignant cells were used for enzyme activity determination, whereas the rectal carcinomas used for analysis contained a much greater proportion of malignant cells, frequently up to 90%. In spite of large coefficients of variation, averaging about 40–50% for these means values, several distinct features may be perceived. First, the enzyme activities of the neoplastic tissue are, in practically all instances, about 1.3–4.2-fold greater than the activities in the corresponding normal colon or rectum and, in some instances, may be shown to be significantly higher. The values for phosphoglucoisomerase, triosephosphate, and glyceromutase in normal rectal tissues are based on the analysis of one specimen, and the comparisons may not be reliable. Second, in the direct glycolytic pathway, phosphofructokinase appears to be the rate-limiting enzyme since it has the least activity of any of the glycolytic enzymes. At this low level of enzyme activity it, along with fructose-1,6-diphosphatase of the reverse pathway, showed the lowest ratios of neoplastic to normal enzyme activity. The extent of the increased glycolytic capacity of the tumors appeared, therefore, to be limited by phosphofructokinase, and changes in the activity of this enzyme could markedly influence the glycolytic rate.

C. *Individual Glycolytic Enzymes in Serum and Tissues of Patients with Cancer*

1. Introduction

Because of the high glycolytic activity of tumors and the assumption that the activities of glycolytic enzymes might be elevated in tumors,

TABLE 2-4

Activities of Glycolytic Enzymes in Normal Colon and Rectum and in Adenocarcinoma of These Tissues[a]

Enzyme and position in glycolytic sequence[c]	Activities as IU per gm of wet weight[b] in					
	Normal colon	Carcinoma of colon	Ratio of neoplastic to normal enzyme activity	Normal rectum	Carcinoma of rectum	Ratio of neoplastic to normal enzyme activity
1. Glucokinase	2.0	3.5	1.8	1.3	3.3	2.5
3. Phosphoglucoisomerase	176	226	1.3	189[d]	240	1.3
4. Phosphofructokinase	1.8	2.5	1.4	1.4	2.0	1.4
5. Fructosediphosphatase	0.8	0.9	1.1	0.6	0.9	1.5
6. Aldolase	1.6	4.4	2.8	1.4	2.9	2.1
7. Triosephosphate isomerase	410	700	1.7	366[d]	580	1.6
8. Glyceraldehydephosphate dehydrogenase	26	73	2.8	25	67	2.7
9. Phosphoglycerate kinase	24	51	2.1	22	50	2.3
10. Phosphoglycerate mutase	30	39	1.3	38[d]	35	0.9
11. Enolase	8.5	16	1.9	7.6	28	4.2
12. Pyruvate kinase	33	69	2.1	28	66	2.4
13. Lactate dehydrogenase	30	65	2.2	21	71	3.4

[a] Based on data of Shonk *et al.* (1965).

[b] Each value represents the average of determinations on 6–12 specimens. The coefficient of variation for the various values ranged from about 20 to 70% and averaged about 40%. One unit is equivalent to the conversion of 1 μmole of substrate in 1 minute.

[c] Trivial names are those employed by Shonk *et al.* (1965).

[d] Represents value of one specimen.

Warburg and Christian (1943) conceived the possibility that the blood leaving the tumor and entering the general circulation might show elevations of serum glycolytic enzymes. Indeed, they found that serum aldolase activity was elevated in rats bearing the Jensen sarcoma. These observations were confirmed by Sibley and Lehninger (1948), who, in addition, extended the studies to human cancer. Of a series of 102 patients with various types of cancer, approximately 20% had elevations of serum aldolase. Baker and Govan (1953) found that the activity of this enzyme was elevated in patients with advanced carcinoma of the prostate, and returned to normal in almost all instances, following estrogen therapy or orchiectomy.

Since these early studies, other enzymes in the glycolytic sequence and, indeed, in other metabolic sequences have been studied with regard to their appearance in human serum and their elevation in patients with cancer. Several factors may be involved in the elevation of a serum enzyme activity in cancer: the damage of membranes of tumor cells or normal cells, so that one or more enzymes pass into the extracellular fluid and then into the circulation; the size of the tumor or organ which is damaged and the concentration of enzymes in the tumor or normal organ; and the rate of disappearance of the enzyme from the circulation, whether by metabolism or by excretion. Elevations in serum enzyme activities may be characterized by varing degrees of specificity, as will be shown in greater detail later.

2. Glucosephosphate Isomerase

Glucosephosphate isomerase, also known as phosphohexose isomerase and phosphoglucose isomerase, catalyzes the reversible interconversion of glucose 6-phosphate and fructose 6-phosphate, and was first described by Lohmann in 1933. It is widely distributed in animals and plants, and highly purified preparations have been obtained from human erythrocytes (Smith and McCants, 1969) and human muscle (Carter and Yoshida, 1969). The latter preparation appeared to be homogeneous by several criteria: schlieren patterns in the analytical ultracentrifuge, and electrophoresis in starch gel and elution profile from column chromatography. Sedimentation–equilibrium studies showed that the enzyme has a molecular weight of 134,000. Similar studies in the presence of guanidine hydrochloride and mercaptoethanol yielded a value of 61,000 and indicated that the enzyme from human muscle was a dimer.

When homogenates of normal and malignant human tissues are subjected to starch block electrophoresis, almost all of the enzyme migrates to the γ-globulin region (Schwartz and Bodansky, 1966a). Occasionally,

however, a small fraction is observed in the α,β-globulin region. When starch gel electrophoresis was employed, over 99% of hemolysates from 3397 unrelated individuals of several population groups showed a typical pattern of at least three zones which migrated toward the cathode (Detter *et al.*, 1968). The most cathodal component was the most strongly staining. The pattern in leukocyte extracts was very similar, but in platelet extract, the most cathodal component showed more staining than the comparable band in the erythrocyte and leukocyte preparations, and the other two bands were relatively less intense. Plasma samples showed only the most cathodal zone, and it was stated that the pattern in other tissues was similar to that in the erythrocytes and leukocytes. In the remaining 1% of homolysates tested, ten different types of electrophoretic patterns could be discerned. Studies of selected families indicated that the variants occurred in individuals who were heterozygous for one or another of a series of rare alleles at an autosomal locus.

Glucosephosphate isomerase is generally the most active enzyme in human tissues (Shonk *et al.*, 1964, 1965) (Table 2-4). The relative activities are based upon the activity of glyceraldehydephosphate dehydrogenase, an enzyme which may be employed as standard because it has very similar activity in several different tissues. The relationship of the activities in the various tissues to that in the serum is of interest since this may be a potential factor of responsiveness of the serum level in various diseases. Bodansky (1954a) found that the average activities in tissues compared with that of serum were lung, 270-fold; brain, 480-fold; bone, 650-fold; liver, 1120-fold; and muscle, 1120-fold.

The elevation of serum glucosephosphate isomerase activity was first studied in metastatic carcinoma of the breast in patients who were receiving various forms of palliative treatment (Bodansky, 1954b). The activity of this enzyme in normal persons was 21 ± 7.0 (SD), in arbitrarily defined units. At various stages of metastatic carcinoma of the breast, the activity could rise as high as 200–400 units. An impressive degree of correlation was found, in general, to exist between increases in this serum enzyme activity and growth of metastatic tumor in bone, as judged clinically and by biochemical methods, particularly by the determination of urinary excretion of calcium. The serum glucosephosphate isomerase activity was also elevated, sometimes quite considerably, in metastatic growth of breast cancer in the liver when the urinary excretion of calcium could not be expected to be, and was not, affected appreciably. The role of this serum enzyme activity was also explored over long periods of time in patients with metastatic carcinoma of the prostate (Bodansky, 1955). Again, a good degree of correlation was found, in general, to exist between elevations in this serum enzyme activity and growth of

tumor, as judged clinically and by biochemical methods, particularly by the changes in the serum acid and alkaline phosphatase activities.

These early observations were amply confirmed and extended in subsequent investigations, not only in patients with carcinoma of the breast (Rose *et al.*, 1961) and prostate (Tan *et al.*, 1963) but also in gastrointestinal carcinoma (Schwartz *et al.*, 1962a); carcinoma of the head, neck, and esophagus (Schwartz *et al.*, 1962b); and in cancer of the lung (West *et al.*, 1962). Elevations of serum glucosephosphate isomerase have also been reported in noncancerous diseases such as tuberculosis (Bodansky, 1954a), infectious hepatitis (Bodansky *et al.*, 1959; Bruns and Jacob, 1954), myocardial infarction (Bing *et al.*, 1957), cardiac failure (West *et al.*, 1961), various myopathies (Schapira *et al.*, 1955), and leukemia (Israels and Delory, 1956; Blanchaer *et al.*, 1958).

The glucosephosphate isomerase activity of body fluids, other than serum, has been investigated in some detail (Thompson *et al.*, 1959; Hulanicka *et al.*, 1963; Brauer *et al.*, 1963). In cerebrospinal fluid, it has been reported to be a more sensitive indicator than either lactate dehydrogenase or glutamate-oxaloacetate transaminase (aspartate aminotransferase) of the presence of secondary and primary tumors of the central nervous system and of meningitis and meningoencephalitis caused by pyogenic, viral, or yeast organisms (Thompson *et al.*, 1959). Brauer *et al.* (1963) observed that the ratio of glucosephosphate isomerase activity in serum to that in effusions was elevated in 72% of patients with neoplastic disease as compared with 45% of patients with effusions resulting from heart failure or cirrhosis.

Although the elevation of glucosephosphate isomerase activity in the serum is not specific for the presence of cancer, the extent of elevation of this enzyme activity is a fairly good criterion of the progress of neoplastic disease and, as we shall point out later, is more sensitive than other serum enzyme elevations in revealing changes in the clinical status of the cancer patient.

3. Aldolase

Aldolase, also known as fructosediphosphate aldolase and fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate-lyase (EC 4.1.2.13), was found by Meyerhof and Lohmann (1934) in muscle and yeast extracts. It mediates the reversible cleavage of fructose 1,6-diphosphate between C-3 and C-4 to yield dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate. It was later realized that a distinctive pathway of fructose metabolism existed in the liver and consisted in the cleavage of fructose 1-phosphate to dihydroxyacetone phosphate and D-glyceraldehyde. In

contrast, the aldolase isolated from muscle tissue exhibits relatively little activity toward fructose 1-phosphate (Leuthardt *et al.*, 1952; Hers and Kusaka, 1953). A third aldolase was isolated from brain (Rensing *et al.*, 1967; Penhoet *et al.*, 1966).

Since the various aldolases (isoenzymes) are found in different proportions in the tissues and serum of patients, it is advisable to consider their properties in some detail. Much of our precise information in this area is based on the aldolases obtained in highly purified form from rabbit tissues (Morse and Horecker, 1968; Penhoet *et al.*, 1969a,b). They may be differentiated by several criteria. Thus, the ratio of activity on fructose 1,6-diphosphate to that on fructose 1-phosphate (the FDP/F-1-P ratio) is approximately 50 for type A (muscle), 1 for type B (liver), and 10 for type C (brain). The Michaelis constants for these 3 types with fructose 1-phosphate as substrate are 5×10^{-3} *M*, 3×10^{-4} *M*, and 4×10^{-3} *M*, respectively (Penhoet and Rutter, 1971). These 3 types are immunochemically distinct and show no cross reactions. They have different amino acid compositions and different primary structures as demonstrated by peptide mapping. However, they are closely related otherwise and possess a considerable degree of homology.

Each of the three aldolases has a molecular weight of about 160,000 and may be dissociated into subunits of about 40,000 (Morse and Horecker, 1968), can be considered as tetramers, and may be more precisely designated as A_4, B_4, and C_4 (Penhoet and Rutter, 1971). Aldolases A and B are present largely in homomeric form in the muscle and liver, respectively, but there are many tissues in which aldolase exists in heteromeric (hybrid) form (Penhoet *et al.*, 1966; Gürtler and Leuthardt, 1969; Foxwell *et al.*, 1966; Tsunematsu and Shiraishi, 1969). Penhoet and Rutter (1971) have studied the properties of the AC hybrids normally found in rabbit brain and of artificially produced BC hybrids. The kinetic constants for the aldolase AC hybrid set are an arithmetic average of those for the homomeric forms, A_4 and C_4. Thus, the FDP/F-1-P ratio is 50 for A_4, 10 for C_4, and essentially the average of these, or 32, for the hybrid form, A_2C_2. Specific antibodies show a less graded inhibition of the catalytic activity of AC and BC hybrids. For example, the activity of A_4 is not inhibited by anti-C, but as soon as one subunit is incorporated in the molecule, as in A_3C, inhibition is almost complete.

The basic information obtained by study of animal tissues appears equally applicable to man. Gürtler and Leuthardt (1969) obtained a 184-fold purified and crystallizable preparation of aldolase from human liver. The FDP/F-1-P ratio was 1.1 and, like other mammalian liver aldolases, was of type B. Table 2-5 shows a compilation of the FDP/F-1-P ratios for various human tissues. It may be seen that the values for

TABLE 2-5

Distribution of Types of Aldolase in Normal Human Tissues Based on Ratio of Rates of Hydrolysis of Fructose 1,6-Diphosphate to That of Fructose 1-Phosphate (FDP/F-1-P Ratio)[a]

	Average values for FDP/F-1-P ratio		
Normal tissue	Data of Tsunematsu and Shiraishi (1969)	Data of Dikow (1969)	Data of Lebherz and Rutter (1969)
Skeletal muscle	54.0	25.5	44
Liver	1.0	1.2	1.2
Kidney	1.7	2.9	4.0
Lung	5.6	—	—
Spleen	5.5	19.5	—
Brain[b]	7.1	—	—
Heart	9.0	13.1	—
Stomach, mucosa	10.0	—	—
Stomach muscle layer	42.0	—	—
Erythrocytes	10.3	—	—
Serum	2.8	—	—

[a] Each value is the mean of determinations on 5 specimens with the exception of serum which is based on determinations of 14 specimens.

[b] This value for brain is distinctly lower than that, 48.9, obtained by Sato *et al.* (1971).

muscle and liver obtained by Tsunematsu and Shiraishi (1969) and those of Lebherz and Rutter (1969) are in good agreement. The value of approximately 50 for the FDP/F-1-P ratio for human skeletal muscle is also in agreement with the ratios obtained for skeletal muscle from other vertebrate species (Lebherz and Rutter, 1969): monkey, 46; rat, 40; perch, 60; and shark, 50. Similarly, the ratios for the liver and for kidney were fairly uniform throughout this vertebrate range, ranging from 1.1 to 3.5 for liver and 3.0 to 7.0 for kidney. The values for the FDP/F-1-P ratios for organs other than the muscle and kidney are between 1 and 50 and can represent either mixtures of aldoases A, B, or C or the presence of various hybrids. Cellulose-polyacetate electrophoresis may be used to detect the presence of hybrids. Human muscle aldolase (type A) and liver aldolase (type B) each gave a single band, cathodic to the origin, with the liver aldolase being more electronegative. Heart had an A band, but also showed some AC hybrids. The brain showed five bands, revealing the entire AC hybrid set (Lebherz and Rutter, 1969).

In 1963, Schapira *et al.* reported that the mean value for the ratio of FDP/F-1-P activities obtained with aldolase from human hepatomas was 5.48, considerably higher than the ratio, about 1.0–1.2, in normal human liver. Table 2-6 shows that the mean values for the FDP/F-1-P ratios in hepatoma, carcinoma of the lung, and carcinoma of the stomach were considerably and significantly higher than the ratios in the corresponding normal tissues. The rise in the ratio was due, not so much to the rise in the rate of action on the substrate fructose 1,6-diphosphate (FDP), as to marked decreases in the rates of action on fructose 1-phosphate (F-1-P) (Tsunematsu and Shiraishi, 1969).

These results raise the question whether the proportion of aldolase A (muscle type) and B (liver type), present in normal tissue, undergoes a shift toward A as malignancy develops. In rats, the FDP/F-1-P ratios were found to be 1.1 in normal liver, 3.6 in fetal liver, and to attain values as high as 16 in hepatomas. Antibody to rat aldolase A inhibited aldolases from normal rat liver by about 10%, and from fetal liver and hepatomas by 50–80%. Conversely, antibody to aldolase B inhibited aldolases from normal liver by 85% and aldolases from fetal liver and hepatoma by 30–60% (Nordmann, 1969). This shift to the muscle or A type aldolase in the liver can be induced in rats by the feeding of a hepatocarcinogenic diet containing 0.06% 3′-methyl-4-dimethylaminoazobenzene. After a 60-day feeding period of this carcinogenic diet, the increased level of type A aldolase persisted during a subsequent observation period of 300 days without showing any reversion to the normal liver pattern (Endo *et al.*, 1970).

Similar results have been obtained in human neoplasms. In a series

TABLE 2-6

Comparison of FDP/F-1-P Ratios in Normal and Neoplastic Human Tissues[a]

Normal tissue	No. of specimens	Ratio (mean + SE)	Types of neoplasm	No. of specimens	Ratio (mean + SE)
Gastric mucosa	5	10.0 ± 0.85	Carcinoma of stomach	8	40.7 ± 4.1[b]
Liver	5	1.0 ± 0.0058	Hepatoma	5	7.9 ± 1.4[b]
Lung	5	5.3 ± 0.30	Carcinoma of lung	5	26.8 ± 3.8[b]

[a] Data of Tsunematsu and Shiraishi (1969).

[b] Significantly higher ($p < 0.005$) than the corresponding normal tissue.

of seven hepatomas, Nordmann and Schapira (1967) found the FDP/F-1-P ratios to range from 2.0 to 13.4, all higher than the value of <1.1 for normal human liver. Generally paralleling this increase was an increase in the degree of inhibition of aldolase activity by ATP from about 25% at FDP/F-1-P ratios of about 2.0 to 58% at a ratio of 13.4. Weinhouse's group (Spolter *et al.*, 1965) has shown that ATP inhibits muscle aldolase. The degree of inhibition by antimuscle aldolase also paralleled the increases in the FDP/F-1-P ratio.

This shift is somewhat more difficult to elicit in human brain tumors. As was noted earlier, type C aldolase is found in brain tissue, frequently as AC hybrids. Tsunematsu and Shiraishi (1969) submitted a mean value of 7.1 for the FDP/F-1-P ratio of 5 normal human brains. Sugimura *et al.* (1969) have found the values of the ratio in cerebral cortex, glioblastoma, and meningioma to be 19.2, 20.8, and 47.6, respectively. Electrophoretic patterns showed a shift from a mixture of A and C bands in cortex or in glioblastoma to only one A band in meningioma. However, in a recent and more extensive study, this group of investigators (Sato *et al.*, 1971) found that the FDP/F-1-P ratio in normal brains was about 50, essentially the same as that for aldolase A (muscle type), and a further shift toward A would not be apparent (Table 2-7). Cellulose acetate electrophoresis patterns (Fig. 2-2) showed that in normal brain aldolase C was present together with aldolase A and their hybrid molecules. Strong bands of aldolase A and the A_3C hybrids were present

TABLE 2-7

Aldolase Activities and FDP/F-1-P Activity Ratios of Normal and Fetal Brains and Brain Tumors[a]

Specimen	No. of cases	Average activity (units/gm protein)		
		FDP	F-1-P	FDP/F-1-P ratio[b]
Normal brain	8	135	3.4	49
Fetal brain	2	12.0	0.3	50
Glioma	9	89	3.2	33
Meningioma	12	56	1.5	53
Hemangioblastoma	2	23	0.4	53

[a] Based on data from Sato *et al.* (1971). Reproduced in part by permission of J. B. Lippincott Company.

[b] These values represent the average of the FDP/F-1-P ratios determined for each individual specimen.

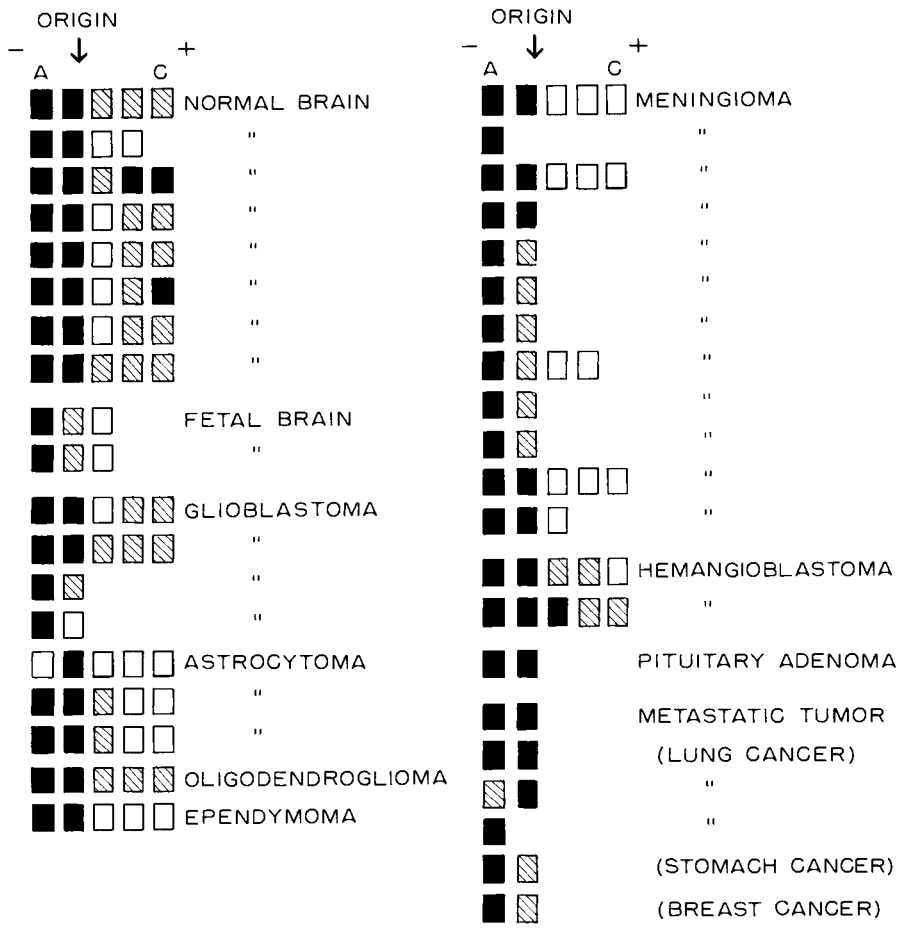

Fig. 2-2 Schematic representation of aldolase isoenzyme patterns of normal and fetal brains and of primary and metastatic brain tumors. From Sato *et al.* (1971). Reproduced by permission of J. B. Lippincott Company.

in each of 8 normal brains; C was present in substantial amounts in 2 of the 8 specimens, moderately in 5, and was absent in 1 (Sato *et* al., 1971). The other hybrids, A_2C_2 and AC_3, were absent or were present in low or moderate amounts. The patterns in a group that included glioblastomas, astrocytomas, hemangioblastomas, 1 oligodendroglioma, and 1 ependymoma showed some diminution in the frequency and itensity of the C_4, AC_3, and A_2C_2 bands. The shift toward the A portion of the electrophoretic spectrum was more marked in a group of meningiomas.

4. Total Serum Aldolase and Its Isoenzymes in Neoplastic Disease

Long before the recognition of its isoenzymic nature, aldolase was found to be elevated frequently in the serum of patients with neoplastic disease. We have already referred (Section II,C,1) to the early work of Warburg and Christian (1943), Sibley and Lehninger (1948), and Baker and Govan (1953). The later studies of Zimmerman and his associates (Rose *et al.*, 1961; Tan *et al.*, 1963; Schwartz *et al.*, 1962a,b; West *et al.*, 1962) extended these observations greatly and determined the incidence of elevations of serum aldolase in various forms of cancer: 70% of 119 patients with gastrointestinal carcinoma (Schwartz *et al.*, 1962a); 45% of 88 patients with carcinoma of the head, neck, or esophagus (Schwartz *et al.*, 1962b); 62% of 126 patients with cancer of the lung (West *et al.*, 1962); 45% of 57 patients with cancer of the breast (Rose *et al.*, 1961); and 75% of 284 patients with metastatic carcinoma of the liver (Tan *et al.*, 1963). However, as we have pointed out earlier for glucosephosphate isomerase, serum elevations of aldolase are not specific for cancer and may occur in other diseases (Bruns and Jacob, 1954). We shall later consider the relationship of changes of serum aldolase activity to changes in other serum enzyme activities in patients with cancer.

It is a fortunate circumstance that the increases in the FDP/F-1-P ratio which occur in neoplasia are readily reflected in the serum, for a potential diagnostic aid is thus made available. Table 2-8 shows that the serum aldolase activity with fructose 1,6-diphosphate as substrate is increased in several types of cancer and in hepatitis. This is, of course, in agreement with the older work in the literature when fructose 1,6-diphosphate was the substrate employed for determination of aldolase. We have summarized this work in the preceding paragraph.

However, the activity of serum aldolase with fructose 1-phosphate as substrate is significantly less in patients with various types of nonhepatic cancer without hepatic metastases than in normal individuals. In cancer with hepatic metastases or in primary hepatomas this activity is normal, and in hepatitis it is markedly elevated. Accordingly, the serum FDP/F-1-P ratios are all significantly and substantially elevated in cancer patients generally, whether with or without hepatic metastases, and in patients with hepatomas. In contrast, in acute infectious hepatitis this ratio is not only not elevated, but also it is even significantly less than in controls. It would thus appear that the serum can reflect the shift toward aldolase A that is characteristic of tumor tissue, probably by entry of this isoenzyme into the circulation. In infectious hepatitis,

TABLE 2-8

Serum Aldolase Activities and FDP/F-1-P Ratios in Normal Individuals, Patients with Cancer, and Patients with Hepatitis[a]

Group	Number	Mean value for activity of		FDP/F-1-P ratio
		FDP-aldolase[b]	F-1-P aldolase[b]	
Normals	14	5.44	2.50	2.8
Cancer without liver metastases	20	8.68[c]	0.78[c]	12.3[c]
Cancer with liver metastases	6	16.4[c]	2.88	7.1[c]
Primary hepatoma	7	16.7[c]	1.50	18.2[c]
Acute hepatitis	10	18.7[c]	14.55[c]	1.3[c]

[a] Based on data of Tsunematsu and Shiraishi (1969). Values for standard error (SE) have been omitted from present tabulation.

[b] Values for serum. Activity expressed as units, with one unit defined as cleavage of 1 μmole of substrate per minute.

[c] Statistically significant (<0.005) with respect to values for normal serum.

there is no shift in the liver tissue toward aldolase A; aldolase B, which is characteristic of the liver, enters the circulation. Indeed, the FDP/F-1-P ratio in the serum decreases from 2.8, which probably represents several tissue sources, to 1.3, approaching the ratio 1.0–1.2, characteristic of liver itself.

5. Lactate Dehydrogenase and Its Isoenzymes in Normal Human Tissues

Lactate dehydrogenase, also referred to as lactic acid dehydrogenase, mediates the reversible interaction of pyruvate and DPNH to form lactate and DPN. It was first identified in washed muscle by Meyerhof (1919) and was crystallized from bullock heart by Straub (1940). It has since been isolated and crystallized from many other animal sources, including human tissues (Nisselbaum and Bodansky, 1961). Its molecular weight is about 140,000. Kaplan (1963) and Markert (1965) showed that the enzyme exists in five molecular forms which represent the five possible combinations in tetramer form of two different units. The five varieties would, therefore, be termed A_4B_0, A_3B_1, A_2B_2, A_1B_3, and A_0B_4 according to Markert (1965), and as M_4, M_3H, M_2H_2, MH_3, and H_4 according to Kaplan (1963). This latter nomenclature indicates that M_4 is the preponderant enzyme in skeletal muscle and H_4 in the heart.

In later literature, these tetramers were also designated as lac-

tate dehydrogenases 1–5: LDH-1, LDH-2, LDH-3, LDH-4, and LDH-5; and the presence of these could easily be revealed on starch gel electrophoresis: In the early publications from the United States, the tetramer LDH-1 (A_4B_0 or MMMM) was identified as the most cathodic, whereas LDH-5 (A_0B_4 or HHHH) was identified as the most anodic (Schwartz and Bodansky, 1966b). However, usage in Europe was the exact converse of this. The Subcommittee on Isoenzymes of the International Union of Biochemistry (IUB) suggested that LDH-1 (A_0B_4, or the heart isoenzyme) be designated as that isoenzyme which moves most rapidly during electrophoresis at pH 8.6 or, in other words, is the most anodic, whereas the other isoenzymes are successively arranged in order of decreasing mobility, and LDH-5 (A_4B_0, or the muscle or liver isoenzyme) is therefore the most cathodic (Latner, 1967). Wherever possible, we shall follow the latter designation.

The distribution of the isoenzymes of lactate dehydrogenase was first determined by Vesell and Bearn (1961), using starch block electrophoresis. Skeletal muscle and liver contained the most substantial fraction of the cathodic isoenzyme, LDH-5, whereas heart tissue was characterized by the highest content of LDH-1, the most anionic isoenzyme. More complete data on the distribution of these isoenzymes in human tissues are shown in Table 2-9.

6. Lactate Dehydrogenase and Its Isoenzymes in Neoplastic Tissues

Kaplan and his associates (Goldman *et al.*, 1964) found a definite shift in the pattern of isoenzymes of lactate dehydrogenase in several types of human malignant neoplasms as compared with the corresponding normal tissues or benign tumors in these tissues. Their procedure was to compare the enzyme velocity obtained in a reaction mixture containing reduced nicotinamide hypoxanthine dinucleotide (NHXDH) and a low concentration of pyruvate with the velocity obtained in a reaction mixture containing reduced nicotinamide dinucleotide (NADH) and a relatively high concentration of pyruvate. The ratios of the velocities were 0.7 for the most cathodic or the pure muscle or liver form (LDH-5) and 2.7 for the most anionic or the pure heart form (LDH-1). The position along this spectrum gives a measure of the relative amounts of these two forms as well as of the relative amounts of the intermediate hybrids. Table 2-10 shows that, in spite of the considerable variability shown by the original data (the coefficients of variation ranged from 20 to 50%), the moiety of LDH-5, the most cathodic isoenzyme, was

TABLE 2-9

Lactate Dehydrogenase Isoenzyme Patterns of Histologically Normal Human Adult Tissues[a]

Tissue	No. of samples	Percent activity and range[b]				
		LDH-1	LDH-2	LDH-3	LDH-4	LDH-5[c]
Prostate	20	2.9 ± 1.2	21.2 ± 3.2	48.1 ± 3.7	24.0 ± 3.0	3.7 ± 1.4
Thyroid	15	5.4 ± 2.3	21.7 ± 2.9	35.2 ± 6.3	26.9 ± 2.8	10.9 ± 4.6
Adrenal	16	3.6 ± 2.1	18.6 ± 4.0	36.6 ± 5.4	32.8 ± 3.8	8.6 ± 4.2
Lung	25	4.5 ± 1.9	13.5 ± 3.7	32.2 ± 5.0	29.6 ± 4.3	20.0 ± 5.9
Spleen	17	5.0 ± 2.3	14.2 ± 4.2	29.0 ± 4.3	31.7 ± 3.6	20.1 ± 6.2
Bone marrow	11	3.6 ± 2.4	13.2 ± 3.5	27.8 ± 3.2	30.2 ± 4.5	25.2 ± 6.7
Leukocyte	37	3.5 ± 1.0	15.4 ± 2.1	22.1 ± 2.0	13.7 ± 3.2	45.3 ± 2.6
Liver	16	1.1 ± 0.6	2.5 ± 3.0	7.2 ± 4.0	12.3 ± 4.9	77.1 ± 8.7
Brain	12	25.2 ± 3.7	28.9 ± 2.0	30.9 ± 3.4	14.1 ± 5.0	0.9 ± 0.5
Pancreas	16	26.8 ± 5.9	31.6 ± 4.9	26.9 ± 4.3	9.4 ± 6.0	5.3 ± 4.4
Kidney	20	33.2 ± 4.5	34.0 ± 4.3	24.2 ± 3.6	7.3 ± 4.9	1.3 ± 1.0
Erythrocyte	35	35.9 ± 1.8	43.7 ± 1.2	14.6 ± 1.0	3.6 ± 1.0	2.1 ± 0.4
Cardiac muscle	30	47.6 ± 4.3	35.1 ± 3.6	13.4 ± 3.2	2.5 ± 1.0	1.3 ± 0.5

[a] After the table of Starkweather *et al.* (1966b). Reproduced by permission of C. V. Mosby Company.

[b] The ranges represent two standard deviations around the mean.

[c] This isoenzyme nomenclature is that recommended by IUB, with LDH-1 as the most anionic and LDH-5 as the most cathodic.

significantly increased in carcinoma of the stomach, large bowel, and lung.

The shift of the pattern of lactate dehydrogenase isoenzymes to the muscle or LDH-5 moiety has been confirmed and extended by other investigators, using a variety of methods. Procházka *et al.* (1968) observed significantly lower values in the percentages of LDH-1 and LDH-2 in gastric carcinoma tissue, as compared with those in normal fundic mucosa with or without gastritis. Conversely, the percentage of LDH-4 and LDH-5 were significantly higher in the carcinomas. Langvad (1968a) studied the distributions of the isoenzyme ratio, LDH-4/LDH-2, in 420 tissue specimens from 36 consecutive cases of cancer of the colon and rectum, with the control tissue being taken from the presumably normal tissue adjacent to the tumor; the isoenzyme ratio was above 1.0 in only 16% of tumor-negative samples, as compared with 90% in

TABLE 2-10

Activities of the LDH-1 and LDH-5 Isoenzymic Moieties of Lactate Dehydrogenase in Normal and Neoplastic Human Tissue[a]

Tissue	Activity of total LDH in[b]		Activity of M-LDH (LDH-5) in[b]		Activity of H-LDH (LDH-1) in[b]	
	Normal	Cancer	Normal	Cancer	Normal	Cancer
Stomach	99	87	21	58[c]	78	29[c]
Large bowel	57	108[c]	33	81[c]	23	29
Lung	23	45	14	32[d]	9	12
Breast	7.3	37	3.9	28.6	3.4	8.7

[a] Based on the data of Goldman *et al.* (1964). See text for method. Each value represents the mean of determinations on 7–25 different specimens. Original report also contains data on thyroid, pancreas, ovary, prostate, and uterus, but mean values are based on a smaller number of determinations.

[b] Units per minute per gram wet tissue. One unit is equal to Δ-OD at 340 nm of 1.000

[c] Significance at $p < 0.01$ in comparison of cancer with normal tissue.

[d] Significance at $p < 0.05$ in comparison of cancer with normal tissue.

tumor-positive samples. Similar results were obtained for bronchogenic carcinoma (Langvad, 1968b).

The significance of a shift in the LDH isozyme pattern from the LDH-1 to the LDH-5 part of the isoenzyme spectrum was first suggested by Goldman *et al.* (1964). LDH-5 (M-LDH) is better able than LDH-1 (H-LDH) to convert pyruvate to lactate at high pyruvate concentrations. This reaction,

$$NADH_2 + CH_3\,COCOOH \rightleftarrows NAD + CH_3{\cdot}CHOH{\cdot}COOH$$

results in the replenishment of nicotinamide adenine dinucleotide (NAD) which is required as a hydrogen acceptor in the oxidation of triosephosphate produced earlier in the glycolytic cycle. The existence of this phenomenon is consistent with the generally increased production of lactic acid by tumors.

Several investigators have suggested that the change in isoenzyme composition may precede histologically apparent alterations and, hence, serve as a diagnostic aid. For example, Langvad's (1968a) data show that in histologically negative tissue adjacent to carcinomas of the colon, the incidence of the isoenzyme ratio, LDH-4/LDH-2 with a value greater than 1.0, was 40% in the 0–1-cm distance from the edge of the tumor, 20% in the 2–5-cm distance, and 9% in the 5–10-cm distance. Langvad's suggestions are reminiscent of that proposed by Sato *et al.*

(1971) concerning the use of aldolase isoenzyme patterns as an aid in the diagnosis of human brain tumors.

7. Serum Lactate Dehydrogenase and Its Isoenzymes in Neoplastic Disease

Prior to the recognition that lactate dehydrogenase existed in several isoenzyme forms, its role in neoplastic disease and its elevation in the serum of patients with neoplastic and other diseases had already been explored (Bodansky, 1960, 1961). In 1954, Hill and Levi found lactate dehydrogenase to be present in human serum and its activity to be elevated in patients with neoplastic disease. The studies of Zimmerman and his associates are representative of many similar investigations in the literature. Elevations of serum lactate hydrogenase occurred in 53% of 119 patients with gastrointestinal carcinoma (Schwartz *et al.*, 1962a); 21% of 88 patients with carcinoma of head, neck, or esophagus (Schwartz *et al.*, 1962b); 53% of 126 patients with cancer of the lung (West *et al.*, 1962); 60% of 57 patients with cancer of the breast (Rose *et al.*, 1961); and 69% of 284 patients with metastatic carcinoma of the liver (Tan *et al.*, 1963). However, elevations in serum lactate dehydrogenase activity have also been reported in patients with myocardial infarction, hepatitis, infectious mononucleosis, infection, anemia, and other nonneoplastic diseases (Bodansky, 1961).

The extent of these elevations have been reviewed by Schwartz and Bodansky (1966b). In a typical study on myocardial infarction, about 47% of the patients showed a serum elevation 2–5 times the upper limit of normal on the first and second days, after which these levels decreased so that on the sixth day only about 14% of the patients showed an elevation between 2 and 3 times the upper limit of normal. No higher values than these were present. In a group of children with infectious hepatitis, only 80% showed elevations of serum lactate dehydrogenase, and none was higher than 3 times the upper limit of normal. Patients with myopathies and granulocytic leukemias had 18 and 13%, respectively, of elevations between five- and eightfold the upper limit of normal.

Many attempts have been made to determine the pattern of distribution of lactate dehydrogenase isoenzymes in the serum and thus provide a basis for interpretation of alteration of this pattern in various diseases (Wróblèwski and Gregory, 1961; Hess and Walter, 1961; Cohen *et al.*, 1966; Starkweather *et al.*, 1966a,b; Wright *et al.*, 1966). Table 2-11 shows the isoenzyme patterns of lactate dehydrogenase (LDH) in normal human serum. These results, obtained by different investigators, are in fairly good agreement although, in evaluating alterations of this pattern in

TABLE 2-11

Percentage of Lactate Dehydrogenase Isoenzymes in Serum of Normal Persons[a]

	Mean ± SD percent of total serum LDH		
Isoenzyme	Wright *et al.* (1966) on 50 normals	Starkweather *et al.* (1966a) on 120 normals[a]	Cohen *et al.* (1966) on 51 normals
LDH-1	32.8 ± 5.7	28 ± 2	33 ± 6.5
LDH-2	46.6 ± 3.3	38 ± 2.5	47 ± 6.7
LDH-3	16.4 ± 4.3	18 ± 2	14 ± 3.8
LDH-4	2.5 ± 1.0	8.7 ± 2.1	6.0 ± 3.1
LDH-5	1.5 ± 0.8	7.6 ± 1.8	

[a] Starkweather *et al.* (1966a) represented their normal values graphically as areas which include two standard deviations about the mean. These have been used to calculate the mean values. Cohen *et al.* (1966) presented the isoenzyme distribution as present in the α-, α_2-, β-, γ-, and γ_2-globulins, and these values have been assumed to coincide with LDH-1 and LDH-5, respectively. The nomenclature here is that recommended by IUB (Latner, 1967) with LDH-1 as the most anionic and LDH-5 as the most cationic isoenzymes, corresponding to the H_4 (heart) and M_4 (muscle), respectively, of Kaplan (1963).

neoplastic disease, it is preferable to utilize as a base the normal values obtained by the same investigator.

In patients with malignant neoplasms, particularly if metastases are present, there are, in addition to the frequent increase in the activity of total serum LDH that we have already described, rises in the percentages of LDH-3, LDH-4, and LDH-5 (Cohen *et al.*, 1966; Starkweather *et al.*, 1966a). Of 39 patients with extensive lung cancer, 25 had both an elevated serum LDH activity and an abnormal isoenzyme pattern; 10 patients had only an abnormal isoenzyme pattern; and 4 were normal with respect to both parameters (Starkweather *et al.*, 1966a). Of 9 patients with limited lung cancer, 2 had both an elevated serum LDH activity and an abnormal isoenzyme pattern; 2 had only an abnormal isoenzyme pattern; and 5 were normal with respect to both the total serum LDH activity and the isoenzyme pattern. The abnormal isoenzyme pattern consisted of a preponderance of patients with percentages of LDH-1 and LDH-2 isoenzymes below the lower limit of normal (mean — 2 SD) and high percentages of LDH-3, LDH-4, and LDH-5.

Of the 48 patients, 3 had LDH-1 values above the upper limit of normal (mean + 2 SD) and 2 patients had elevated levels of LDH-2. In contrast, approximately 20 of the 48 patients had elevated LDH-3, LDH-4, and LDH-5 components, with some values being twice as high as the upper limit of normal.

Computerized discriminant analyses have been applied to data on assays for total and isoenzyme activities of serum lactate dehydrogenase. These have indicated the usefulness of these assays for differential diagnosis of infectious hepatitis, infectious mononucleosis, myocardial infarction, and lung cancer (Glick, 1969).

D. Comparison of Glycolytic and Other Serum Enzyme Activities in Human Cancer

We have already described briefly the extent of elevations of glucosephosphate isomerase, aldolase, and lactate dehydrogenase. These are only several of the enzymes in the glycolytic cycle and of other metabolic sequences whose presence in the serum was sought following the formulation by Warburg and Christian (1943) that growth of tumor might be reflected by enzyme changes in the peripheral blood. We have already noted previously (Section II,C,1) that the elevation of enzyme activity of the blood may depend on several factors: the damage of membranes of tumor cells so that there is a leakage of enzymes into the circulation; the size of the tumor or organ which is damaged and the concentration of enzymes in the tumor or the particular organ effected; and the rate of disappearance of the enzyme from the circulation, whether by metabolism or excretion.

The glycolytic enzymes and enzymes in other metabolic sequences have been termed "ubiquitous" enzymes since they are present in almost every tissue of the body and, hence, may pass into the circulation when tissues are damaged by the presence of cancer or other disease. Table 2-12 shows a comparative compilation of the incidence of serum elevations of eight different enzyme activities in several major types of neoplastic disease. It may be seen that the incidence of glucosephosphate isomerase elevation is highest in each of these neoplastic diseases. Glutamic oxaloacetic and pyruvic transaminases (aspartate and alanine aminotransferases, respectively) are the least sensitive indicators. The others are of intermediate sensitivity. Accordingly, among the ubiquitous enzymes listed in Table 2-12, glucosephosphate isomerase would be the serum enzyme determination of choice in following the growth or regression of tumor and in determining the effectiveness of certain types of therapy (Bodansky, 1954b, 1955, 1965).

TABLE 2-12

Incidence of Serum Elevations of Ubiquitous Enzymes in Cancer[a,b]

Group	No. of patients	Percent elevations of							
		GPI	ALS	LD	ASP	ICD	MD	GTD	ALT
Gastrointestinal carcinoma (stomach, colon, pancreas)	119	74	70	53	35	40	37	30	19
Carcinoma of head, neck, and esophagus	88	51	45	21	16	21	10	15	9
Cancer of lung	126	72	62	53	15	24	25	28	7
Carcinoma of breast	57	70	45	60	—	40	40	35	—
Metastatic carcinoma of liver	284	84	75	69	51	53	62	47	44

[a] From summary of literature by Bodansky (1965).

[b] The abbreviations used here have been recommended by several official bodies in Great Britain (Baron *et al.*, 1971). These abbreviations together with the Enzyme Commission number of the IUB and recommended trivial names are as follows: GPI, glucosephosphate isomerase (EC 5.3.1.9); ALS, fructosediphosphate aldolase (EC 4.1.2.13); LD, lactate dehydrogenase (EC 1.1.1.27); ASP, aspartate aminotransferase (EC 2.6.1.1); ICD, isocitrate dehydrogenase (EC 1.1.1.42); MD, malate dehydrogenase (1.1.1.37); GTD, glutathione reductase (EC 1.6.4.2); and ALT, alanine aminotransferase (EC 2.6.1.8).

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3

Enzymes in Cancer: The Phosphohydrolases

I. Introduction

The phosphohydrolases, the enzymes that hydrolyze phosphoric monoesters, have been utilized for a considerable number of years in the diagnosis and management of patients with cancer. The three enzymes in this group of greatest relevance to our subject are acid phosphatase (EC 3.1.3.2), alkaline phosphatase (EC 3.1.3.1), and 5′-nucleotidase (EC 3.1.3.5).

II. Acid Phosphatase

A. Introduction

The existence of acid phosphatase was first revealed in 1925 when Demuth noted that human urine was capable of hydrolyzing what was then termed "hexose diphosphate" with optimal activity occurring at a pH of approximately 5.0. Subsequently, it was found that this enzyme was present particularly in the tissues of the human genital system (Kutscher and Wörner, 1936) and that its activity in the serum could reflect the existence of carcinoma of the prostate (Gutman and Gutman, 1938a,b). The enzyme was designated as "acid" phosphatase. Subse-

quently, de Duve (Applemans *et al.*, 1955; de Duve, 1963) found that acid phosphatase was a characteristic component of a new intracellular component, the lysosome, and that it thus played a role in intracellular metabolism. The possibility that there might be differences among the acid phosphatases of different tissues was explored (Abul-Fadl and King, 1949) and the existence within a given tissue or cell of more than one molecular form, or isoenzyme, has been established (Lundin and Allison, 1966; Hopkinson, 1968). The activities of acid phosphatase in platelets (Zucker and Borelli, 1958) and in normal and abnormal leukocytes (Valentine and Beck, 1951; Li *et al.*, 1970) have been described as indicators of corresponding pathological states.

Acid phosphatase has been purified from human prostate (London and Hudson, 1953; Boman, 1958; Ostrowski and Rybarska, 1965), erythrocytes (Tsuboi and Hudson, 1954), and placenta (Di Pietro and Zengerle, 1967). The degrees of purification have been as high as 4900-fold for human prostatic acid phosphatase (London and Hudson, 1953) and approximately 1500-fold for human erythrocytic acid phosphatase (Tsuboi and Hudson, 1954).

B. Properties of Tissue Acid Phosphatases

1. Isoenzymes

As we have already noted, acid phosphatase exists as more than one isoenzyme. Sur *et al.* (1962) subjected a concentrated aqueous extract of human prostate to starch gel electrophoresis in citrate buffer at pH 6.2 and obtained at least 13 active zones. These were recovered from the gels in four groups according to their mobilities. All four had the same pH optimum of 5.5 and, within experimental error, the same Michaelis–Menten constant. These results have been amplified and confirmed by subsequent investigators, with gel electrophoresis and isoelectric focusing yielding as many as 8–20 bands (Lundin and Allison, 1966; Smith and Whitby, 1968; Ostrowski *et al.*, 1970). However, treatment with neuraminidase tends to reduce and even abolish the electrophoretic heterogeneity and indicates rather clearly that the large number of isoenzymes differ from each other in the number of neuraminic acid residues attached to essentially the same protein molecule. Column chromatography or electrophoresis of human erythrocyte hemolysates has resulted in the definition of more than one zone of acid phosphatase activity (Hopkinson *et al.*, 1963; Georgatos, 1965). Different electrophoretic patterns or phenotypes in humans reflect the existence of alleles, or contrasting genes situated at the same locus in homologous chromosomes (Hop-

kinson, 1968). Chromatography of a high-speed supernatant fraction of human placentas on Sephadex G-200 yielded three peaks of acid phosphatase activity. The molecular weights, estimated by sucrose density gradient centrifugation, were >200,000, 105,000, and 35,000 (Di Pietro and Zengerle, 1967).

2. Kinetics

Many studies have been made on the relative rates of hydrolysis of phosphoric monoesters, on the Michaelis constants, and on other kinetic aspects of acid phosphatases from different tissues and, indeed, on the isoenzymes of an acid phosphatase from the same tissue (Bodansky, 1972). Some of these results may be noted briefly here. With highly purified human erythrocytic acid phosphatase, the relative rates of action on various phosphoric acid monoesters were phenyl phosphate, 100; α-glycerophosphate, 33; β-glycerophosphate, 2; adenosine 5′-phosphate, 3; and glucose 1-phosphate, 1. The relative actions of purified human prostatic acid phosphatase on these substrates were, respectively, phenyl phosphate, 100; α-glycerophosphate, 69; β-glycerophosphate, 84; adenosine 5′-phosphate, 60; and glucose 1-phosphate, 19. Thus, prostatic acid phosphatase had a broader spectrum of actions on these phosphoric monoesters (Tsuboi and Hudson, 1955; Nigam *et al.*, 1959).

The values of the Michaelis constant, K_m, for prostatic acid phosphatase were 2.4 mM with β-glycerophosphate as substrate and 0.15 mM with phenyl phosphate (Tsuboi and Hudson, 1955). Under the same conditions, Nigam *et al.* (1959) obtained K_m values of 4.0 mM and 0.75 mM for these two substrates, respectively.

The effect of compounds that might either accelerate or inhibit acid phosphatase has been explored in great detail. As long ago as 1949, it was observed that even for crude preparations, fluoride in 0.01 M concentration had only a slight inhibitory effect, about 8%, on erythrocytic acid phosphatase, but exerted a marked inhibition, 96%, on prostatic acid phosphatase. L(+)-Tartrate (0.01 M) also had a marked differential effect, inhibiting 94% of prostatic acid phosphatase activity and none of erythrocytic acid phosphatase. These marked inhibitions hold for purified preparations as well (Tsuboi and Hudson, 1955; Nigam *et al.*, 1959).

The three isoenzymes of human placental acid phosphatase also show some interesting differences with p-nitrophenyl as substrate (Di Pietro and Zengerle, 1967). Thus, two of these isoenzymes had a pH optimum in the vicinity of pH 5.5, whereas the pH optimum of the third was at pH 4.0. The Michaelis constant, K_m, with p-nitrophenyl phosphate as substrate was 1 mM for isoenzyme III and 7 mM for isoenzyme

II. At 0.02 *M*, L(+)-tartrate had the following inhibitory effects on the three isoenzymes: I, 41%; II, 90% and III, zero. Fluoride at 0.05 *M* had inhibitory effects of 51, 23, and 5%, respectively. An interesting property of isoenzyme III, not shared by either I or II, was the stimulation of its activity by various purines. 6-Ethylmercaptopurine (1 m*M*) increased the velocity by 168%.

3. Subcellular Localization of Acid Phosphatase

With rare exceptions (Reith *et al.*, 1964), normal human tissues are seldom available in amounts adequate for studies of intracellular distribution. Accordingly, most of our information on acid phosphatase is based on investigations in animals and has been inferred to hold in man as well. In 1951, Berthet and de Duve initiated a series of investigations which resulted in the concept of a new intracellular organelle, the lysosome, that is present in many cell types but is especially abundant in the liver and kidney. Lysosomes are surrounded by a single membrane and contain not only acid phosphatase but also many other hydrolytic enzymes such as β-glucuronidase, aryl sulfatase, cathepsin, ribonuclease, and deoxyribonuclease. These structures have been postulated to be the site of intracellular digestion accompanying pinocytosis and phagocytosis.

The attempt to determine whether acid phosphatase was present only in the lysosome is burdened by the fact that the separation of this structure is attended by damage to it or cross contamination with other subcellular fractions. Although some of the older investigations resulted in a recovery of approximately 40–50% of the acid phosphatase in the lysosomes (de Duve *et al.*, 1955), it has been estimated that, in general, approximately 70–80% of the acid phosphatase can be recovered in the lysosomal fraction. Sawant *et al.* (1964) obtained a purified lysosomal preparation that was contaminated to some extent with microsomal fractions, as manifested by determination of marker enzyme activities, but electron microscopic examination of this preparation showed about 95% of intact lysosomes and about 5% of mitochondria. Microsomal fragments were seldom visible.

Shibko and Tappel (1963) attempted to determine the extent to which acid phosphatase is derived from lysosomes broken during the fractionation procedure or whether some acid phosphatase is actually localized in other subcellular structures. They found that the chromatographic, electrophoretic, and kinetic properties of acid phosphatase in a highly purified lysosomal fraction were essentially the same as those in the soluble fraction, except for the degree of inhibition by fluoride. It would

appear, therefore, that practically all the acid phosphatase is localized in the lysosomes, at least in the case of rat liver.

The activity of acid phosphatase and, to a lesser extent, those of other hydrolyases have been used as one criterion for the presence of lysosomes in other tissues. Kidney, spleen, and pancreas contain acid phosphatase capable of being centrifuged in the fraction corresponding to lysosomes. The heart contains little of this enzyme (Bodansky, 1972).

The prostate is known as a rich source of acid phosphatase, but attempts to determine the intracellular distribution of acid phosphatase must take into account the presence of this enzyme in the extracellular secretion. Employing centrifugal methods, Siebert *et al.* (1955) found that, of the total acid phosphatase present in bull prostate homogenate, 0.7% was in the nuclear fraction, 41% in the mitochondrial fraction which presumably included the lysosomal component, and 84% in the microsomal and supernatant components. The finding that the sum of these activities exceeded that in the homogenate probably represents removal of inhibitors during separation of these fractions. Similar results have been obtained in the rat prostate (Bertini and Brandes, 1965).

Human semen is also a very rich source of acid phosphatase. It is made up of the secretory fluids produced in the epididymis, vas deferens, ampulla, seminal vesicle, the prostate and the bulbourethral (Cowper's) and urethral (Littre's) glands. It has been observed that in bull and ram semen certain lysosomal enzymes, including acid phosphatase, are sequestered into membranelike droplets which are shed from the spermatozoon during maturation but persist in the seminal plasma (Dott and Dingle, 1968).

C. Alteration of Serum Acid Phosphatase Activity in Disease

1. Normal Values for Serum Acid Phosphatase

Several methods have been employed for determining serum acid phosphatase, and even for each method slight modifications have been proposed at various times. The Gutman method (Gutman and Gutman, 1940) employs phenyl phosphate as substrate, as in the method for alkaline phosphatase (King and Armstrong, 1934); units of activity have been expressed as King–Armstrong or KA units and have been defined as the number of milligrams of phenol liberated at 37°C and pH 4.9 in 1 hour by 100 ml serum. The normal ranges by this method, or by slight modifications of it, and expressed as the mean ± standard deviation are 1.2 ± 0.39 (Gutman and Gutman, 1940); 1.8 ± 0.8 (Fish-

man *et al.*, 1956); 2.70 ± 0.57 (Day *et al.*, 1956); and 1.2 ± 1.2 (Benotti *et al.*, 1946).

Another procedure that gained wide use is that of A. Bodansky (personal communication, 1948) which depended on the hydrolysis of β-glycerophosphate for 2 hours at 37.5°C at pH 5.0. The activity was expressed in units, as the number of milligrams of inorganic phosphorus liberated as phosphate in 1 hour by 100 ml serum. The normal ranges, obtained by two different groups and again expressed as the mean ± standard deviation, are 0.19 ± 0.048 units (Bodansky and Bodansky, 1952) and 0.28 ± 0.12 units (Marshall and Amador, 1969). For convenience, these have been designated as Bodansky (B) units. Several other methods for determination of acid phosphatase activity based on the hydrolysis of other phosphoric esters are available. These include the use of *p*-nitrophenyl phosphate and the liberation of *p*-nitrophenyl (Hudson *et al.*, 1947), phenolphthalein diphosphate and the release of phenolphthalein (Huggins and Talalay, 1945), β-naphthyl phosphate (Seligman *et al.*, 1951) or α-naphthyl phosphate (Babson *et al.*, 1959), and the diazotization of the liberated naphthol.

It should be realized that, in this field of methodology as in others, the rapid onrush of automation may replace the techniques we have just described. However, much of the clinical biochemical correlations we depend on have been gained by the use of these techniques, and it is appropriate, therefore, to review briefly the findings that have been obtained in various types of human cancer.

2. Serum Acid Phosphatase Activity in Carcinoma of the Prostate

Carcinoma of the prostate is today one of the three most frequent causes of death from neoplastic disease in men in the United States (Gilbertsen, 1971). A total of 16,848 deaths from this cause was reported in 1968 (Silverberg and Holleb, 1973). Table 3-1 summarizes several studies showing that from about 75 to 90% of patients with carcinoma of the prostate and skeletal metastases have elevations of serum acid phosphatase activity. Some of these activities were sensationally high as in 5 cases with elevations greater than 1000 KA units reported by Sullivan *et al.* (1942), or as in the high level of 520 B units reported by Woodard (1952). The incidence of elevations in cases without skeletal metastases, as determined roentgenographically, but possibly with metastases to soft parts was much lower. In general, the elevations were slight. In the cases reported by Sullivan *et al.* (1942), no activity was greater than 4.0 KA units. In patients with metastases to distant soft parts, the inci-

TABLE 3-1

Incidence of Elevation of Serum Acid Phosphatase Activity in Carcinoma of the Prostate[a]

Type of extension in carcinoma of prostate	Sullivan *et al.*		Herbert		Woodard		Marshall and Amador	
	No. of patients	Incidence of elevations (%)	No. of patients	Incidence of elevations (%)	No. of patients	Incidence of elevations (%)	No. of patients	Incidence of elevations (%)
With skeletal metastases	130	85	35	89	107	74	23	83
Without skeletal metastases	70	11	47	42	—	—	—	—
With local invasion	—	—	—	—	51	31	—	—
With metastases to distant soft parts	—	—	—	—	9	78	27	56
Intracapsular	10	0.0	—	—	20	5	57	46

[a] The upper limits of normal determined and used by the various investigators were as follows: Sullivan *et al.* (1942), 3.0 KA units; Herbert (1946), 4.0 KA units; Woodard (1952), 0.98 B unit; and Marshall and Amador (1969), 0.51 B unit.

dence was substantial, 78% of 9 cases reported by Woodard (1952) and 56% in those reported by Marshall and Amador (1969). The elevations of these cases ranged up to 18.3 B units in Woodard's series (1952) and up to about 10 B units in the study of Marshall and Amador (1969).

The frequency of elevations in serum acid phosphatase activity is negligible in benign prostatic hypertrophy or prostatitis. Sullivan *et al.* (1942) found none in 75 cases of the former and none in 10 cases of the latter. Similar results have been obtained by others (Day *et al.*, 1956; Woodard, 1952).

The acid phosphatase in the serum consists of moieties from other tissues as well as the prostate. On the assumption that the prostatic moiety might be elevated in early stages of prostatic carcinoma while the total acid phosphatase activity was still normal, Fishman and Lerner (1953) applied the earlier observation of Abul-Fadl and King (1949) that L(+)-tartrate specifically inhibited prostatic phosphatase. The activity in the presence of 0.02 *M* L(+)-tartrate was subtracted from the total acid phosphatase to denote the inhibited or prostatic phosphatase in the serum. Evidence was obtained in some cases that the prostatic fraction was elevated while the total serum acid phosphatase activity was normal and that these elevations were the herald of clinically evident disease and clearly elevated total serum acid phosphatase activities (Fishman *et al.*, 1953). In a summation of their studies of 91 patients with proven prostatic carcinoma, Fishman *et al.* (1956) obtained the following incidences for the elevation of total and prostatic acid phosphatase activities: 35 and 84%, respectively, for the group as a whole; 31 and 87%, respectively, in 39 untreated cases; and 38 and 81%, respectively, in 52 treated cases. The incidence of elevations of total acid phosphatase was 25 of 53 cases, or 47%, in patients with bone metastases; 2 of 12, or 16%, in patients with soft tissue metastases; and 4 of 26, or 15%, in patients with no metastases. It is difficult to reconcile the incidences for total acid phosphatase activity in these various groups with those obtained by other investigators as listed in Table 3-1. These incidences are also much lower than the overall values obtained by Bodansky and Bodansky (1952) in a review of the literature up to 1951, according to which total acid phosphatase was elevated in 81% of 349 cases with bone metastases and in 24% of 218 cases without such metastases. Whatever the cause for the discrepancy between these results and those of Fishman *et al.* (1956) for the incidence of elevations of total acid phosphatase, it should be noted that Fishman *et al.* (1956) obtained higher values for the incidences of elevations of the prostatic moiety. When serum prostatic acid phosphatase activities were determined during the course of the patient with prostatic carcinoma, these values generally paralleled the exacerbation or remission of the disease.

In 1941, Huggins and Hodges observed that treatment of patients with metastatic carcinoma of the prostate by orchiectomy or estrogen administration lowered elevated acid serum phosphatase activity. For example, in one patient, the serum acid phosphatase decreased from 26 KA units immediately preoperatively to 5 KA units within a period of 7 days after operation. This type of observation has been amply confirmed since then (Marshall and Amador, 1969; Fishman *et al.*, 1953). However, there have been reports, particularly in more recent years, that the level of serum acid phosphatase activity may not always bear a clear relationship to the apparent clinical progress of the disease or the extent of metastases at autopsy (Bodansky, 1955). In patients who have already had therapeutic procedures such as orchiectomy and stilbestiol administration, high levels of serum acid phosphatase activity may develop and remain refractory to further treatment. On the other hand, extensive metastases may develop with relatively small rises in the serum acid phosphatase activity. Other factors may also influence the level of acid phosphatase. These include the possibility that the enzyme may not be readily released from metastases, that elevations in body temperature may increase its metabolic degradation and thus lower its level in the blood and, finally, that impaired liver function may decrease the metabolic degradation of acid phosphatase and lead to high levels (Bodansky, 1972). In evaluating elevations of acid phosphatase in patients, it is well to recognize that certain clinical maneuvers or states, such as massage, palpation, or pressure on the prostate, may result in adventitial but sudden elevations of the enzyme activity (Hock and Tessier, 1949; Daniel and Van Zyl, 1952; Bonner *et al.*, 1954).

3. Elevations of Serum Acid Phosphatase Activity in Nonprostatic Cancer and Other Diseases

Serum acid phosphatase activity has been reported to be elevated in low incidence and to a slight or moderate degree in tumors with metastases to the liver or skeleton, Paget's disease, hyperparathyroidism (Sullivan *et al.*, 1942), in thrombocytosis (Zucker and Woodward, 1962), myeloproliferative disorders (Bases, 1962), and Gaucher's disease (Tuchman *et al.*, 1956).

The elevations in these conditions are much more frequent with phenyl phosphate than with β-glycerophosphate as substrate. Thus, with the former substrate, the incidences of elevations in neoplastic disease other than prostatic cancer were 19% in patients with skeletal metastases, 2% in patients with liver involvement, 6% in those without either bone or liver involvement, and 10% in primary bone tumors. In the category of nonneoplastic disease of the bone, elevations were present in 21%

of 96 patients with Paget's disease and in 33% of 9 patients with hyperparathyroidism (Sullivan *et al.*, 1942). Using the Bodansky procedure with β-glycerophosphate as substrate, Woodard (1952) was unable to obtain any elevations in a large group of 425 patients which included such conditions as infectious or metabolic disorders and oestitis deformans, and in 332 cases of primary and metastatic neoplastic disease, other than prostatic carcinoma.

A somewhat unusual observation has appeared in the literature that the determination of serum acid phosphatase in the presence of 0.0002 *M* Cu^{2+} and with phenyl phosphate as substrate yielded about a 20% incidence of elevated activities for patients with miscellaneous diseases such as acute or chronic inflammations, osteoporosis, hypertension, and diabetes mellitus (Reynolds *et al.*, 1956). Of interest was the occurrence of a 74% incidence in female patients with metastatic carcinoma of the breast and a 46% incidence in male patients with nonprostatic metastatic carcinoma. These observations have not yet been pursued or confirmed.

The presence of acid phosphatase in human platelets (thrombocytes) has been recognized since 1953 (Alexander, 1953). It would appear that approximately 60% of the acid phosphatase in serum arises from the liberation of this enzyme from platelets as a result of clotting (Zucker and Woodard, 1962). The normal concentration of thrombocytes in the peripheral circulation is approximately 250 to 350×10^3 per mm^3, and it may be expected that the serum acid phosphatase activity would be decreased in thrombocytopenia and elevated in thrombocytosis. Zucker and Woodard (1962) reported a series of 12 patients with thrombocytopenia secondary to a variety of neoplastic conditions such as carcinoma of the breast and acute and chronic leukemia. The platelet counts ranged between 2,000 and 60,000. The mean value for the serum acid phosphatase activity was 0.123 unit, significantly less than the values of 0.226 ± 0.0126 B units for normal women and 0.278 ± 0.0270 B units for normal men. Thrombocytosis, or the occurrence of greater than the normal number of platelets in the circulation, is a manifestation of increased activity of the hematopoietic system and may occur in association with neoplastic disease. In three cases of chronic granulocytic leukemia and one case of carcinoma of the bladder, the platelet counts were greatly elevated, ranging from 680×10^3 to 2300×10^3 per mm^3. Determinations made at various times during the hospital stay and treatment of these patients ranged from 0.39 to 1.68 B units and averaged 1.00 B unit, considerably elevated above the normal values of 0.226 B unit for normal women and 0.278 B unit for normal men.

The activity of acid phosphatase and its isoenzymes has been studied

in the leukocytes of the various leukemias and lymphomas. However, we shall consider this area in conjuction with a review of other biochemical aspects of these neoplastic diseases in Chapter 9.

III. Alkaline Phosphatase

A. Introduction

In 1912, Grosser and Hussler found that several mammalian tissues, such as kidney, intestine, lung and liver, contained an enzyme capable of hydrolyzing glycerophosphate. No phosphatase activity was found to be present in skeletal muscle, cardiac muscle, or human blood. Some years later, Forrai (1923) confirmed the presence of this enzyme in human tissues, but again failed to find it in human serum. It was not until 1924 when a relatively sensitive colorimetric method for phosphate ion was available that Martland *et al.* (1924) were able to demonstrate the presence of alkaline phosphatase in human blood and serum. This finding initiated a vast series of studies into the diagnostic potentialities of this enzyme in human disease and, more generally, on its role and nature in living tissues, studies which proceed to an unabated degree to this day.

Serum alkaline phosphatase activity is elevated in two main groups of disease—those affecting the bone and the bone-forming tissues and those involving the structural and functional integrity of the liver. In the former group of disorders, the level of the serum alkaline phosphatase activity reflects the intensity of the cellular activities mobilized in laying down skeletal tissue and evoked in response to a disturbance in the equilibrium between orderly deposition and resorption of bone. In disease of the hepatobiliary tract, the level of the enzyme activity generally reflects the patency of the excretory biliary channels, either extrahepatic or intrahepatic. In rare instances, as will be noted later, the serum alkaline phosphatase activity is elevated as a result of primary neoplasms at other sites.

B. Purification and Properties of Alkaline Phosphatase

1. Purification

As we shall presently see, alkaline phosphatase may differ from tissue to tissue and, occasionally, within one tissue there may be more than

one molecular form. Many attempts have been made to obtain alkaline phosphatase in highly purified and, if possible, in crystalline form. A 10,000-fold purified product was prepared from swine kidneys (Mathies, 1958), and several years later, Malamy and Horecker (1964) obtained a crystallized preparation of alkaline phosphatase from *E. coli*. Of more relevance are the purifications and properties of alkaline phosphatase from human tissues (Smith *et al.*, 1968; Eaton and Moss, 1968; Harkness, 1968; Ghosh, 1969). Placental alkaline phosphatase was purified by procedures involving butanol treatment, precipitation and extraction with ammonium sulfate, exposure to heat, alcohol fractionation, molecular sieving with Sephadex G-200, TEAE-cellulose anion exchange chromatography, continuous curtain electrophoresis, and equilibrium dialysis. The enzyme was found to exist in more than one form separable on Sephadex gel filtration and two of these had molecular weights of 70,000 and 200,000 (Ghosh, 1969). Harkness (1968) reported that the content of zinc increased during purification of placental phosphatase, and the concentration in the crystalline preparation, which had a molecular weight of 125,000, indicated the presence of 2 or 3 atoms of zinc per enzyme molecule.

2. Isoenzymes of Alkaline Phosphatase

It has long been appreciated that the alkaline phosphatases from various tissues may differ from each other, as determined by inhibition with taurocholate (Bodansky, 1937), various amino acids (Bodansky, 1948), and stereospecifically by L-phenylalanine (Fishman *et al.*, 1963). Other procedures which serve as differential criteria are gel filtration (Estborn, 1964), heat sensitivity (Moss and King, 1962; Posen *et al.*, 1969), irreversible inactivation by urea (Butterworth and Moss, 1967), exposure to low pH (Scutt and Moss, 1968), effect of neuraminidase on electrophoretic mobility (Moss *et al.*, 1966), and immunochemical properties (Schlamowitz and Bodansky, 1959; Sussman *et al.*, 1968). Table 3-2, based on the summation by Moss (1969), shows that the phosphatases can be divided into three classes: (1) placental, (2) intestinal, and (3) nonplacental and nonintestinal phosphatases such as those from liver and bone. Within this third group, there are also some differences, but not as marked as between this group as a whole and either of the first two groups.

Some additional data in this connection are of interest. According to Posen *et al.* (1969), the extent of inactivation of alkaline phosphatases after incubation at 56°C and pH 7.4 for 15 minutes is for bone, 72–96%, with a mean of 88%; liver, 75–92%, with a mean of 84%; intestine, 44–83%,

TABLE 3-2

Differences between Three Main Categories of Human Alkaline Phosphatases[a]

Properties	Placental phosphatase	Intestinal phosphatase	Nonplacental, nonintestinal (liver, bone, etc.) phosphatases
Catalytic properties			
1. Substrate specificity	Hydrolyzes β-glycerophosphate and phenyl phosphate at similar rates (others more active on phenyl phosphate)	Hydrolyzes AMP and PP_i faster with respect to *p*-nitrophenyl phosphate than nonintestinal enzymes	Similar relative rates of hydrolysis of various substrates by enzymes within this group
2. Inhibition			
(a) By bile acids	—	Not inhibited	Inhibited
(b) By L-phenylalanine	Inhibited	Inhibited	Not inhibited
Molecular properties			
1. Resistance to denaturation			
(a) Time for half-inactivation at 55°C	Completely stable	60 minutes	Varies slightly for different phosphatases (bone 5–10, liver 30–40 minutes)
(b) Urea concentration for irreversible inactivation	8 *M*	6–7 *M*	Varies with source of enzyme (liver 3 *M*, bone and kidney <3 *M*)
(c) Exposure to low pH	—	More stable than nonintestinal enzymes	Liver phosphatase more stable than bone phosphatase.
2. Effect of neuraminidase on electrophoretic mobility	Retarded	Not retarded	Retarded
3. Immunochemical	Antigenically distinct (partial cross-reaction with intestinal enzymes)	Antigenically distinct (partial cross-reaction with placental enzymes)	Antigenically distinct from placental and intestinal enzymes. Antiserum to liver phosphatase does not cross-react with bone and kidney phosphatases

[a] From Moss (1969). Reproduced by permission of the New York Academy of Sciences.

with a mean of 68%; and placenta, 8 to —5%, with a mean of 0%. The rate of inactivation of human alkaline phosphatases in acid depends upon the pH, the extent increasing with the acidity of the reaction (Scutt and Moss, 1968). For example, liver phosphatase is completely inactivated at pH 2.6 and 0°C within 2–3 minutes, whereas only 60% is inactivated at pH 3.1 during 10 minutes. Intestinal alkaline phosphatase is more rapidly inactivated. Some degree of reversibility exists. For example, after incubation for about 1–2 minutes at pH 2.1, complete reactivation occurs at pH 7.2, whereas incubation at the low pH for about 5 minutes and reactivation at the higher pH restores the activity from 30 to 80% of the original activity. The reactivated intestinal phosphatase differs from the original in its Michaelis constants and in its inhibition constants with arsenate. In contrast, the liver enzyme resembles the original.

Schlamowitz and Bodansky (1959) prepared antibodies in the rabbit to human bone and intestinal phosphatases. Each of these antibodies precipitated under suitable conditions the homologous antigen almost completely, and cross-reacted to a very small extent, 0–4%, with the heterologous antigen. The use of antirabbit γ-globulin antibodies enhanced the degree of cross-reaction between the antibody to human bone phosphatase and the phosphatases from intestine, kidney, and liver. Using antibodies prepared in the sheep to purified human liver and the placenta, Sussman *et al.* (1968) showed that antibody to liver phosphatase and antibody to placental phosphatase were specific for their respective homologous antigens and did not react with alkaline phosphatase from bone, neutrophils, kidney, or intestine. These results indicated that there were at least three antigenic types of alkaline phosphatases: one derived from liver, one from placenta, and one or more from other organs. This immunochemical classification, it may be noted, differs from that of Schlamowitz and Bodansky (1959).

We have so far been considering the distinctions between alkaline phosphatase from different human tissues, and it is to these different tissue phosphatases that the term "isoenzyme" has usually been applied. But there are also heterogenous alkaline phosphatases within a single tissue, and these, too, may be designated as isoenzymes. Using the vertical starch gel electrophoresis method of Smithies (1959), and supernatants obtained after centrifugation at 100,000 g for 30 minutes, Hodson *et al.* (1962) obtained the following patterns: Liver alkaline phosphatase gave one heavy anionic band; the bone enzyme yielded one slower moving, fairly intense band and a second closely spaced but fainter band; intestinal phosphatase yielded one less anionic, intense band. At about the same time, Moss and King (1962) used extracts of human

tissues that had been prepared by butanol treatment and then concentrated in order to contain 400–500 KA units per 100 ml. Electrophoresis was carried out on horizontal starch gels by the method of Smithies (1959) in the discontinuous citrate–borate buffer of Poulik (1957) at pH 8.65. That liver and kidney phosphatases have three electrophoretic bands and intestinal and bone phosphatases, two bands is shown in Fig. 3-1. The bands are numbered from 1, the most anionic, to 3, the cationic band. The relationship of the alkaline phosphatase activities of the major bands to the minor ones was determined quantitatively after maceration and elution of the starch gel. L. Fishman (1974) has recently subjected alkaline phosphatase of various human tissues to acrylamide disc gel electrophoresis with Triton X-100 in the sample and the gel matrix. Although more distinct bands were obtained, the relative rates of migration were essentially the same as those obtained by Moss and King (1962) (Fig. 3-1).

The various bands within a single tissue had essentially the same stability to heat and the same Michaelis constants as determined with β-naphthyl phosphate as substrate. Moss and King (1962) suggested that the main phosphatase zone probably corresponds to a free enzyme protein and that the subsidiary zones represent moieties whose electrophoretic mobility has been modified in some way. Factors which may influence the mobility of the enzyme protein molecule may include aggregation or disaggregation, modifications of the molecule by postmortem autolysis, and attachment of the phosphatase protein to sialic acid.

The enzyme neuraminidase splits off sialic acids which are ubiquitously distributed in tissues and have been identified as constituents of lipids, polysaccharides, and mucoproteins. Sialic acids occur as *N*- and *O*-acyl derivatives of a 9-carbon, 3-deoxy-5-amino sugar called "neuraminic acid." Neuraminidase treatment was found to lower the electrophoretic mobility of liver and kidney but not that of intestinal phosphatase (Moss *et al.*, 1966; Butterworth and Moss, 1966). Treatment with neuraminidase reduced the mobility of the faster of two electrophoretic components of placental alkaline phosphatase (Fishman *et al.*, 1968a).

3. Kinetics

As is to be expected, the Michaelis constant, K_m, varies with several factors, including the nature of the substrate and the pH. It is, therefore, of little value to make any exhaustive comparisons between the results of different investigators. Using β-naphthyl phosphate as substrate and the optimal pH for each isoenzyme, Moss and King (1962) found little

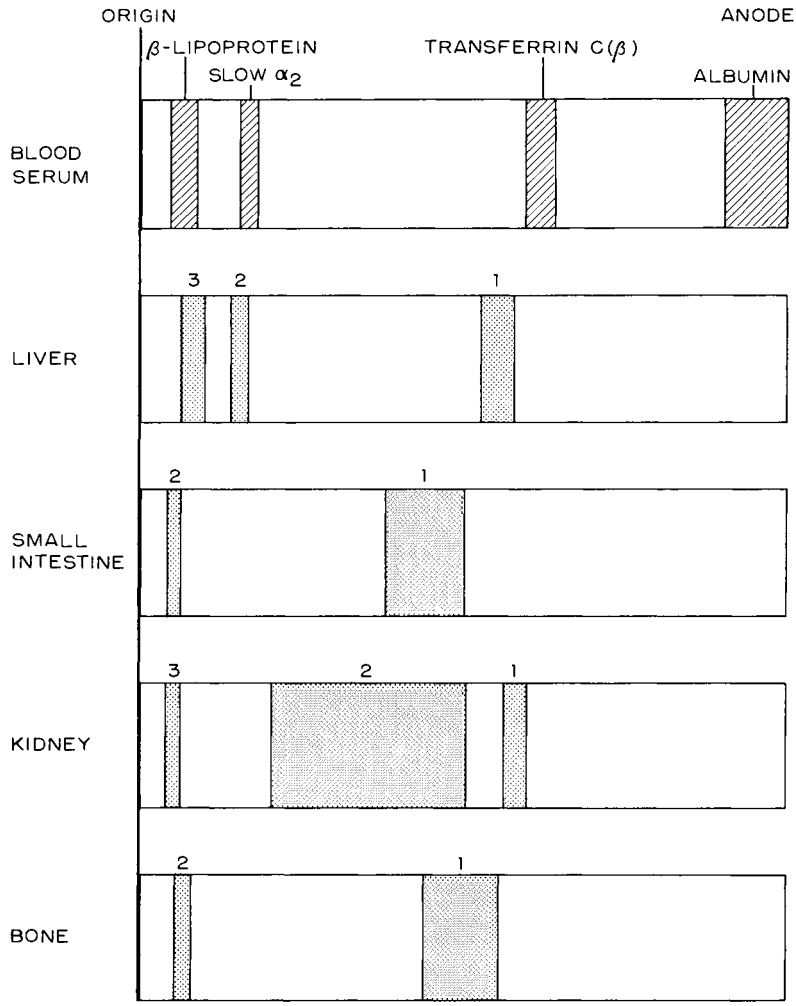

Fig. 3-1 Location of alkaline phosphatase fractions after starch gel electrophoresis of concentrated butan-1-ol extracts, compared with that of principal protein fractions of normal blood serum. From Moss and King (1962). Reproduced by permission of The Biochemical Society. Diagonally shaded areas represent serum proteins. Dotted areas represent bands of alkaline phosphatase activity.

variation between the K_m values for the electrophoretic bands of a particular tissue phosphatase. The average K_m values for the different human tissue isoenzymes, expressed as mM, were bone, 0.114; liver, 0.068; kidney, 0.101; and intestine, 0.094. With β-glycerophosphate as substrate and a reaction pH of 9.3–9.5, the K_m values were of a different order

of magnitude: bone, 0.9 m*M*; liver, 0.6 m*M*; and intestine, 1.2 m*M* (Bodansky and Schwartz, 1964). The K_m values for placental alkaline phosphatase with phenyl phosphate as substrate has been variously given as 1.2 m*M* at pH 10 (Ahmed and King, 1960) and 2.3 m*M* at pH 9.8 and 17.0 m*M* at pH 10.6 (Fishman *et al.*, 1968a).

4. Isoenzymes in Serum

In 1962, Hodson *et al.* were able to visualize the presence of various zones of alkaline phosphatase activity upon electrophoresis of normal human serum. One anionic band, corresponding to the mobility of the liver enzyme, was present in each serum. But there were also sera which revealed, in addition, the presence of a slower moving and less anionic band close to the liver band and corresponding to a bone alkaline phosphatase, and still other sera in which there was a heavy slower moving band, corresponding to the band for intestinal alkaline phosphatase. These findings were soon followed by the observation that the distribution of these bands was influenced by genetic factors, and the association between blood group types and serum alkaline phosphatase isoenzymic phenotypes was determined (Arfors *et al.*, 1963; Walter *et al.*, 1970).

The patterns of the serum alkaline phosphatase isoenzymes, as determined by acrylamide and starch gel electrophoresis, have recently been summarized in a group of 56 normal individuals (Green *et al.*, 1972). Of 50 individuals for whom starch gel electrophoretic patterns were available, 10 had two bands for the liver and bone isoenzymes, and 40 had three bands for liver and intestine. Acrylamide gel electrophoresis separated bone and liver isoenzymes more effectively but did not elicit the intestinal bands as well. Of 48 for whom this type of electrophoresis was performed, 46 had two bands, bone and liver, and only two had a liver–bone–intestine pattern. The liver position of the enzyme in every subject was seen as the fastest moving compact band and had relatively low heat inactivation, whereas the bone band was slower, less compact, and had a high heat inactivation.

It will be recalled that the purification of human placental phosphatase revealed the presence of two isoenzymes (Ghosh, 1969). Neither of these is seen in the serum of the normal male or the nonpregnant female. The pattern of appearance of these bands in the serum during pregnancy and after parturition has been studied by Fishman and his associates (1972).

A fairly comprehensive scheme for the quantitative determination of alkaline phosphatase isoenzymes from bone, liver, intestine, and placenta in serum has been presented by Fishman (1969). This includes the

determination of the "Regan" isoenzyme which, as we shall presently note, is found in the serum of patients with cancer. The activity of one aliquot of serum is determined at pH 9.8, with 18 m*M* phenyl phosphate as substrate and 0.005 *M* L-phenylalanine. The subtraction of this activity from the control determination with 0.005 *M* D-phenylalanine represents the L-phenylalanine sensitive alkaline phosphatase (LPSAP). A second aliquot is heated for 16 minutes at 55°C prior to measurement at pH 9.8 with 18 m*M* phenyl phosphate, again in the presence of D- and L-phenylalanine. The bone isoenzyme is completely inactivated by the heating whereas, as can be seen from Table 3-3, liver and intestine are only partially inactivated. Placental and Regan isoenzymes are unaffected. A third aliquot of serum is heated for 5 minutes at 65°C to destroy the isoenzymes other than placental and Regan. Suitable calculations permit the estimation of each moiety.

C. Alteration of Serum Alkaline Phosphatase in Cancer

1. Normal Values for Total Serum Alkaline Phosphatase

Most of the data dealing with total serum alkaline phosphatase activity in cancer have been gathered by the use of the method of A. Bodansky (1933) and of King and Armstrong (1934) or of slight modifications of these procedures. The units in the method of A. Bodansky (1933) are equivalent to the milligrams of inorganic phosphorus liberated as phosphate from a standard β-glycerophosphate–diethylbarbiturate buffer mixture by 100 ml serum in 1 hour at 37°C. The pH was originally stated to be 8.6 by a colorimetric method, but is actually 9.1–9.2, as determined electrometrically (Bodansky, 1961). The mean normal values by this method are 2.6 ± 0.6 (SD) units in adults and 7.7 ± 2.2 (SD) in children. The units in the King–Armstrong method are equivalent to the milligrams of phenol liberated from a standard phenyl phosphate–diethylbarbiturate buffer mixture, pH 9.0, by 100 ml serum in 30 minutes at 37°C. The mean normal value in adults by the King-Armstrong method is 7.8 ± 2.2 (SD) units (Bodansky and Bodansky, 1952).

In recent years, the serum alkaline phosphatase method has been automated, and it has become customary to express the unit of activity in terms of moles of substrate cleaved per unit of time under stated conditions. Thus, a current automated method which utilizes nitrophenyl phosphate as substrate expresses the units of activity as the number of micromoles of phenol liberated per minute per liter of serum. The

TABLE 3-3

Biochemical and Immunological Characteristics of Various Human Isoenzymes of Alkaline Phosphatase[a]

Conditions for alkaline phosphatase measurement	Phenyl phosphate (m*M*)	18	18	72		
	pH	9.8	9.8	10.7		
			Heat inactivation			
Tissues		% Inhibition by L-phenylalanine	16 minutes at 55°C (%)	5 minutes at 65°C (%)	Order of anodal migration on starch gel	Reaction with antiserum to placental isoenzyme SA20
Liver		7.7	50–70	100	1	0
Bone		10.3	90–100	100	2	0
Intestine		77.0	50–60	100	4	0
Placenta		79.0	0	0	3	+
Regan isoenzyme		79.0	0	0	3 (diffuse)	+

[a] From Stolbach (1969). Reproduced by permission of the New York Academy of Sciences.

normal range is 57 ± 13 (SD) units, about 20-fold the magnitude of the normal range expressed in Bodansky units (1972).

2. Elevation of Total Serum Alkaline Phosphatase Activity in Cancer

Most of the early and now classic studies on the alteration of serum alkaline phosphatase activity in disease were performed by the method of A. Bodansky (1933). The upper limit of normal by this method may be considered as 3.8 units, or two standard deviations above the mean value (Bodansky and Bodansky, 1952). Elevations of serum alkaline phosphatase activity are observed in two large classes of neoplastic disease—those affecting the skeletal system and those affecting the liver. Table 3-4 summarizes the ranges of values observed in a series of neoplastic diseases. A few of these may be more fully illustrated.

Intrahepatic metastases arise with the following frequencies from primary sites in other tissues: breast, 61%; lung, 40%; stomach, 45%; ovary, 52%; kidney, 27%; pancreas, 63%; prostate, 22%; and colon, 55% (Abrams

TABLE 3-4

Serum Alkaline Phosphatase Activities in Neoplastic Disease[a]

Condition	Mean (BU)[b]	Range (BU)	Percent abnormal: Mildly elevated 4–12 BU	Percent abnormal: Markedly elevated >12 BU
Normal adults	2.6	1.4–3.8	—	—
Normal children	7.7	3.3–12.1	—	—
Intrahepatic metastases	9.9	2.1–65	49	37
Malignant biliary obstruction	14.0	3.9–70	21	76
Hyperparathyroidism with skeletal changes	23	6–45	29	71
Osteogenic sarcoma in adults >16 years	8.3	2.1–28.5	69	15
Bone metastases from carcinoma of breast	10.8	3.3–38	58	25
Bone metastases from carcinoma of prostate	20.7	3.4–147	—	89[c]
Multiple myeloma	3.6	1.7–5.2	46	0

[a] From summary of literature by Bodansky (1962; 1965).
[b] Bodansky units.
[c] Includes some mildly elevated values.

et al., 1950). Of a series of 160 cases with primary cancer at these sites and without clinical evidence of icterus or any evidence of bone disease, 99 had intrahepatic metastases, as proved by direct visualization and biopsy, autopsy, or needle biopsy. The remaining 61 cases served as controls. The group with intrahepatic metastases had serum alkaline phosphatase values ranging from 2.1 to 65 B units (Mendelsohn and Bodansky, 1952) with a mean of 9.9 units and with 78% exceeding the upper limit of normal. In contrast, the values in the control group of cancer patients without metastases ranged from 1.7 to 5.4 units, with an average of 3.1 units and with only 10% of the cases exceeding the upper limit of normal.

Gutman (1959) gathered from the literature 150 cases of obstruction of the extrahepatic biliary tract by carcinomas of the pancreas, common bile duct, or gallbladder. In 141 cases, or 94%, serum alkaline phosphatase levels were in excess of 10 Bodansky units or 30 KA units. Complete and protracted occlusion of the common bile duct consistently leads to pronounced rises in this serum enzyme activity.

The skeleton is also a common site for metastases from tumors primary in other tissues. Approximately 75% of carcinomas of the breast and about 65% of carcinomas of the prostate metastasize to bone (Bodansky, 1961). The large majority of metastases from breast cancers are osteolytic, with very little admixture of an osteoblastic process. Most of the serum alkaline phosphatase levels in these cases are, therefore, only slightly elevated (Table 3-4). Griboff and his associates (1954) noted an inverse relationship between changes in the serum calcium and serum alkaline phosphatase levels; decreases in the latter frequently heralded the onset of hypercalcemia and exacerbation of the disease. This observation is in agreement with the concept that in the absence of hepatic involvement an elevated serum alkaline phosphatase reflects some degree of an osteoblastic process and that the return of this enzyme activity toward normal levels represents abatement of this process with increasing osteolysis, increased urinary excretion of calcium, and a rise in the serum calcium.

The majority of metastatic bone lesions associated with carcinoma of the prostate are osteoblastic as judged both roentgenographically and upon microscopic examination of autopsy material (Elkin and Mueller, 1954). Although values as high as 147 B units have been recorded, they usually range from 15 to 40 units. Table 3-4 shows a mean value of 20.7 units. The fluctuations in any particular case are dependent upon several factors such as the degree of hepatic involvement, the course of the disease, and the effect of treatment. Detailed examination of reports which contain sequential data reveals that a favorable effect following orchiectomy or treatment with estrogens is frequently associ-

ated with an initial rise in serum alkaline phosphatase activity, followed by a steady decrease toward normal levels (Bodansky, 1961).

Elevations of serum alkaline phosphatase activity also occur in nonneoplastic hepatobiliary disease such as infectious and serum hepatitis, drug-induced hepatotoxicity, xanthomatous biliary cirrhosis, and nonicteric granulomatous and infiltrative diseases. Nonneoplastic diseases of the skeleton are also characterized by elevations of serum alkaline phosphatase activity. Some extremely and even sensationally high levels have been recorded in marked infantile rickets and in advanced, widespread Paget's disease (Gutman, 1959). Although the kidney and the intestinal epithelium are also rich sources of alkaline phosphatase, there seem to be few reported instances of diseases of those organs that are characterized by marked or moderate elevations of this enzyme activity and which cannot be elucidated by an effect on either the skeletal or the hepatobiliary systems. Such elevations have been reported in chronic ulcerative colitis (Boden *et al.*, 1959), congestive heart failure (Felder *et al.*, 1950), and pulmonary infarction (Nikkilä, 1959).

3. Serum Alkaline Phosphatase Isoenzymes in Cancer

Less distinctive than finding an isoenzyme in serum that is characteristic of, and specific for, cancer is the attempt to determine whether the isoenzyme composition of serum alkaline phosphatase is altered in cancer. Fennelly and his associates (Dunne *et al.*, 1967; Fennelly *et al.*, 1969) employed several techniques in the attempt to explore this aspect. The first was that of Flodin and Killander (1962) who found that fractionation of human serum on Sephadex G-200 led to elution of three main groups of proteins in order of decreasing molecular size. These were designated as peaks A, B, and C, respectively, by Fennelly *et al.* (1969).

The mean values and standard deviations for the percentage of phosphatase in the 19 S peak (peak A) in the various groups of patients were as follows: 11 controls, 1.73 ± 2.8; 11 patients with nonmalignant skeletal disease, 1.4 ± 2.04; 11 patients with metastatic skeletal disease, 6.4 ± 5.73; and 13 patients with metastatic liver disease, 36.2 ± 11.8 (Fennelly *et al.*, 1969). Statistical evaluation of these data showed significant differences between the values for the nonmalignant and metastatic skeletal disease ($p < 0.05$) and also between the nonmalignant and metastatic liver disease ($p < 0.01$).

In the studies of Fennelly *et al.* (1969), the mean values and the standard errors for the residual serum alkaline phosphatase activities after treatment with 2 *M* urea were $32 \pm 2.4\%$ in 19 controls; $21.6 \pm 4.4\%$

in 8 patients with nonneoplastic bone disease; 31.8 ± 2.5% in 12 patients with neoplastic metastatic bone disease; 45 ± 1.8% in 13 patients with nonneoplastic liver disease; and 44.8 ± 2.1% in 16 patients with neoplastic metastatic liver disease. There were no statistically significant differences between neoplastic and nonneoplastic disease of the liver or of the skeleton. The residual values for both groups of patients with liver disease were significantly higher than those with bone diesease as well as those in the control group.

As was noted in Table 3-3, 0.005 *M* L-phenylalanine inhibits the various alkaline phosphatase isoenzymes to the following extent: liver, 7.7%; bone, 10.3%; intestine, 77.0%; placenta, 79.0%; and Regan isoenzyme, 79.0%. In 1965, Fishman *et al.*, utilizing the inhibition by L-phenylalanine, determined the mean value for the proportion of intestinal-like phosphatase in the serum of 64 patients with cancer to be 21.4%, but not significantly lower than the mean value of 39.3% in 36 normal persons. The mean value in 33 cases of hepatic cirrhosis was 44.5%, significantly higher than the values for normal persons and for patients with cancer. It may be realized that the calculations upon which these values were based did not take into account the approximately 8–10% inhibition of the bone and liver phosphatase isoenzymes. The existence of the placental-like or Regan isoenzyme was not recognized at that time, but if it were present in any of the sera of patients with cancer, it would have been analyzed as the intestinal fraction.

In 1968, Fishman *et al.* (1968c) reported that the serum of a patient with bronchogenic carcinoma contained a placental-like isoenzyme that was also found at autopsy to be present in high concentrations in the tumor and its metastases. As we have already noted (Table 3-3), the tumor phosphatase was inhibited to the extent of about 75% by 0.005 *M* L-phenylalanine, was not inactivated by heating at 55°C for 16 minutes, and resembled placental alkaline phosphatase with regard to electrophoretic migration to the anode. The total serum alkaline phosphatase in this patient showed the same characteristics, though obviously not to the same degree, because of admixture with other isoenzymes in the serum. Fishman designated this isoenzyme, in accordance with the name of the patient, as the Regan isoenzyme and soon reported its presence in serum in an additional 16 patients (Fishman *et al.*, 1968b). The existence of similar placental-like isoenzymes of alkaline phosphatase in tumor tissue and serum has since been reported in a patient with pleural carcinoma (Nakayama *et al.*, 1970) and in a case of a tumor of the bile duct (Jacoby and Bagshawe, 1971). The production of these placental-like phosphatase isoenzymes by the tumor may be considered as an instance of the derepression of the genome of the cancer cells,

a mechanism that has been frequently invoked to explain the ectopic polypeptide production in cases of human cancer (Fishman *et al.*, 1968c) or the occurrence of α-fetoprotein in hepatocellular cancer (Warnock and Reisman, 1969).

The method of determination of Regan isoenzyme has previously been indicated. In a series of 520 cases of cancer, Stolbach (1969) reported the occurrence in 17 patients with this isoenzyme in serum, or an incidence of 3.3%. The levels of Regan isoenzyme activity ranged from 0.14 to 42.3 placental isoenzyme units, where this unit is somewhat different from the KA unit, being equal to the milligrams of phenol liberated by 100 ml of serum at 37.5°C from a standard phenyl phosphate buffer at pH 10.7 in only 15 minutes (Fishman *et al.*, 1972). Indeed, one KA unit is equivalent to 3.53 placental units. Although, in general, high levels of Regan isoenzyme were frequently associated with high total alkaline phosphatase levels, there was no necessary connection. For example, one patient with an adenocarcioma of the colon and a total serum alkaline phosphatase level of 80 KA units had a Regan isoenzyme component of only 0.29 placental isoenzyme units. In the 6 patients with normal total alkaline phosphatase activity, the level of Regan isoenzyme ranged from 0.14 to 0.8 placental units. In a study of 323 patients with 30 different types of cancer, Nathanson and Fishman (1971) found positive identification of the Regan isoenzyme in serum in 39 patients, or 12% of the total. The incidences in various types were as follows: 5 of 23, or 22%, in carcinoma of the ovary; and 7 of 51, or 14%, in bronchogenic carcinoma. The question naturally arises whether the Regan isoenzyme is also found in patients with nonneoplastic disease. In a series of 81 patients with nonmalignant disease and elevated total serum alkaline phosphatase activity, Regan isoenzyme was present in the sera of 9 patients or 11% of the group. These included 2 of 8 patients with Laennec's cirrhosis, 2 of 4 patients with ulcerative colitis, 3 of 7 patients with peripheral vascular disease, and 1 of 2 patients with hydronephrosis. It has been suggested that the positive findings in cirrhosis and ulcerative colitis might be heralds of a predisposition to cancer in these conditions (Nathanson and Fishman, 1971), but it is difficult to account for the positive findings in peripheral vascular disease and hydronephrosis on such a basis, or for a similar low incidence of findings in patients with manifest cancer. Obviously, more extensive studies are necessary to determine the specificity of the Regan isoenzyme in cancer.

In connection with this problem, Cadeau *et al.* (1974) have recently shown that the incidence of increased serum placental-like phosphatase activities in patients with cancer was not significantly greater than in patients with non-neoplastic disease. However, there was a significantly

increased incidence, about 15%, in females with genitourinary and breast tumors that were active at the time of the study.

IV. 5′-Nucleotidase

A. Introduction

In the course of studying the deamination of nucleotides in heart and skeletal muscle in 1934, Reis raised the question whether the liberation of phosphate from nucleotides resulted from the action of nonspecific phosphatase or whether a special group of nucleotidases was responsible for this cleavage. Subsequent studies established, with a high degree of probability, the existence of an enzyme in animal and human tissues that was capable of hydrolyzing 5′-nucleotides, such as 5′-adenosine monophosphate (5′-AMP) and 5′-inosine monophosphate (5′-IMP), much more rapidly than other phosphoric esters, such as 3′-adenosine monophosphate (3′-AMP) or β-glycerophosphate (Bodansky and Schwartz, 1968). This enzyme, 5′-nucleotidase, is widely distributed, being present in bacteria, plants, and many animals, including human tissues. The current systematic designation is 5′-ribonucleotide phosphohydrolase, EC 3.1.3.5. We shall use the recommended trivial name, 5′-nucleotidase.

B. Preparation and Properties

1. Purification

5′-Nucleotidase has been purified 5000-fold from *E. coli*, 50-fold from bull seminal plasma, 64-fold from bovine pituitary gland, twofold from calf intestine (Bodansky and Schwartz, 1968), and tenfold from human liver (Song and Bodansky, 1966).

2. Kinetics

The kinetics of 5′-nucleotidase from bull semen are of considerable interest. In the presence of 0.01 *M* Mg^{2+} and at 37°C, two pH optima, one at pH 7.5 and a second at pH 9.2–9.4, characterize its action on 5′-AMP. In the absence of Mg^{2+}, only one pH optimum at about 7.5 is present (Bodansky and Schwartz, 1968). In the presence of Mg^{2+}, the appearance of the second optimum at about 9.0–9.5 is dependent on the temperature of the reaction. It is absent at 10°C, becomes slightly

discernible at 25°C, and increasingly evident at 37°C and temperatures up to at least 50°C (Levin and Bodansky, 1966). The double pH optimum was present with guanosine 5′-phosphate (5′-GMP) and inosine 5′-phosphate (5′-IMP) as substrates. Only one pH optimum, that at pH 9.0–9.3, was present with deoxyadenosine 5′-phosphate (dAMP), deoxyguanosine 5′-phosphate (dGMP), and pyrimidine 5′-nucleotides as substrates. A mechanism for these actions in terms of binding sites was proposed by Levin and Bodansky (1966).

The values for the Michaelis constant, K_m, were generally of a low order of magnitude, ranging from about 1.8 to 12.0×10^{-5} M for the various ribonucleotides at optimal pH. There was some dependence on the particular pH optimum, 7.5 or 9.2, and on whether Mg^{2+} was absent or present. With the deoxyribonucleotides as substrates, the K_m values were substantially higher. The Michaelis–Menten equation for K_m can be cast into a mathematical form which expresses explicity the velocity at any time, t, in terms of K_m, the maximal velocity, V_{max}, and the concentration of substrate at that time, t (Dixon and Webb, 1958). For enzymes with very low values for K_m, as is the case with 5′-nucleotidase, the reaction velocity remains constant and the reaction is of zero order for a considerable portion of the reaction, namely, about 60–70%.

Relatively little work has been done on human 5′-nucleotidases. However, since serum 5′-nucleotidase activity appears to be elevated rather specifically in diseases of the liver, the enzyme from this organ has received some attention (Song and Bodansky, 1966). It resembled the kinetic properties of the bull seminal plasma 5′-nucleotidase very closely with respect to the presence of a double pH optimum, the pattern of hydrolysis of various nucleotides and other organic phosphate esters, and the existence of a relatively low K_m value for the hydrolysis of AMP.

3. Isoenzymes

There is little evidence for the existence of isoenzymes in 5′-nucleotidase. The possibility that the double pH optimum characteristic of bull seminal plasma 5′-nucleotidase might represent isoenzymes was explored. Fractional ammonium sulfate precipitation, starch paste electrophoresis, column chromatography with diethylamino cellulose, alternate freezing and thawing for 10 times, denaturation with guanidine and heating at 64° to 65°C for various periods of time failed to result in any resolution (Levin and Bodansky, 1966). More recently, it was reported that a preparation of 5′-nucleotidase from smooth muscle of small intestine had three pH optima (Burger and Lowenstein, 1970), and efforts to resolve this preparation into isoenzymes, each with a particular

pH optimum, were also unsuccessful. No information appears to be available at present to determine whether, like alkaline phosphatase, 5′-nucleotidases of different human tissues differ sufficiently from each other to warrant the designation of isoenzymes.

C. *Alteration of Serum 5′-Nucleotidase Activity in Cancer*

1. Normal Values for Serum 5′-Nucleotidase

Several procedures for the determination of serum 5′-nucleotidase have been utilized in order to determine the alterations in the activity of this enzyme in disease. In the method of Dixon and Purdom (1954), a unit of 5′-nucleotidase activity was defined as equivalent to the liberation in 1 hour at 37°C of 1 mg phosphorus as inorganic phosphate by 100 ml serum from a 2.4 m*M* AMP solution, buffered at pH 7.4. Correction was made for liberation of phosphorus by nonspecific phosphatase by determining the extent of action on β-glycerophosphate at pH 7.4. The normal range was 0–1.6 units. Kowlessar *et al.* (1961) attempted to correct for the effect of nonspecific phosphatase by introducing Mg^{2+} in order to give a final concentration of 3 m*M* Mg^{2+} in the β-glycerophosphate reaction mixture and 2.4 m*M* in the AMP reaction mixture. The unit of activity was defined in the same manner as Dixon and Purdom (1954). The mean value of serum 5′-nucleotidase activity in a group of 65 normal persons was 1.0 ± 0.3 (SD).

Campbell (1962) proposed a procedure that was dependent upon the difference in the rates of action of serum on AMP at pH 7.5 in the absence and presence of Ni^{2+}. Mn^{2+} was also present to act as activator. The reactions were carried out at 37°C, and the units of activity were expressed as micromoles of substrate utilized per minute per liter of serum. The normal range was 2–12 units. The procedure was modified slightly by Schwartz and Bodansky (1965) who obtained a range in 15 presumably normal blood bank donors of 3.2–11.6 units with a mean of 7.1 ± 2.4 (SD) units.

Several modifications of the preceding methods or new procedures have been proposed (Persijn *et al*, 1969; Belfield and Goldberg, 1969). These are based on the inclusion of a large excess of phenyl phosphate or β-glycerophosphate in the reaction mixture to compete with AMP for action by nonspecific alkaline phosphatase. Adenosine deaminase is also included in the reaction mixture so that the adenosine liberated by the action of 5′-nucleotidase is converted to inosine. The rate of reaction is measured by following the decrease in absorbance at 265 nm.

2. Elevations of Serum 5′-Nucleotidase Activity in Cancer

Most of the studies so far performed indicate that serum 5′-nucleotidase is elevated in patients with hepatic disease, but is not affected in those with skeletal disease (Dixon and Purdom, 1954; Kowlessar *et al.*, 1961). Very rarely are there slight elevations in Paget's disease (Schwartz and Bodansky, 1965) fractures and even in patients with cardiovascular, gastrointestinal, endocrine, pulmonary, and other diseases (Eschar *et al.*, 1967). However, it is possible that these elevations reflect slight and unrecognized hepatic involvement. Table 3-5 shows that serum 5′-nucleotidase is elevated in neoplastic hepatobiliary disease but not in neoplastic skeletal disease. It may be seen that the elevations in hepatobiliary disease, whether neoplastic or nonneoplastic, are generally of the same order of magnitude. Thus, in the study by Kowlessar *et al.* (1961), a group of 24 patients with acute viral hepatitis had a range of 1.5–19.3 units, as compared with a range of 0.4–2.0 units in normal individuals. A group of 18 patients with biliary cirrhosis had a range of 6.1–49.3 units and a mean of 23.8 ± 14.8 (SD) units.

The elevation of serum 5′-nucleotidase activity in neoplastic hepatobiliary involvement has received further detailed consideration. This

TABLE 3-5

Serum 5′-Nucleotidase Activities in Skeletal and Hepatic Neoplastic Disease

Investigator	Group		5′Nucleotidase (units)[a]	
	No.	Description	Mean ± SD	Range
Kowlessar	65	Normal subjects	1.0 ± 0.3	0.4–2.0
et al.	21	Neoplasia with skeletal metastases	0.9 ± 0.24	0.2–1.6
	36	Neoplasia metastatic to liver	12.5 ± 6.1	3.3–36.1
Dixon and	54	Miscellaneous controls	—	0–1.6
Purdom	13	Osseous disease	—	0–1.6
	22	Hepatobiliary disease	—	1.7–36
Belfield and	250	Normal males	5.4 ± 4.4	0–24
Goldberg	267	Normal females	5.0 ± 4.8	0–25
	16	Metastatic bone disease	10 ± 11	0–37
	32	Hepatic metastatic disease	67 ± 139	0–800

[a] The units in the procedures of Dixon and Purdom (1954) and of Kowlessar *et al.* (1961) have been previously defined. The units for 5′-nucleotidase employed by Belfield and Goldberg (1969) are equivalent to nanomoles AMP hydrolyzed per minute per milliliter serum under their stated conditions.

enzyme activity was elevated in 27 of 31 patients with cancer studied by Schwartz and Bodansky (1965); 4 patients had no elevation of serum 5′-nucleotidase and slight or moderate elevation of serum alkaline phosphatase activity. Skeletal survey showed lesions which were chiefly osteolytic. Slight to moderate enlargement of the liver was present in 2 of the 4 cases. Autopsy was performed in 2 of these cases, and 1 had liver metastases. The slightly to moderately elevated serum alkaline phosphatase activities might have reflected some osteoblastic component in the skeletal involvement or some minimal liver involvement. In other words, normal serum 5′-nucleotidase values could be obtained in cases with hepatic involvement. Four of the 31 patients had very high serum 5′-nucleotidase values, ranging from 168 to 389 units, and moderate to high serum alkaline phosphatase activities. Smith *et al.* (1966) found elevated serum 5′-nucleotidase activities in all of a series of 11 patients with proven tumor of the liver. The serum alkaline phosphatase was elevated in only 6 of these patients, and the extent of elevation in these cases was of a lower order of magnitude than the increase in the serum 5′-nucleotidase activity. It would appear, therefore, that 5′-nucleotidase is a fairly specific indicator of hepatic disease and that, in neoplastic hepatic disease, when skeletal lesions are absent, it is frequently a more sensitive indicator than serum alkaline phosphatase.

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4

Miscellaneous Enzymes in Human Cancer

I. Introduction

This chapter is concerned with a discussion of those enzymes which, although belonging to metabolic sequences other than those which we discussed in the preceding chapters, have been reported to be elevated in the serum or urine of patients with cancer or other diseases. Some of these, when first introduced, held out considerable promise as aids in the diagnosis and management of patients with cancer, although subsequent work lessened their impact. We shall describe these in some detail. In addition, we shall note more briefly other enzymes whose usefulness has been explored.

II. Amylase

A. Introduction

Amylase exists as several types, the α-amylase, the β-amylase, and the glucoamylase (International Union of Biochemistry, 1965). The one that is of interest here is the α-amylase which has been designated more precisely as α-1,4-glucan 4-glucoanohydrolase (EC 3.2.1.1) and splits α-1,4-glucosidic bonds of the polysaccharide molecule. Starch, which

is the usual substrate, is hydrolyzed to maltose. The action is manifested in several successive changes: a decrease in the viscosity, indicating the splitting of the carbohydrate chain; loss of the capacity of amylase to give a blue color with iodine; and appearance of reducing groups. These changes have been utilized in developing methods for assay of amylase activity (Somogyi, 1938; Van Loon *et al.*, 1952; Babson *et al.*, 1970). That of Somogyi (1938) was used most extensively in exploring the diagnostic role of amylase in human disease, including cancer. The activity was expressed as Somogyi units, and these were defined as the number of milligrams of reducing substance liberated from the standard starch mixture by 100 ml serum in 30 minutes at 40°C. The normal value is 105 ± 26 (SD) units (Bodansky and Bodansky, 1952).

B. Amylase Activity of Tissues

Although no complete analysis of human tissues for amylase is available, several studies on animals show that the enzyme activities of the salivary gland and pancreas are extremely high, as compared with those of other tissues (Wiberg and Tuba, 1955; McGeachin *et al.*, 1958). For example, if the activity of the rat pancreas is set at 100, the relative activities for other tissues in the rat are liver, 0.07; duodenum, 2.3; kidney, 0.03; muscle, 0.01; and lung, 0.02 (McGeachin *et al.*, 1958). In spite of the high concentrations of amylase in the parotid gland and pancreas, several older as well as recent studies have shown that extirpation of these organs does not result in any lowering of the normal serum and urinary amylase activity. Nothman and Callow (1971) recently confirmed this in man after subtotal pancreatectomy and in dogs after complete pancreatic extirpation. When the pancreatic ducts were ligated, urinary and serum amylase activity became markedly elevated. Hepatectomy resulted in a transient rise of the serum amylase during the first few hours, succeeded by a decrease to about half the preoperative level. These results point to the liver as a major source of serum amylase in the normal individual.

C. Alterations of Serum Amylase Activity in Cancer and Other Diseases

Most of the alterations in serum amylase activity are associated chiefly with nonneoplastic diseases of the pancreas, the liver, and the kidney. In 1941, Heifetz *et al.* reported a series of 36 patients with acute pancreatitis; only three had values below 200 Somogyi units, the upper limit of normal; most had values above 500 units, and the highest was 3600

units. The rises to very high levels may be sudden, and the recession to normal levels may be rapid (Elman, 1942). Chronic recurrent pancreatitis is associated frequently with serum amylase elevations of modest degree (Muether and Knight, 1949), but these may be elevated to very high levels, above 1000 units, during exacerbations. Elevations may occur in a small percentage of other intraabdominal diseases such as perforated gastroduodenal ulcers and intestinal obstruction (Burnett and Ness, 1955). It has been postulated that in these condition amylase leaks out from the intestinal lumen into the peritoneal cavity and is then absorbed into the circulation. Liver disease may be characterized frequently by low levels of serum amylase (Gray *et al.*, 1941; Bhutta and Rahmann, 1971). Renal impairment, particularly when associated with azotemia, has been repeatedly, though not invariably, characterized by moderate elevations of serum amylase activity (Heifetz *et al.*, 1941). Berk *et al.* (1970) have reported a series of 20 cases of miscellaneous disease with persistently elevated serum amylase activity, normal renal function, and normal urinary amylase activity. For example, in one case, the serum amylase activity was determined 45 times during a period of 3 years and 8 months and ranged, during this period, from 200 to 3440 Somogyi units. This condition has been designated as "macroamylasemia" and has been postulated to result from the presence of a macromolecular complex of amylase, which is not readily filtered by the kidney.

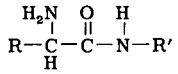
Alterations of serum amylase activity in neoplastic diseases, unassociated with the conditions we have been describing, are rare but may nonetheless be striking. In 7 cases of carcinoma of the pancreas studied by Heifetz *et al.* (1941), only 3 had definitely elevated serum amylase activities, 2 between 200 and 499 Somogyi units and 1 higher than 500 units. Many carcinomas of the pancreas cause obstructions of the pancreatic duct, but obstruction without disruption of the ductules and acinar cells would not lead to high serum amylase levels (Grossman, 1955; Byrd and Sawyer, 1957). Comfort and his associates (1943) reported a patient with carcinoma of the pancreas and extensive metastases who had values rising from 4,000 to 25,600 units. Examination of the autopsy specimen showed that a large, firm nodular acinar cell carcinoma compressed and partially obstructed the common bile duct and the duct of Wirsung, and the exceedingly high values may have resulted from excessive production of amylase. Weiss *et al.* (1951) studied a patient with bronchogenic carcinoma in whom the serum amylase ranged spectacularly from 5,450 to 16,000 Somogyi units. No basis was found for these values. The serum amylase activity was 246 units in another case of bronchogenic carcinoma studied by these investigators, and less than 102 units in 3 other cases. Additional instances of pronounced hyper-

amylasemia in patients with carcinoma of the lung have continued to be described since the report by Weiss *et al.* (1951), most recently by Ammann *et al.* (1973). The latter observed that the mobility pattern, obtained by ion exchange chromatography, of the amylase in the serum, urine and tumor tissue resembled that of salivary amylase. They suggested that amylase, produced ectopically in some carcinomas of the lung, was the cause of the pronounced and persistent hyperamylasemia.

III. Leucine Aminopeptidase

A. Introduction

The enzyme, leucine aminopeptidase (EC 3.4.1.1), was named after the early studies of this enzyme on leucyl aminopepetides (Smith, 1951; Smith and Spackman, 1955), but its action is not confined to leucyl compounds and acts broadly on the CO–NH site of compounds of the following type:



An enzyme closely related to or identical with leucine aminopeptidase is widely distributed in microorganisms, plants, and various animal tissues. Using L-leucyl β-naphthylamide as substrate, Green *et al.* (1955) obtained the following activities, expressed as micrograms of β-naphthylamine liberated per hour per milligram human tissue: brain, 3.0; duodenum, 2.8; striated muscle, 2.7; kidney, 2.4; spleen, 2.3; and liver, 2.0. Low values, 0.3–2.0 units, were also obtained for other human tissues such as uterus, esophagus, thymus, and aorta.

Leucine aminopeptidase appears to exist in several molecular forms. Beckman *et al.* (1966) reported that all human tissue extracts showed the presence of a zone with fast mobility. In brain, red cells, and serum from normal individuals, this was the only component. Other tissues had, in addition to the common fast moving component, the following: (1) slower moving component in heart, lung, liver and spleen; (2) slower components in kidney and intestine, with those in kidney staining quite strongly; and (3) slow components in placenta.

Several methods for the determination of serum leucine aminopeptidase are available (Bodansky, 1971a). The assay most commonly used is based on the cleavage of the substrate, L-leucyl-β-naphthylamide and the diazotization of the released β-naphthylamine with *N*-(1-naphthyl)

ethylenediamine to give an azo dye with maximal absorption at 560 nm (Bodansky, 1958). The units of activity were expressed as equal to the Klett colorimetric reading. Subsequently, other investigators defined the unit more rigorously as the formation of 1 nmole of β-naphthylamine produced in 30 minutes at 37°C under stated conditions of pH and substrate concentration (Bodansky, 1971a).

B. Serum Leucine Aminopeptidase Activity in Cancer and Other Diseases

In spite of the fact that leucine aminopeptidase is distributed in many human tissues and its concentration in the liver is not particularly high, it is only diseases of this organ and of the pancreas that are reflected in elevated levels of this enzyme in serum. One of the earliest studies was that of Fleisher and his associates (1957) who expressed the enzyme activity as micromoles of substrate split per hour by 1 ml serum at 38°C. Using substantial numbers of patients in each group, they obtained the following mean values, expressed as units: normals, 0.98; portal cirrhosis, 1.67; postnecrotic cirrhosis, 5.61; chloropromazine jaundice, 6.06; neoplasms of liver, 4.42; and acute hepatitis, 79.5. The distribution of enzyme elevations in 51 patients with neoplastic disease of the liver was 23% in the normal range; 31% in 1.1–2.0 × normal range; 18% in the 2.1–3.0 × normal range; and the remainder, 28%, at higher levels.

Several other studies show that hepatobiliary disease, whether neoplastic or not, leads to elevations of serum leucine aminopeptidase (Rutenberg *et al.*, 1958; Arst *et al.*, 1959; Kowlessar *et al.*, 1961). This feature can best be illustrated by the results of Kowlessar *et al.* (1961). These investigators, employing the method of Goldberg and Rutenberg (1958), obtained the following mean values and standard deviation values for normals or for various groups with extrahepatobiliary disease: 65 normal subjects, 128 ± 31; 20 cases of Paget's disease, 119 ± 20; 21 cases of skeletal metastatic carcinoma, 125 ± 19; and 61 cases with carcinoma, nonmetastatic to liver or bone, 121 ± 15. In contrast, in 10 cases of carcinoma of the head of the pancreas with obstruction of the extrahepatic biliary tract, the mean serum level of the enzyme was 441 ± 166; 24 cases of viral hepatitis, 318 ± 92; 8 cases of hepatitis with intrahepatic cholestasis, 1000 ± 93; 18 cases of primary biliary cirrhosis, 642 ± 275; 10 cases of choledocholithiasis with jaundice, 431 ± 296; 4 cases of carcinoma of the biliary tree, 511 ± 134; and 10 cases of lymphosarcoma with hepatic infiltration, 258 ± 82 units. With the exception of pregnancy (Arst *et al.*, 1959), elevations of serum leucine aminopeptidase do not seem to occur in any extrahepatic condi-

tion or disease. The elevations reported for carcinoma of the head of the pancreas or in leukemia can be attributed to involvement of the liver.

IV. Serum Arylamidase

A. Introduction

Arylamidase (EC 3.4.1.2) is capable of splitting very readily a number of amino acid arylamides such as alanine-β-naphthylamide. The enzyme that acts on this substrate may also be designated as alanine aminopeptidase (ANAase). Haschen *et al.* (1966) reported that this enzyme had five electrophoretic peaks of activity, ranging from peak 1, the most anionic, to peak 5, the most cathodic. Figure 4-1 shows that liver contained almost exclusively peak 1, whereas other tissues were characterized as follows: peak 2, pancreas; peak 3, kidney; peak 4, ileal mucosa; and peak 5, duodenal mucosa (Peters *et al.*, 1968) In contrast to arylamidase, leucine aminopeptidase showed only one peak for these various organs, its location corresponding in electrophoretic mobility to peak 2 of the arylamidase. In normal serum, arylamidase exists only as isoenzyme-1 (Beier *et al.*, 1969).

B. Serum Arylamidase Isoenzymes in Pancreatic and Hepatic Disease

Beier *et al.* (1969) indicated that the activity of isoenzyme-1 was increased in intra- or extrahepatic cholestasis, whereas isoenzyme-2, ordinarily absent from the serum, was present in pancreatic disease. This observation offered a possibility for differentiating between these two groups of disease and, indeed, indicated the more interesting possibility of aiding in the diagnosis of pancreatic carcinoma in its early stages. However, Schlaeger and Katterman (1971) could not confirm this. Of 20 patients with well-defined acute pancreatitis, isoenzyme-2 was absent from the sera of 8 patients. Of 5 patients with pancreatic carcinoma, only 2 had isoenzyme-2. Moreover, isoenzyme-2 was also present in hepatic disease: in 10 of 11 cases with cholestasis and in each of 5 patients with hepatic neoplasms. More recently, Peters *et al.* (1973) have shown that 35% of a large series of patients with hepatobiliary disease, including metastatic neoplastic disease, had serum components in addition to the liver isoenzyme-1. In contrast, only 3% of the sera of patients with other diseases showed multiple bands.

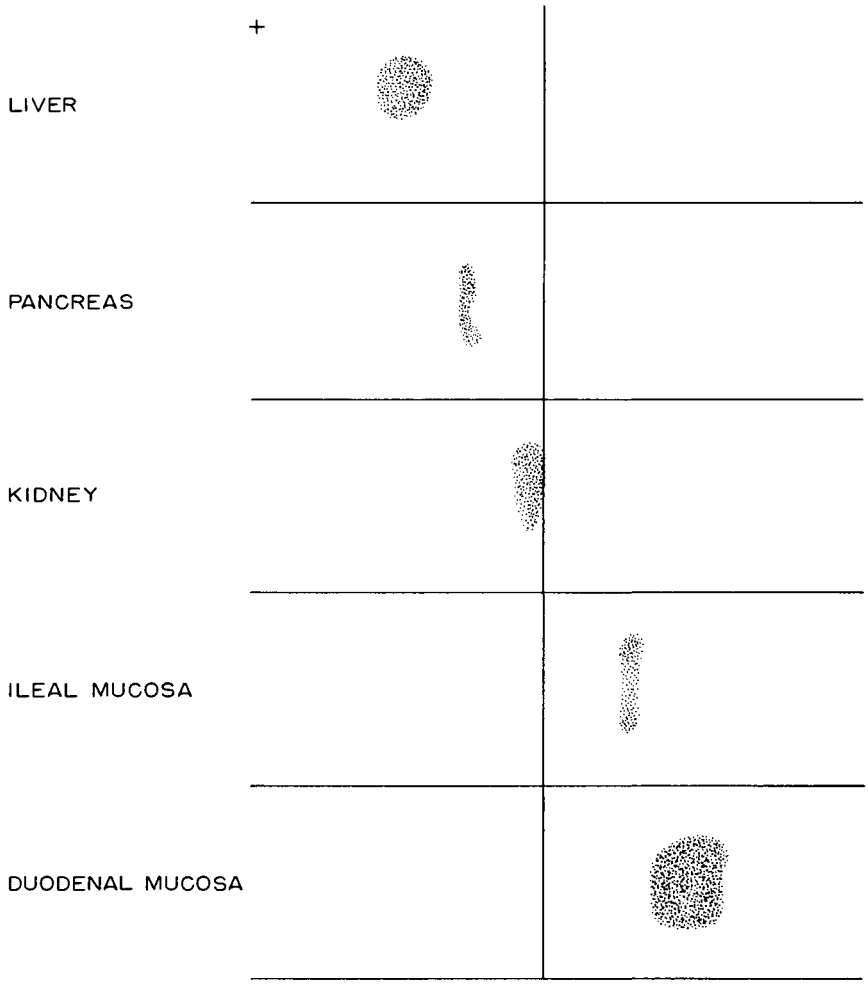


Fig. 4-1 Electrophoretic behavior of arylamidase (ANA) isoenzymes on agar gel. Isoenzymes in liver, pancreas, kidney, ileal mucosa, and duodenal mucosa. From Peters *et al.* (1968). Reproduced by permission of Elsevier Publishing Company.

V. Aspartate and Alanine Aminotransferases

A. Introduction

Aspartate aminotransferase (EC 2.6.1.1) and alanine aminotransferase (EC 2.6.1.2) were formerly known as glutamic-oxaloacetic transaminase

and glutamic-pyruvic transaminase, respectively. They have also been designated as aspartate and alanine transaminases, and abbreviated as AST and ALT, respectively (Baron *et al.*, 1971). As these names indicate, the enzymes catalyze the metabolic process by which the α-amino group of one amino acid is transferred to the carbon skeleton of another amino acid. Thus, aspartate aminotransferase catalyzes the reaction:

$$\text{L(+)-Aspartate} + \alpha\text{-ketoglutarate} \rightleftarrows \text{oxaloacetate} + \text{L(+)-glutamate}$$

Similarly, alanine aminotransferase catalyzes the reaction:

$$\text{L(+)-Alanine} + \alpha\text{-ketoglutarate} \rightleftarrows \text{puruvate} + \text{L(+)-glutamate}$$

In the present discussion, we shall concern ourselves chiefly with aspartate aminotransferase.

The enzyme, aspartate aminotransferase, has been the subject of considerable investigation. Jenkins *et al.* (1959) purified the enzyme from pig heart before it was recognized that it had two molecular forms. The preparation had a molecular weight of 110,000 and 2 residues of coenzyme, predominantly pyridoxal phosphate, per molecule of enzyme. The overall reaction has been formulated on the basis of two half-reactions. Aspartate reacts with the pyridoxal forms of the enzyme to pass through intermediate forms and result in the formation of oxaloacetate and the pyridoxamine form of the enzyme. The latter then reacts with ketoglutarate to pass through intermediate stages to form glutamate and the pyridoxal form of the enzyme. All reactions are reversible (Velick and Varva, 1962).

As we have just noted, aspartate aminotransferase exists as two isoenzymes—a cationic isoenzyme present in the mitochondria and an anionic isoenzyme present in the supernatant. The kinetics and other properties of these have been studied in some detail in several species, including man (Boyd, 1961; Nisselbaum and Bodansky, 1964). The influences governing the relative proportion of these in the cell as well as the mechanisms involving the interaction with substrate and coenzyme have also been investigated by Ivanov and Karpeisky (1969).

B. Aspartate and Alanine Aminotransferases in Human Tissues

Aspartate aminotransferase activity in serum and tissues may be measured in several ways, but the method most frequently employed has been the coupled spectrophotometric procedure of Karmen (1955). This is based on the interaction of α-ketoglutarate with aspartate to form glutamate and oxaloacetate, and the reduction of the resulting oxaloacetate by NADH in the presence of excess malic dehydrogenase. Under

the standardized conditions of the reaction, the number of Karmen units per milliliter of serum was defined as 1000 times the change in absorbance at 340 nm produced per minute by 1.0 ml of serum in the 3-ml reaction mixture.

Using the coupled reaction just described, Wróblèwski and LaDue (1956) obtained the following values for the content of aspartate aminotransferase in human tissues expressed as kilounits (10^3 units) per gram of wet tissue: heart, 156; liver, 142; skeletal muscle, 99; kidney, 91; pancreas, 28; spleen, 14; lung, 10; and serum, 0.02. The corresponding values for alanine aminotransferase were heart, 7.1; liver, 44; skeletal muscle, 4.8; kidney, 19; pancreas, 2; spleen, 1.2; lung, 0.7; and serum, 0.16. It may be seen from these values that, although the highest concentrations of aspartate aminotransferase were in the liver and heart, substantial activities were present in other tissues also. It may be expected, as has been extensively demonstrated, that the highest elevations of serum aspartate aminotransferase activity may occur in diseases of the heart or liver, but that diseases affecting other tissues also show elevations.

Procedures used in the preparation of the aspartate aminotransferase from human heart and human liver indicated that the mitochondrial isoenzyme was largely, if not completely, cationic, and that the supernatant isoenzyme was practically entirely anionic (Nisselbaum and Bodansky, 1964). Accordingly, the assumption was made that electrophoresis of aspartate aminotransferase from these and other human tissues obtained at autopsy could be used to identify the mitochondrial and supernatant isoenzymes. Using starch gel electrophoresis, Schwartz and Bodansky (1966) obtained two main peaks, a γ-globulin and an α,β-globulin region. Most of the enzyme in the γ-globulin region was cathodic to the origin and, indeed, to the human serum γ-globulin used as a marker. The average values for the fraction of cationic component was 49% in 8 livers, 68% in 6 hearts, 68% in 5 samples of skeletal muscle 64% in 3 lungs, 61% in 2 kidneys, 34% in 2 breasts, 32% in 2 brains, 23% in 2 pancreases, 73% in 2 prostates, and 8% in 4 samples of erythrocytes. The average values for the activity of the total aspartate aminotransferase activity, expressed as kilounits per gram of wet tissue, were as follows: heart, 164; liver, 138; skeletal muscle, 36; lung, 4.2; kidney, 73; breast, 2.1; prostate, 10; brain, 37; pancreas, 8.5; and erythrocytes, 2.1 (Schwartz and Bodansky, 1966). A number of the values, as in liver and heart, agree fairly well with those previously submitted by Wróblèwski and LaDue (1956), but those of skeletal muscle and pancreas appear to be much lower.

Very few values are available for the total aspartate aminotransferase

activity of neoplastic tissues and for the isoenzyme distribution. In 3 hepatocarcinomas, the average value was 65.5 ± 45.7 kilounits per gm wet tissue, lower, but not significantly so, than the average 138 ± 89 kilounits per gm for 8 livers without tumor. The values for 1 breast carcinoma was 22 kilounits per gm, as contrasted with an average value of 2.1 ± 1.2 kilounits per gm for 2 breasts without tumor (Schwartz and Bodansky, 1966).

C. *Serum Aspartate Aminotransferase in Cancer*

Approximately 20 years ago, serum aspartate aminotransferase achieved great utility as a laboratory aid in the diagnosis and management of myocardial infarction and of hepatic diseases. Since discussion of these are not relevant to our main purpose here, the reader may be referred to several of the original and review articles in this field (Karmen *et al.*, 1955; Wróblèwski *et al.*, 1956; Bodansky *et al.*, 1959; Wróblèwski, 1959). Serum asparate transaminase activity is elevated in other conditions, such as the myopathies, albeit to a lower degree. In general, the determination of serum alanine aminotransferase activity has not added much to the diagnostic aid offered by a determination of the aspartate aminotransferase activity. Thus, it is less sensitive than the latter in reflecting myocardial infarction and other conditions such as Laennec's cirrhosis or various myopathies (Wróblèwski, 1959). In contrast, in infectious hepatitis in children, the alanine aminotransferase activity was much more sensitive (Bodansky *et al.*, 1959).

Molander (1958) pointed out that serum aspartate aminotransferase activity was elevated in metastatic or primary neoplastic involvement of the liver, but not in metastic bone disease. The degree of peak elevations was shown to range from 40 to 250 units and was considered as approximately proportional to the degree of liver cell injury. Schwartz *et al.* (1963) studied patients with prostatic carcinoma in whom the activity of serum aspartate aminotransferase together with those of glucosephosphate isomerase, isocitrate dehydrogenase, alkaline and acid phosphatases, and aspartate aminotransferase was followed sequentially and concurrently for periods ranging from 1 to 4 months. The changes in these serum enzyme levels were correlated in each patient with conventional biochemical parameters and with alterations in clinical status, either spontaneous or induced, produced by testosterone proprionate or ethinyl estradiol. Serum glucosephosphate isomerase and isocitrate dehydrogenase levels changed much more sensitively than the other serum enzymes in response to exacerbation or remission of the disease.

Aspartate aminotransferase activity was particularly unresponsive. We have previously noted (Chapter 2) that, of the various serum enzyme activities that have been studied in patients with cancer, aspartate and alanine aminotransferase showed the lowest incidence of elevations in patients with cancer.

D. Isoenzymes of Serum Aspartate Aminotransferase in Cancer

We have already noted that, upon electrophoresis, tissue aspartate aminotransferase (AST) activity was distributed in two main peaks, the γ-globulin and the α,β-globulin regions. The activity in the former region constituted the cationic or mitochondrial isoenzyme, and that in the latter represented the anionic or supernatant isoenzyme. Starch block electrophoretic patterns of the sera of 4 normal individuals and 34 patients with cancer were studied (Schwartz and Bodansky, 1966). In the normal individuals with serum AST activities ranging from 17 to 32 units, the enzyme activity was completely confined to the α,β-globulin region and represented the anionic or supernatant isoenzyme.

The electrophoretic patterns of the serum AST in the cancer patients fell into three groups. The first group consisted of 17 patients with carcinomas, primary in various organs such as the breast, cervix, larynx, biliary tree, or tongue. Two melanomas were also represented. Most of these patients had hepatic, pulmonary, brain, or bony metastases. The rest had such complications as serum hepatitis, hemochromatosis, or renal failure The serum AST activity ranged from 23 to 625 Karmen units. Yet all of these activities consisted of only the anionic isoenzyme peak. Three patients with serum or infectious hepatitis whose serum AST activities ranged from marked elevations of 1060–2475 units also exhibited only the anionic isoenzyme in their sera.

The sera of 10 patients with cancer showed the presence of 2 isoenzymes of aspartate aminotransferase. The total serum enzyme activity ranged from 42 to 16,000 units and the proportion of the cationic, mitochondrial enzyme ranged from 4 to 40% of the total AST activity. These patients either had massive liver metastases, viral hepatitis superimposed upon their basic neoplastic disease, or were in shock, usually 1 or 2 days before death. The emergence of the cationic isoenzyme in the second group of patients appeared to be associated with an acute phase in the patient's course, and its disappearance paralleled the subsidence of this phase.

In addition to these 2 groups, a third group, consisting of 6 patients, exhibited the usual anionic isoenzyme, but, in addition, a faster compo-

nent in the albumin region. This was small, 2–8% of the total enzymic activity in 4 of the patients, but somewhat more substantial, 24 and 42% in 2 others. Three of these 6 patients also exhibited a third isoenzyme in the cationic region, ranging from 9 to 41% of the total activity.

The appearance of the cationic component of AST in the sera of patients may be explained either by the direct passage of the mitochondria into the serum or by liberation *in vivo* of the cationic component from the mitochondria in a soluble form and the subsequent passage of this form into the serum.

VI. Additional Serum Enzymes

Several other serum enzymes have been studied with respect to the alteration of their activities in disease and, more particularly, in cancer. These may be briefly summarized. Straub and his associates (1957) reported that adenosine deaminase, the enzyme that catalyzes the deamination of adenosine to inosine, was elevated in the serum of 92% of a series of 527 cancer patients. Such a high frequency would appear to have substantial diagnostic value. However, Schwartz and Bodansky (1959) found that, as with other serum enzymes, the level of the enzyme activity was related not to the presence of the tumor, but to its growth as manifested by the clinical status of the patient. In a group of 55 patients with various types of cancer, only 8 patients, or 15%, had values higher than the upper limit of normal, as defined by the mean value plus 2.5 standard deviations.

Ornithine carbamyltransferase (EC 2.1.3.3) is involved in urea formation and catalyzes the reversible formation of phosphate and citrulline from carbamyl phosphate and ornithine. The activities of various human tissues, expressed as micromoles of $^{14}CO_2$ liberated by 1 gm of tissue or body fluid in 24 hours when incubated with [^{14}C]carbamyl citrulline under standardized conditions were liver, 2500; small intestine, 347; large intestine, 5.8; stomach, 4.7; gallbladder, 3.5; lung, 1.5; spleen, 0.71; heart muscle, 0.15; kidney, 0.04; blood cells, 0.06; and serum, 0.04 (Reichard, 1960). Obviously, because of its high activity in the liver, this enzyme possesses diagnostic potentialities for diseases of that organ. Highly elevated activities have been found in most patients with infectious hepatitis (Reichard, 1961; Bodansky, 1971b) but the alterations in patients with cancer of the liver, either primary or secondary, were not marked. Of 17 patients in this group, 6 had normal values, 3 slightly elevated, 3 moderately elevated, and only 5 greatly elevated (Reichard, 1961). In another series of 10 cases with liver metastases, 100% had moderately

elevated values, 1.1–2.0 times the upper limit of normal (Bodansky, 1971b). It is apparent that there is no clear distinction between neoplastic disease and other diseases of the liver.

Cholinesterase (EC 3.1.1.8), the enzyme that hydrolyzes acylcholine to acetic acid and choline, is present in serum and its serum activity is decreased in patients with hepatobiliary disease (Antopol *et al.*, 1938). McArdle (1940) observed the range in 40 normal adults to be 51–121 units, with a mean value of 78 units. In 71 patients with hepatic disease, the mean was 36 units and the range from 10 to 70 units. Twenty-two of these patients had hepatic metastases, and the mean value was 41 units with a range from 23 to 61 units. Here, too, there was no distinction between neoplastic and other diseases of the liver.

Of the enzymes in the citric acid cycle that may appear in the serum, isocitrate dehydrogenase has received most attention. This enzyme catalyzes the reversible interaction between D-isocitrate and NADP, and was found to be present in human serum by Wolfson and Williams-Ashman (1957). Impressive elevations of this enzyme activity are found chiefly in acute viral hepatitis and in extensive neoplastic disease of the liver (Sterkel *et al.*, 1958). This enzyme activity may be a more sensitive indicator of the progress of disease in metastatic cancer of the prostate than either acid phosphatase or aspartate aminotransferase (Schwartz *et al.*, 1963).

The presence of magnesium ion in a final, optimal concentration of 0.003 *M* leads to a distinct and readily measurable serum deoxyribonuclease activity (Wróblèwski and Bodansky, 1950). The mean values in a group of 50 patients with cancer was 0.15 arbitrarily defined units, significantly less than the average values of 0.21 unit in a group of 34 patients in noncancerous disease and of 0.30 unit in a group of 30 normal individuals. Migliarese (1958) found that serum ribonuclease was increased in patients with untreated cancer; the mean value was 155 units for 48 cancer patients as compared with 95 units for 75 apparently healthy subjects. Treatment caused a decrease in serum enzyme activity. With the exception of bronchopneumonia, other diseases were not associated with elevated values.

VII. Summary

There are several other enzymes which we have not considered in this chapter since it appears to be more appropriate to do so in connection with the specific type of neoplasia. For example, we shall examine the subject of lysozyme in our chapter on leukemias (Chapter 9). The

studies reviewed in the past three chapters indicated the manifold ways in which the activities of enzymes in tissues, formed elements of the blood, and serum are altered in neoplastic processes of the human organism. Studies of serum enzymes have been of two kinds. The first of these includes the enzymes which are confined in substantial concentrations to very few tissues, or preferably, to one tissue. Damage to the tissue, overproduction of the resident enzyme, obstruction to its secretion, or excretion through normal channels result in serum elevations that are fairly specific for the tissue and often for the pathological process. The alkaline and acid phosphatases have proved their value in this connection, particularly in neoplastic diseases. The presence of ornithine transcarbamylase and isocitrate dehydrogenase in high concentrations in the liver are associated with high serum levels in diseases of that organ. As we shall note in Chapter 9, the main sources of lysozyme are the blood monocytes and granulocytes, and leukemias involving these cells result in high levels of serum and urinary lysozyme. But there are also enzyme activities such as those of 5′-nucleotidase and leucine aminopeptidase which are rather specifically elevated in the serum in diseases of a particular organ, in this case the liver. Yet the tissue does not have a particularly high content of the corresponding enzyme. The mechanism of this type of association remains to be studied.

The second sector of serum enzyme studies has been concerned with the enzymes such as those of the glycolytic sequence that are intimately involved in major metabolic processes and which are widely distributed in the tissues of the body. In general, these enzymes are neither tissue- nor disease-specific. However, because of relative differences in their concentrations in various organs, the size of the organs, the presumable differences in rates of synthesis, degradation, or excretion of these enzymes, study of the alteration of these serum enzyme activities is applicable, in conjunction with clinical and other laboratory information, to the diagnosis and management of patients with certain types of cancer.

We have also noted that a serum enzyme may represent a mixture of isoenzymes and that the composition of this mixture may be characteristic of certain diseases, including cancer. For example, it will be recalled from Chapter 2 that the isoenzymes of aldolase may be characterized by the ratio of the activity on fructose 1,6-diphosphate to that on fructose 1-phosphate. Whereas the values for this ratio is about 2.8 in the sera of normal individuals and 1.3 for patients with acute hepatitis, it is characteristically much higher in patients with cancer. We shall also note that the isoenzyme pattern of leukocytic acid phosphatase varies with the type of leukemia (Chapter 9, Section II). Further studies of isoenzyme patterns in the enzymes of serum and formed elements of

the blood may reveal specific relationships between these patterns and diseases and thus aid greatly in the diagnosis and management of patients with cancer.

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5

The Neoplastic Immunoglobulinopathies

I. Introduction

The role of immunoglobins is related to malignancy in two important ways. First, although immunoglobulins have been defined as proteins of animal origin endowed with known antibody activity, this definition also includes other proteins which are the result of unbalanced proliferative disorders of the cells that are normally involved in the synthesis of immunoglobulins (World Health Organization, 1964). Among the neoplastic conditions falling into this group are multiple myeloma, Waldenström's macroglobulinemia, heavy (H) chain disease, chronic lymphocytic leukemia, lymphosarcoma, reticulum cell sarcoma, and Hodgkin's disease. These may also be designated as neoplastic gammopathies, in accordance with Waldenström's usage of this term for conditions associated with disturbed γ-globulin formation (1961).

The second way in which malignancy is related to immunological factors is that the incidence of malignancy among patients with immunological deficits appears to be much higher than would be expected on the basis of chance alone (Good and Finstad, 1969; Gatti and Good, 1970a,b, 1971; Good, 1971, 1973; Kersey *et al.*, 1973). We shall discuss instances of this association later on in this chapter.

II. Human Immunoglubulins

A. Cellular Basis for Antibody Formation

The bone marrow stem cells give rise to 3 types of cell precursors that are involved in the immune response. These are the blood monocyte (immature macrophage), the B (bursal) cell precursor, and the T (thymus) cell precursor (Eisen, 1973). The immunobiological mechanisms involved in the elaboration of the B and T cells and in their functions have been most comprehensively formulated by Good and his associates (Hoyer *et al.*, 1968; Good and Finstad, 1969; Gatti and Good, 1970a; Good, 1971, 1973). As Fig. 5-1 shows, the immune mechanism may be conceived to consist of two main branches, namely, the bursal and the central thymus systems. The former develops by budding from the intestinal epithelium. The central thymus system originates as an epithelial structure arising from the third and fourth pharyngeal pouches and becomes a lymphoid organ. Eisen (1973) has noted that the B cells give rise successively to small lymphocytes, large lymphocytes, and then plasma cells. The T cell precursor divides rapidly in the thymus into a mature T cell and passes through the stages of being a small and large lymphocyte. In any case, as Fig. 5-1 indicates, the lymphoid cells are released from these 2 central organs into the bloodstream and are then reassembled in the peripheral lymphoid tissue. The lymphocytes which are thymus-dependent in origin control cellular immunity, whereas the bursa-dependent plasma cells synthesize serum antibodies (Good, 1971).

The 2 cell types have been shown to have widely different functions and can be distinguished from each other in a number of ways as, for example, by electrophoretic mobility, serologically by the presence or absence of specific surface antigen markers, *in vitro* reactivity to various lectins, and, in the human, by the properties in T cells of forming rosettes with sheep red blood cells (Wybran *et al.*, 1971). Although it has been stated that the B and T cell types are morphologically indistinguishable (Eisen, 1973), recent studies of peripheral human lymphocytes with the scanning electron microscope have shown that the B lymphocytes have a complex surface architecture with multiple microvilli covering almost the entire surface. In contrast, the T lymphocytes have a generally smooth surface without surface projections (Polliack *et al.*, 1973). A variety of methods have shown that T cells constitute between 85 and 90% of lymphocytes in the blood, lymph, and lymph nodes of the mouse (Eisen, 1973). The electron microscopic scanning method gives a similar distribution for human peripheral blood lymphocytes, namely,

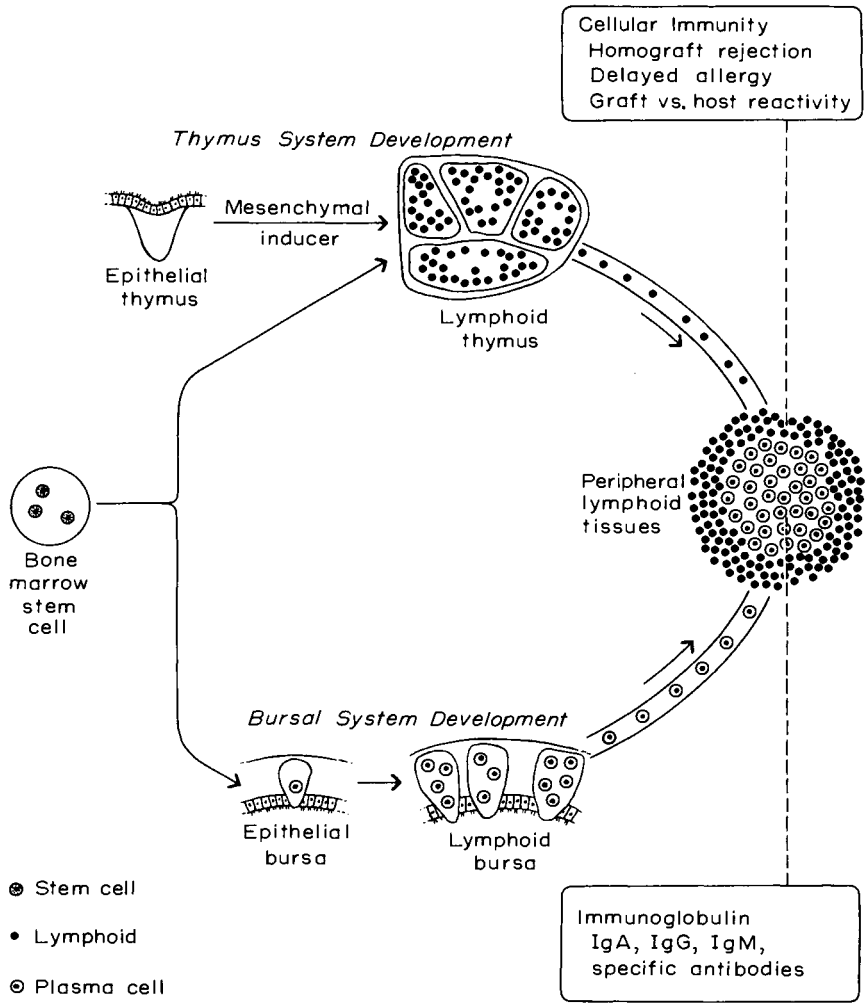

Fig. 5-1 The two branches of the immune mechanism (Good, 1971). Reproduced by permission of Sinauer Associates, Inc.

7.6–34% with an average of 20% for the villous B cells and 65–92% with an average of 80% for the relatively smooth T lymphocytes.

The third type of cell precursor that arises from the bone marrow stem cell is the monocyte which develops into the mature macrophage. This highly phagocytic cell, about 15–20 μm in diameter, does not form antibodies, but does cooperate with T cells in activating the response of B cells to many immunogens.

The entrance of an antigen (immunogen) into the body sets into motion a train of events which involves certain cells in the formation of antibodies (immunoglobulins) and results finally in the appearance of these antibodies in the serum. Several formulations of the mechanisms involved in the cellular activities necessary to produce antibodies have been proposed during the past 70 years, but the one that appears to be most satisfactory and currently accepted is the clonal selection theory (Burnet, 1959; Eisen, 1973). This formulation proposes that an immunologically responsive cell such as a lymphocyte can respond to only one antigen or perhaps to several closely related ones, even though the cell has not previously experienced this antigen. An individual's lymphocytes may thus be viewed as a greatly diversified pool of cells, some of which respond to one antigen, others to a second antigen, still others to a third, etc. After its entrance into the body, an antigen binds to an antibodylike receptor on the surface of the corresponding lymphocyte which is then stimulated to multiply and generate a clone of differentiated cells. The small B lymphocytes synthesize and secrete immunoglobulins. The specific binding of antigen by the receptors on the cell surface stimulates transformation of small lymphocytes into large lymphocytes which can also secrete antibodies and which can further differentiate into the mature plasma cell, most active of all lymphoid cells in the synthesis and secretion of immunoglobulins. We may also note that the T cells play a helping role by regulating the proliferation and differentiation of B cells into plasma cells. There is evidence that the macrophages cooperate with T cells in aiding the response of B cells to many immunogens (Eisen, 1973).

B. Structure and Classification of Immunoglobulins

The chemical structure of the immunoglobulins has been elucidated largely through the efforts of two groups of investigators, Edelman and his associates (Edelman, 1959; Edelman and Poulik, 1961; Edelman and Gall, 1969) and Porter and his-coworkers (Porter, 1959, 1962; Fleischman *et al.*, 1962).

Several representations have been submitted for the general structure of the human immunoglobulin molecule (Edelman and Gall, 1969; Hobbs, 1971; Smith *et al.*, 1971). For example, according to Smith *et al.* (1971), the structure of IgG has the following properties:

(a) Two identical light (L) polypeptide chains, each with a molecular weight of about 22,500 daltons and two identical heavy (H) chains, each with a molecular weight of about 53,000 daltons, are linked by disulfide bonds and noncovalent interactions.

(b) This molecule folds into three compact domains which are linked by the heavy-chain hinge region. Several proteolytic enzymes preferentially cleave the molecule in or near this region to give two fragments with antibody specificity of the parent molecule (Fab) and one crystallizable fragment (Fc).

The memorandum issued by the World Health Organization in 1964 supplied a basis for the nomenclature of the various immunoglobulins and helped to discard the several synonyms which had previously been used for each of the immunoglobulins. The major classes of human immunoglobulins are now known as γG or IgG, γA or IgA, and γM or IgM. The minor classes present in human plasma are IgD and IgE.

Each class of immunoglobulins has a characteristic type of heavy chain, such as the γ (gamma) for IgG and the α (alpha) for IgA. The characteristics and main structural features of the various classes of human immunoglobulins have been compiled by Fleischman (1966) and by Edelman and Gall (1969). For example, the IgG class of human immunoglobulins has a heavy chain in the γ class, with subclasses γ_1, γ_2, γ_3, and γ_4 distinguished by their antigenic determinants. As will be noted below, differences in the subclasses are reflected in variations in the amino acid sequences of the carboxyl terminal parts of the γ chain. The IgG class contains two major classes of light chains, κ (kappa) and λ (lambda), which are also present in the other immunoglobulins. The molecular weight of the light chains is about 22,500. The immuoglobulins in the IgG class have a sedimentation constant ($s_{20,w}$) of 6.5–7.0, a molecular weight of 150,000, and a carbohydrate content of 2.9%.

As with all polypeptides, the immunoglobulin polypeptide chain has terminal amino and carboxyl groups and, hence, may be divided into an amino terminal region and a carboxyl terminal region. The former has been designated as the variable (V) region and the latter as the constant (C) region. These regions are defined by the presence or absence of sequence variation and by sequence homology. The possibility of variation in the amino acid sequences allows for the tremendous heterogenity of immunoglobulins. The various features that we have been describing are illustrated in the schematic structure of IgG (Fig. 5-2).

C. Production and Metabolism of Immunoglobulins

We have already indicated briefly the way in which the B and T cells and macrophages are involved in the induction of antibody synthesis. These roles are shown more fully in Table 5-1.

The way in which the antibody response is evoked by the macrophage phagocytized antigen is indicated by the findings that a soluble fraction

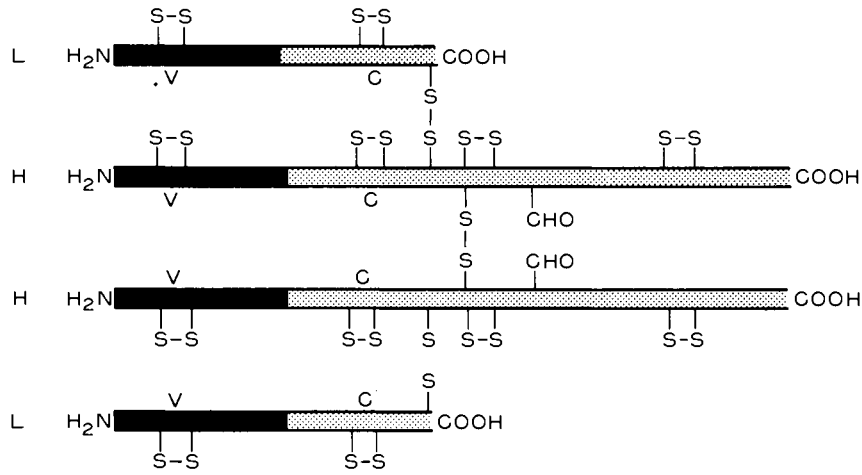

Fig. 5-2 Schematic structure of IgG. L, light chains; H, heavy chains; and CHO, carbohydrate unit. The variable constant portions of the chains, with respect to amino acid sequences, are indicated by the labels V and C, respectively.

from such macrophages can induce antibody synthesis in recipient lymphoid cells in tissue culture and that this soluble fraction is inactivated by treatment with RNase. These findings suggest that transfer

TABLE 5-1

Roles of B and T Cells and of Macrophages in Induction of Antibody Synthesis[a]

Property	B cells	T cells	Macrophages
Role	Differentiate into Ag-secreting cells	Accessory cells	Accessory cells
Specificity[b]	Restricted	Restricted	Unrestricted
Memory[c]	Yes	Yes	No
Proliferation stimulated by Ag binding	Yes	Yes	No
Inactive in unresponsive individuals[d]	Yes (sometimes)	Yes (sometimes)	No
Helping function	No	Release stimulators for B cells	Bind Ag on surface facilitating B-cell binding to Ag

[a] From Eisen (1973). Reproduced by permission of Hoeber Medical Division, Harper and Row, New York.

[b] Variety of antigenic determinants bound per cell.

[c] That is, priming by an Ag increases the number of cells able to respond later to that Ag.

[d] Genetically unresponsive or made tolerant.

of information from cells which have phagocytized antigen to the lymphocyte is mediated by RNA (White *et al.*, 1973).

The general mechanisms involved in protein synthesis apply, of course, to the synthesis of antibody protein. Employing the ascitic form of murine plasmacytoma 5563, Askonas and Williamson (1966) showed that the synthesis of H and L chains were conducted by 2 different types of polyribosomes. Since H and L chains are formed on separate mRNA molecules, a mechanism must exist for the balanced production of the 2 types of chains and for assembly of the immunoglobulin molecule. It has been suggested that the L chain is completed first and released from the polyribosomes to yield a small pool of free chains which combine with partially synthesized H chains. Before H chains can be released from their polyribosomes, they must be combined with L chains (White *et al.*, 1973). Carbohydrate is subsequently attached to the H chains.

Several estimates have been offered on the rate of synthesis of immunoglobulins in humans. Martin (1969) proposed the following values, expressed as milligrams formed per kilogram per day: IgG, 20–40; IgA, 207–55; IgM, 3.2–16.9; and IgD, 0.3–1.49. Waldmann (1969) has submitted the following average rates: IgG, 33.0; IgA, 24.0; IgM, 6.7; and IgD, 0.4.

It should be realized that the rates of synthesis are controlled not only by the pool size and serum concentrations of the immunoglobulins but also by the bacterial and viral environment and the magnitude of the antigenic exposure. For example, the rate of synthesis of the different classes in immunoglobulins in mice raised in a germfree environment is one-fiftieth to less than one three-hundredths of that in normally exposed mice (Sell and Fahey, 1964).

As soon as the immunoglobulins are synthesized, the cells containing them find their way into, and are distributed in, the tissues and blood and become subject to catabolism. The quantitative aspects of these processes may be estimated by the intravenous administration of ^{131}I-labeled IgG or other immunoglobulins and the determination of the following parameters: plasma volume, retained radioactivity, half-life, fractional catabolic rate, distribution in intravascular compartment, and total body pool (Solomon *et al.*, 1963a,b; Martin, 1969; Waldmann, 1969; Hobbs, 1971). The values obtained by Waldmann (1969) are shown in Table 5-2.

D. Concentrations of Immunoglobulins in Human Serum

A number of methods have been employed for the quantitative determinations of immunoglobulins, principally in serum. These have ranged

TABLE 5-2

Physical and Metabolic Properties of Human Immunoglobulins[a]

Immunoglobulin	Sedimentation coefficient	Molecular weight	Biological activity	Distribution (% of total body pool, intravascular)	Total circulating pool (mg/kg)	Fractional catabolic rate (% of intravascular pool/day)	Half-life (days)	Synthetic rate (mg/kg/day)
IgG	7 S	160,000	Major antibacterial antiviral activity in serum	45.0	494.0	6.7	23.0	33.0
IgA	7 S (9,11,13 S)[b]	170,000[c] 385,000[d]	Serum antibodies and Major immunoglobulin of external secretions	42.0	95.0	25.0	5.8	24.0
IgM	18 S	900,000	Antipolysaccharide antibodies; initial antibodies after protein antigen administration	76.0	37.0	18.0	5.1	6.7
IgD	7 S	180,000	Unknown	75.0	1.1	37.0	2.8	0.4
IgE	8 S	200,000	Reaginic antibody role in allergic diseases	51.0	0.02	89.0	2.3	0.02

[a] From Waldmann (1969). Reproduced by permission of the New England Journal of Medicine.
[b] 11 S in external secretions.
[c] As serum monomer.
[d] In secretions.

TABLE 5-3

Concentrations of Immunoglobulins in Normal Human Serum[a]

Investigator	IgG	IgA	IgM	IgD	IgE
Wintrobe (1967)	800–1500	56–193	39–117	3	—
Fahey (1965)	1240 ± 220	390 ± 90	120 ± 35	3	—
Martin (1969)	800–1600	140–420	50–190	0.5–40	0.01–0.14[b]
Waldmann (1969)	1210	260	93	2.3	0.05
Hobbs (1971)	947	248	94	3	0.025[b]

[a] Number of patients not stated except in Hobbs' series (1971) of 54 males and 53 females. Values in mg/100 ml.

[b] Values in μg/100 ml. Value of Waldmann (1969) for IgE appears unusually high.

from the precipitin method of Kabat *et al.* (1948) to later procedures such as those of Claman and Merrill (1964), Mancini *et al.* (1965), Merrill *et al.* (1967), or Vergani *et al.* (1967). The serum concentrations of the different classes of immunoglobulins represent a balance among the dynamic processes of synthesis, distribution, and catabolism and, more specifically, among the rates at which these proceed under various conditions. Table 5-3 shows values obtained by several groups of investigators.

The effect of age upon immunoglobulin levels in serum is naturally of interest. Van Furth *et al.* (1965) reported that immunoglobulin synthesis in the fetus starts at approximately 20 weeks of gestation. Serum IgA concentrations rise slowly from a level of 17–34 mg per 100 ml during the first year of life to attain adult levels of about 150 mg per 100 ml by about 12 years. Levels of IgG rise from about 400 mg per 100 ml to reach a maximum of about 1000 mg per 100 ml by about 8 years of age, and serum IgM concentration reach adult levels by the age of about 1 year for boys and 2 years for girls (Allansmith *et al.*, 1968).

III. Immunoglobulins in Cancer

The reader will recall that the definition of immunoglobulins adopted by the World Health Organization (1964) included related proteins for which antibody activity has not been demonstrated as, for example, myeloma proteins and Bence Jones proteins. It has long been appreciated that electrophoresis of sera from patients with myelomatosis, macroglobu-

linemia, or an allied lymphoreticular dyscrasia revealed the presence of discrete, tightly packed bands (Gutman, 1948; Riva, 1957; Martin, 1969). The term "paraprotein" has been frequently used to refer to such proteins which presumably do not normally occur in the serum (Heremans, 1959; Hobbs, 1969, 1971). Martin (1969) decried the use of this term, noting that there are few syndromes for which this statement clearly holds, and recommended reference to, and characterization of, the abnormal protein occurring in a specific syndrome.

We have already quoted Burnet's (1959) theory to the effect that one plasma cell produces a single immunoglobulin. This has been generally confirmed, although approximately 2% of plasma cells appear capable of producing two immunoglobulins simultaneously (Hobbs, 1971). As Waldenström (1962) pointed out, if a single plasma cell continued to divide to form a clone of cells, all the daughter cells try to produce the same immunoglobulin. If all the molecules have exactly the same structure, they will also have the same electrophoretic mobility and will run as a single narrow band. This band will contain immunoglobulin determinants of a single subclass of H chain and/or a single subclass of L chain. Hobbs (1971) has pointed out that when a narrow band is found upon electrophoresis and is identified as resulting from a single type of immunoglobulin, it may be designated as "paraprotein," a term not used by others (Waldenström, 1961; Martin, 1969). The presence of such proteins indicates that a monoclone is growing in the patient and that the patient is harboring a tumor capable of producing immunoglobulin.

The synthesis of whole immunoglobulin and of L and H chain synthesis is delicately balanced in the well-differentiated plasma or lymphoid cell. In malignancy, the plasma or lymphoid cells appears to become dedifferentiated and an imbalance develops in the synthesis of L and H chains (White *et al.*, 1973). Putnam and Miyake (1958) found that when [^{14}C]glutamate was injected into a patient with myelomatosis, the Bence Jones protein had a much higher specific activity than the myeloma protein, indicating a *de novo* synthesis of the former.

Hobbs (1971) suggested that certain biochemical features are of value in the prognosis of malignant and benign immunocytomas. Of 517 patients with malignant immunocytomas, 84% excreted immunoglobulin fragments, 98% showed suppression of formation of normal immunoglobulins, 92% has narrow electrophoretic (abnormal) serum protein levels greater than 1 gm per 100 ml, and 99% of those that were untreated showed a progressive rise in this level. The corresponding fractions in a group of 112 benign immunocytomas were 0, 10, 15, and 1%, respectively.

IV. Multiple Myeloma (Myelomatosis)

A. Introduction

Multiple myeloma is essentially a malignancy of the plasma and closely related cells and, more rarely, the reticulum cells and the lymphocytes. It results in local tumors, particularly in bones, as well as in disseminated infiltration of the bone marrow and other tissues. The characteristic cell in this disease has been called the myeloma cell, and is a variation of the normal plasma cell. A Wright's stain shows an abundant, strongly basophilic, nonhomogeneous cytoplasm which has a granulated appearance as a result of the endoplasmic reticulum. Occasionally, inclusions of various kinds may be seen in the cytoplasm (Engle and Wallis, 1967).

The tumor cells may be localized in a single tumor mass in the medullary cavity of the bone in an area of red marrow formation such as the skull, mandible, ribs, vertebrae, sternum, or of the bones of the arm or leg. In some instances, multiple tumor masses are found. Extramedullary sites of solitary and multiple tumors include many tissues such as the pleura, thyroid gland, ovary, stomach, intestines, kidney, and the lymph nodes. Patients with disseminated multiple myeloma may have tumor cells in any part of the body.

In 1947, Aegerter and Robbins estimated that a total of about 1000 cases of multiple myeloma had been reported until that time. With more refined techniques for detection of multiple myeloma proteins and a greater awareness of the disease, the incidence has been estimated for various geographic areas as ranging from 1.4 to 3 cases per 100,000 living population per year (MacMahon and Clark, 1956; Martin, 1961) or a worldwide yearly incidence of tens of thousands of cases. Hobbs (1971) has stated that the incidence is about 1% in persons over 70 years of age. The number of deaths in the United States resulting from this tumor for the year 1965 was 1727 males and 1508 females (World Health Organization, 1970).

The incidence of outstanding clinical findings and symptoms in multiple myeloma was given by Adams *et al.* (1949) as weight loss, 68%; gastrointestinal symptoms, 62%; fever, 52%; pallor, 47%; bleeding, 39%; neurological disturbances, 35%; palpable liver, 29%; and palpable spleen, 9%. The reported incidence of bone pain ranged from 68 to 87% (Adams *et al.*, 1949; Snapper *et al.*, 1953; Carson *et al.*, 1955). A later review of 98 myeloma patients gave a somewhat different distribution of clinical symptoms and findings: weight loss, 23%; back pain, 33%; chest pain,

14%; joint pain, 14%; fever, 6%; and neurological disturbances, 10% (Owen, 1965).

B. General Biochemical Aspects

There are several interesting biochemical aspects in multiple myeloma (Bodansky and Bodansky, 1952). Marked elevation of serum alkaline phosphatase activity is rare, and the incidence of mild elevations, ranging from 4 to 12 Bodansky units, is about 40–50% (Bodansky, 1965). The concentration of serum calcium is frequently increased to values above 13 mg per 100 ml and occasionally to as much as 18–20 mg per 100 ml. However, the concentration of serum phosphorus is within the normal range, except when there is renal impairment. These aspects will be considered more fully later in this chapter. Of concern to us here is the nature of the serum abnormal proteins that are elaborated in this disease.

C. Serum Proteins in Myelomatosis

Total serum protein is increased in most patients with myeloma, and these increases may be very impressive, attaining a final concentration of as much as 18–19 gm per 100 ml, as compared with an average concentration in normal individuals of 7.2 gm per 100 ml (Bodansky and Bodansky, 1952). Using simple electrophoresis, typing by means of immunoelectrophoresis and quantitative measurement by means of radial immunodiffusion, Hobbs (1971) investigated 691 patients with abnormal proteinemia. On the basis of x-ray examination of the bones, biopsy, and presence of a sharp spike or homogeneous peak in the serum electrophoretic pattern, 420, or 61%, of these patients were diagnosed as having myelomatosis. The incidence of various clinical and biochemical features in a large group of patients with myelomatosis of different classes and one protein peak (monoclonal type) is shown in Table 5-4. The mean values for the concentrations of myeloma protein were 4.3 gm per 100 ml for class IgG and 2.8 gm per 100 ml for class IgA, as compared with normal levels of about 1.0 gm and 0.2 gm per 100 ml, respectively (Table 5-3). The frequencies of other findings can be seen in Table 5-4. Other types of myelomatosis are rare, with incidences of 1.5% for IgD, 0.5% for IgM, and 0.1% for IgE (Hobbs, 1971). It is of interest that in about 1% of patients with typical clinical myelomatosis, no abnormal proteins could be detected in serum or in 300-fold concentrated urine.

Before we proceed to a fuller discussion of the chemical nature of Bence Jones (BJ) proteins, it may be of value to note briefly the bio-

TABLE 5-4

Incidence of Clinical and Biochemical Features at the Time of Diagonsis in 212 Patients with Myelomatosis of Different Classes[a]

Clinical feature	Class		
	Only IgG	Only IgA	Only Bence Jones
No. of patients	112	54	40
Mean serum level myeloma protein (gm/100 ml)	4.3	2.8	±
Mean doubling time of myeloma protein (months)[b]	10.1	6.3	3.4
Lytic bone lesions (%)	55	65	78
Hypercalcemia (%)	33	59	62
Serum urea >79 mg/100 ml (%)	16	17	33
Hospital admissions because of infection (%)[c]	60	33	20
Detected amyloidosis (%)[d]	0.5	7	10

[a] From Hobbs (1969), with modifications. Reproduced by permission of Blackwell Scientific Publications Ltd.

[b] Time required for serum concentration of myeloma protein to double.

[c] In 48 patients with IgG, 21 with IgA, and 20 with Bence Jones meylomata, the course was followed for up to 3 years.

[d] Incidences were based on 228 patients with IgG, 102 patients with IgA, and 94 patients with Bence Jones myelomata.

chemical–clinical correlations in the series of patients studied by Hobbs (1969) and shown in Table 5-4. The IgG class had a significantly higher serum level of abnormal protein than the IgA class ($p = <0.01$) and a higher mean doubling time than either the IgA or BJ proteinuria class ($p = <0.02$), but the incidences of lytic lesions, of hypercalcemia, and of serum urea elevations were less than the BJ class ($p = <0.02$ or <0.001). The incidence of hypercalcemia in the IgG class was also less than in the IgA class. The IgG patients also had the most severe immune paresis, as manifested not only by the higher incidence of depressed normal serum immunoglobulins but also by the high rate (60%) of infections requiring hospitalization. This group also had the following findings: apparent hyponatremia, 8%; cryoglobulinemia, 2%; and viscosity syndrome, 4%. The last of these may lead to a variety of clinical symptoms and findings such as severe lassitude, impaired phagocytosis, impaired platelet function, anemia, distention of retinal veins with visual impairment, coma, renal failure, and simulation of congestive heart failure (Hobbs, 1971). Renal damage may also result from amyloidosis, hypercalcemia, or blockage of distal tubules by abnormal protein.

D. Bence Jones Proteins

The presence of a characteristic protein in the urine of patients with multiple myeloma was first reported in detail by Bence Jones in 1848. This protein, named after its discoverer, is characterized by its precipitation, as the urine is heated, at a temperature of 40°–50°C and its re-solution, as heating is continued, at temperatures approaching 100°C. With this simple procedure, the following incidences of this proteinuria in multiple myeloma have been reported: 65% of a group of 425 cases recorded in the literature (Geschickter and Copeland, 1928); 53% in a series of 83 cases (Bayrd and Heck, 1947); and 47% in a series of 61 patients (Adams *et al.*, 1949).

Employing a combination of electrophoresis and immunoelectrophoresis, Pruzanski and Ogryzlo (1970) reported an incidence of 70% in a series of 157 patients whose urine was available for testing. The proteinuria is not a constant finding, nor is it a unique finding, for it is also observed in macroglobulinemia and other neoplastic diseases and, occasionally and to a lesser degree, in nonneoplastic disease. Hobbs (1967) has concentrated urines up to 500-fold before subjecting them to electrophoresis, and by this method has been able to detect Bence Jones protein at concentrations of only 1 mg per 100 ml.

Our knowledge of the structure of Bence Jones proteins, which remained somewhat obscure for many years, has advanced considerably during the past two decades (Korngold and Lipari, 1956; Ponstingl *et al.*, 1971; Edelman and Gally, 1962; Fahey, 1963; Schwartz and Edelman, 1963; Stein *et al.*, 1963). The primary structures are fairly well known, and steps toward establishing the secondary and tertiary structures have been initiated (Seon *et al.*, 1971).

Bence Jones proteins are of two immunological types, κ and λ (Korngold and Lipari, 1956). The protein consists solely of free L or light polypeptide chains which are evidently synthesized in excess of heavy or H chains and are then excreted (White *et al.*, 1973). The L chains of myeloma globulin and Bence Jones protein isolated from the same patient are identical, as judged by comparison of two-dimensional high-voltage electrophoresis of their tryptic digests (Schwartz and Edelman, 1963). As in the case of myeloma proteins, the Bence Jones proteins from different patients have never been found to be identical (Stein *et al.*, 1963). For example, Quattrocchi *et al.* (1969) purified 102 Bence Jones proteins by gel filtration, digested them with trypsin, and analyzed them by peptide mapping. No two mappings were the same, and in a few instances in which there was great similarity, differences in amino acid sequence were elicited. In spite of this difference in chemical struc-

ture, there are certain broad common physicochemical features. The majority of Bence Jones proteins have sedimentation constants between 2.44 and 4.40 Svedberg units and diffusion coefficients ($D°_{20,w}$) from 4.7 to 9.8 $\times$ 10^{-7} cm^2/second (Pruzanski and Ogryzlo, 1970). The molecular weights have been listed variously as ranging between 22,000 and 44,000 (Engle and Wallis, 1967) and from 24,000 to 90,000 (Pruzanski and Ogryzlo, 1970). This indicates that Bence Jones proteins may exist as monomers, dimers, trimers, or tetramers.

The difference in the Bence Jones proteins from patient to patient resides in the amino acid sequence. This has now been elucidated for the Bence Jones globulins from a number of patients (Engle and Wallis, 1967; Edelman and Gall, 1969; Ponstingl *et al.*, 1971). The L chains have a sequence of about 214 amino residues. The carboxyl terminal residue of about 107 amino acids is the constant or C region of the L chain and, except for an occasional residue, has the same amino acid sequence in all Bence Jones proteins. In contrast, the amino terminal half, also consisting of about 107 amino acid residues, differs considerably from one patient to another, and has, therefore, been termed the variable or V region of the L chain (Lehninger, 1970). It is this variability which has been the basis for the proposal to divide the κ and λ types into subgroups (Engle and Wallis, 1967; Ponstingl *et al.*, 1971; Milstein, 1967).

The variability in the amino terminal portion allows for great variety in Bence Jones proteins. For example, if any of 40 sites may be occupied by either of two amino acids, then 2^{40} or approximately 10 billion different Bence Jones proteins are possible—enough to supply every person on earth likely to develop multiple myeloma with a different protein for many centuries to come. If these sites can be occupied by any of several amino acids, the possibilities for variety are truly infinite. The same situation holds, of course, for the serum immunoglobulins in multiple myeloma (Lehninger, 1970).

V. Other Immunoglobulinopathies

A. Waldenström's Macroglobulinemia

The disorder, macroglobulinemia, was first described by Waldenström in 1944. Substantial series of cases have been presented since then (Hobbs, 1971; MacKenzie and Fudenberg, 1972). It represents an uncontrolled proliferation of lymphocytoid cells that are intermediate

between lymphocytes and plasma cells and that are capable of synthesizing IgM immunoglobulin. The marrow, blood, nodes, and tissues show infiltration of lymphoid and plasma cells. The condition manifests itself usually in the fifth or sixth decades of life, and the presenting symptoms and clinical findings are lassitude, purpura, enlargement of lymph nodes and spleen, and various neurological effects.

The molecular weight of macroglobulin is about 800,000, and the sedimentation constant, $s_{20,w}$, is about 19 (White *et al.*, 1973). Approximately 50% of cases have serum IgM levels between 3 and 10 gm per 100 ml. Subnormal concentrations of IgG are present in about 5% of cases, and subnormal concentrations of IgA in about 60%. Serum viscosity is frequent in macroglobulinemia and is simply related to increasing serum concentration, with clinical symptoms becoming manifest at levels above 3 gm IgM per 100 ml. The viscosity syndrome is not specific for IgM as it may occur, though less frequently, in conditions characterized by unusual forms or complexes of IgG, IgA, or Bence Jones proteins. The higher viscosity accounts for such clinical symptoms and findings as lassitude, bleeding, petechiae, anemia, renal failure, and simulation of congestive heart failure (Hobbs, 1971).

Reports vary widely concerning the incidence of Bence Jones proteinuria in macroglobulinemia, from rare instances (Engle and Wallis, 1967) to 95% (Hobbs, 1971). Pruzanski and Ogryzlo (1970) reported proteinuria in 6 of 15 patients who were tested. Gross and Epstein (1964) reported a case of macroglobulinemia in which the serum contained Bence Jones protein in addition to IgM. The L chains of the serum IgM component and the Bence Jones protein in the urine had similar thermosolubility properties, sedimentation constants, and peptide maps. Urinary proteins other than Bence Jones protein have also been reported in cases of macroglobulinemia (Pruzanski and Ogryzlo, 1970).

B. Heavy-Chain Diseases

In most instances of myeloma or macroglobulinemia, there is excessive and synchronous production of L chains and one class of H chains which result in the formation of the characteristic protein. In those patients in whom L chains are produced exclusively or in excess of H chains, these are excreted in the urine as Bence Jones proteins. The possibility that the reverse situation might hold and that H chains might be produced asynchronously and be excreted in the urine was recognized by Franklin *et al.* (1964) who described the first case of this disorder in 1964. Reports by others rapidly followed and are still being issued (Osserman and Takatsuki, 1964; Wager, *et al.*, 1969; Ballard *et al.*, 1970;

Lönnroth *et al.*, 1971). These cases have different types of H chain and, in 1971, Hobbs summarized 17 cases of γ-chain disease, approximately 20 cases of α-chain, and about 4 cases of μ-chain.

The clinical findings of H-chain disease resemble, in general, those of malignant lymphoma or Hodgkin's disease, although there are some special points of interest. Lymph node enlargement is frequently present, but may disappear and reappear again. Splenomegaly is consistently present, and hepatomegaly is present in most cases. Edema and redness of the uvula, originally considered as characteristic of γ-chain disease, has been found in other types of lymphoid neoplasia and in other H-chain diseases. The lymph nodes, liver, spleen, and bone marrow of patients with H-disease are infiltrated by large immature "reticuloendothelial cells," immature plasmacytic and lymphocytic elements, and eosinophils (Osserman and Takatsuki, 1964).

The biochemical findings provide a distinctive background for this disorder. In almost all cases, electrophoresis of the serum and urine show a broad-spiked base of β mobility for protein. The early studies (Franklin *et al.*, 1964; Osserman and Takatsuki, 1964) showed that the protein in the serum and urine did not have the characteristics of Bence Jones protein, had molecular weights ranging from 52,000 to 55,000 and that sedimentation constants, $s_{20,w}$, ranging from 3.6 to 3.8, were related immunochemically to each other but were unrelated to L chains. The concentrations of total serum protein were within the normal range or slightly decreased. The concentrations of α_1, α_2, and β-globulin were within the normal range, and those of albumin and of γ-globulin tended to be decreased. The abnormal peak lay between the β-globulin and γ-globulin regions, tended to include the β-globulin peak and exceed the ordinary concentration of β-globulin.

A condition known in the Middle East as Arabian lymphoma of the gut was recognized to be α-H-chain disease (Seligmann *et al.*, 1968, 1969), and Hobbs (1971) described 2 cases with the interesting biochemical finding that the serum alkaline phosphatase reflected an excess of intestinal isoenzyme. Clinically, these patients had an abdominal lymphoma with manifestations of severe malabsorption, associated with a diffuse and predominantly plasmacytic infiltration of the small intestine. Serum electrophoresis showed a broad abnormal band of β mobility comprising approximately 40% of the total protein. The absence of L chains in this protein was demonstrated immunochemically. Analytical ultracentrifugation showed the heavy chain to have an $s_{20,w}$ value of 3.2, with a tendency to polymerize and to yield $s_{20,w}$ values ranging from 4 to 11. The molecular weights ranged from 36,000 to 38,000. The proportion of carbohydrate in the globulin molecule was high, up to 6.6%. Proteinuria

ranged from 5 to 180 mg per 100 ml and consisted of the same IgA-related globulin. The protein belonged to the IgA-1 subclass as determined by specific sera.

The first case of a mu (μ) variant H-chain disease seems to have been reported in a preliminary note by Forte *et al.* (1969) and more fully by Ballard *et al.* (1970). By the end of 1971, a total of 3 cases had been reported (Lee *et al.*, 1971). The clinical findings were hepatosplenomegaly in all 3 cases, and definite pathological fractures in one and probably in another case. Lymphodenopathy was absent in all 3 cases. The disease had been present for 6–21 years, and had been previously diagnosed in all these 3 cases as chronic lymphocytic leukemia.

None of the 3 cases revealed any monoclonal component or spike on electrophoresis of the serum (Lee *et al.*, 1971), and all showed hypogammaglobulinemia. Immunoelectrophoresis revealed an abnormal negatively charged IgM fragment in all 3 cases. Bence Jones proteinuria was present in 2 of the 3 patients. Lee and his co-workers (1971) have suggested that lack of proper coupling of L and H chains in cells, rather than an overproduction of heavy chains, constitutes the biochemical defect in μ-chain disease.

C. Lymphomas

It may be pointed out that the presence of abnormal proteins in the serum and urine of patients with neoplastic tumors of the lymphoid tissue is related to the histogenesis of the cells in this tissue (Wintrobe, 1967; Willis, 1967; Krauss and Sokal, 1966; Alami *et al.*, 1969). In a series of 5 patients with lymphocytic lymphosarcoma, one with Hodgkin's disease, and one with reticulum cell sarcoma, distinct γ-globulin spikes were visible upon cellulose acetate electrophoresis of serum. The concentrations of this peak ranged from 1.44 to 4.56 gm per 100 ml. With two exceptions, ultracentrifugation yielded macroglobulins in the 15.5 S to 18 S range. The serum of 1 patient with lymphocytic lymphosarcoma which had two γ peaks yielded a 7 S protein comprising 47% of the total serum protein (Krauss and Sokal, 1966).

The occurrence of abnormal serum proteins in lymphomas is uncommon. Hobbs (1971) found these serum proteins in 3 cases out of 124 consecutive patients with Hodgkin's disease, in 26 of 207 patients with lymphosarcoma, in one of 45 with reticulosarcoma, and in none of 31 cases with giant follicular sarcoma.

Relatively little information is available concerning the urinary excretion of protein in the lymphomas. Lindström *et al.* (1969) reported an excretion of free L chains above 100 mg/day, the upper limits of

normal, in 7 of 30 patients with lymphoma and in 3 of 23 patients with Hodgkin's disease. The κ type was predominant with the ratio of κ/λ averaging about 12.5:1. The heat test for Bence Jones protein was negative. Alami *et al.* (1969) have reported the case of a patient with lymphosarcoma whose serum showed two sharp peaks in the γ region. They were of the same immunoglobulin class, IgG, but of different L chain antigenic types. Both components had the same molecular size. The urine showed a low molecular weight protein (1.8 S) of κ type; the electrophoretic mobility was in the β region.

D. Leukemias

In 1954, Rundles *et al.* found by electrophoresis abnormal protein constituents in the sera of 6 of 35 patients with various types of leukemia. Four of these 6 had lymphocytic leukemia, one atypical lymphocytic leukemia and one myelogenous leukemia. Many similar reports have appeared in the subsequent years (Osserman and Takatsuki, 1963; Lindström *et al.*, 1969; Hobbs, 1971). Hobbs observed abnormal serum proteins in 3 of 84 cases with chronic lymphatic leukemia, none of 43 cases with chronic myeloid leukemia, none of 57 cases with acute leukemia, and one of 5 patients with chronic myelocytic leukemia. Excretion of L chain proteins was greater than 100 mg/day, the upper limit of normal, in 5 of 11 patients with acute and subactute myelogenous leukemia, in 4 of 7 patients with chronic myelogenous leukemia, and in 2 cases of monocytic leukemia (Lindström *et al.*, 1969).

VI. Biochemical Consequences of Abnormal Proteinemia

The association between abnormal proteins in the immunoglobulinopathies and amyloidosis has been well recognized. Of 35 cases presenting symptoms attributable to amyloidosis, such as nephrosis, bilateral carpal tunnel syndrome, heart failure, thrombosis, malabsorption, and macroglossia, 33 were later shown to have a malignant immunocytoma (Hobbs, 1971). Approximately 8% of all patients with myelomatosis had amyloidosis. It has been shown in one instance that amyloid was derived from the amino terminal segment of an immunoglobulin κ chain (Glenner *et al.*, 1970).

It has long been known that renal function may be impaired in patients with multiple myeloma (Geschickter and Copeland, 1928; Adams *et*

al., 1949; Bayrd and Heck, 1947). More generally, several types of renal damage as a result of abnormal proteins are possible (Hobbs, 1971): (a) blockage of the distal tubules by casts containing abnormal proteins, with giant cell formation; (b) amyloid deposits with resulting nephrosis and azotemia and a predisposition to renal vein thrombosis; (c) an acquired Fanconi syndrome (Costanza and Smoller, 1963; Handley and Arney, 1967); (d) nitrogen retention without Bence Jones proteinuria in IgA myelomatosis; (e) nephrosis without Bence Jones proteinuria in IgA myelomatosis; (f) pyelonephritis, presumably as a result of immune paresis; (g) hypercalcemia; and (h) renal failure in the viscosity syndrome or in cold aggregation.

Low concentrations of plasma sodium, that is, below 120 mEq/liter, are encountered in about 8% of patients with IgG myelomatosis. Although sodium is distributed in the plasma water, the measurement of concentration is usually made on the basis of volume of whole plasma. The concentration of sodium in the plasma water may be normal, but because of the high concentration of proteins, the concentration of sodium, calculated as usual per 100 ml of plasma, is apparently low. In addition, a high isoelectric point of the paraprotein may allow it to act as a base at normal blood pH (Hobbs, 1971).

The term "cryoglobulinemia" refers to the presence in plasma or serum of proteins which precipitate upon cooling at 4°C for 24 hours. The precipitate redissolves when the serum plasma is returned to 37°C. Gradual lowering of the temperature and noting the temperature at which a precipitate begins to appear offer a basis for distinguishing between various types of protein. According to Hobbs (1971), most cryoproteins that cause symptoms precipitate at temperatures above 28°C. The symptoms are manifested as skin lesions which are flat areas of necrosis, marginated by erythema, and result from simple occlusion of peripheral vessels. Hobbs (1971) has reported that, in some 1000 consecutive cases of the presence of abnormal protein in blood and urine, only a minority precipitated between 4° and 20°C. Six cases of multiple myeloma were observed that had Bence Jones proteins precipitating in the cold, under 20°C, and causing no symptoms in the patients.

We have already referred to the viscosity syndrome which was first recorded for macroglobulinemia by Waldenström (1961), but has been found in approximately 4% of cases with IgG-myelomatosis, and occasionally with IgA-myelomatosis and with Bence Jones proteinemia. The high viscosity of plasma or serum in these cases seems to depend not so much on the high concentration of protein (IgM), as is the case in macroglobulinemia, but rather on unusual forms of IgG, IgA, or Bence Jones protein, or on complexes of these with lipoproteins, or on poly-

merization (Hobbs, 1969). Smith *et al.* (1965) reported 2 patients with multiple myeloma who had mucous membrane bleeding and retinal vascular disturbances which were related to serum hyperviscosity. The paraproteins in these 2 cases were shown to be acid-dissociable IgG myeloma globulin aggregates of greater than 7S.

VII. Immunological Deficits in Malignancy

There is now a considerable body of data, both old and more recent, indicating an association between immunological deficits and malignancy. For example, Jim (1957) studied the distribution of the various serum protein components in a series of 50 patients with chronic lymphocytic leukemia. The concentration of serum γ-globulin ranged from 0 to 48.9% of the total serum protein, as compared with an average value of 17.3% in 15 normal subjects. No values for the normal ranges of γ-globulin or for the total serum protein were given. Jim (1957) stated that hypogammaglobulinemia was present in approximately one-third and hypergammaglobulinemia in about one-fourth of the patients. Of 7 males who were studied by Brem and Morton (1955) and who had seriously depressed concentrations of serum γ-globulin, as determined electrophoretically, 2 had long histories of respiratory infections and developed chronic lymphocytic leukemia late in life. In 3 other patients, lymphadenopathy and splenomegaly were prominent features, and the diagnosis of giant follicular lymphoma was either made or seriously considered on the basis of lymph node biopsy. However, autopsy in one case and clinical course in the two who remained living did not support this diagnosis.

In a most-detailed study on 61 patients with chronic lymphocytic leukemia, Creysell *et al.* (1958) found a bimodal distribution of the serum γ-globulin levels. One of these populations consisting of 32 cases had a mean value of 0.516 ± 0.2 (SD) gm per 100 ml; the second, consisting of 24 cases had a mean value of 1.063 ± 0.148 (SD) gm per 100 ml, well within the normal range. The remaining few cases exhibited hypergammaglobulinemia. A statistically significant correlation existed between hypogammaglobulinemia and the incidence of infections.

Page *et al.* (1963) reported 2 cases of interest in connection with the association of immunological deficits and malignancy. The first was a child who developed recurrent bouts of upper respiratory and gastrointestinal infections at the age of 6 months. Serum electrophoresis at the age of 3 years revealed absence of the γ-globulin fraction. The child died at the age of 4 years, 8 months, and a diagnosis of malignant

lymphoma was based on the findings in the lymph nodes, liver, and kidney. The second patient was the brother of a boy with congenital agammaglobulinemia, and his serum γ-globulin levels were followed from birth. These dropped precipitously to 10 mg per 100 ml at 1 year, and rose to 160 mg per 100 ml at 3 years of age. Immunoelectrophoresis was done at intervals, and each time showed very low levels of γ-globulin and complete absence of β_2A- and β_2M-globulin. At the age of 4, he developed a large mediastinal mass, and examination of peripheral blood and of the bone marrow led to the diagnosis of acute lymphocytic leukemia.

The relationship of cancer to immunodeficiency has been treated more fully by Good and his associates (Good and Finstad, 1969; Gatti and Good, 1970a,b, 1971; Good, 1973; Kersey *et al.*, 1973). In a comprehensive literature survey on cancer in patients with immunodeficiency diseases, Gatti and Good (1971) estimated that the frequency of malignancy in patients with primary immunodeficiencies is approximately 10,000 times greater than that of the general age-matched population. Good and Finstad (1969) suggested that malignancy and immunity can be considered as antithetical adaptive processes and that "successful malignant adaptation reflects in some way defective immunologic adaptation."

These concepts may now be illustrated in specific human diseases. Earlier in this chapter (Section II,A), we considered the roles of the bursal (B) and the central thymus (T) systems in immunity, as formulated by Good (1971). Sex-linked (Bruton's) agammaglobulinemia is an example of an abnormality of the B cell system. The disorder is associated with absence of immunoglobulin-secreting plasma cells, usually with few or no B lymphocytes, and with unusual susceptibility to infection with encapsulated pathogenic bacteria. Of 50–60 patients with this condition, 5 developed malignancy, all of which were either leukemias or lymphomas (Kersey *et al.*, 1973). Ataxia-telangiectasia, a heredofamilial, progressive ataxia associated with occulocutaneous telangiectasia (dilation of the capillary vessels and minute arteries) involves both the T cell and B cell systems. Of an estimated 450 individuals who have been reported with this syndrome, 45 have been noted as having developed cancer (Kersey *et al.*, 1973). This number included 5 epithelial malignancies, such as gastric adenocarcinoma and ovarian dysgerminoma; 29 lymphoreticular tumors, such as Hodgkin's disease, lymphosarcoma, and reticulum cell sarcoma; 7 leukemias; 2 mesenchymal malignancies; and 2 nervous system tumors. Other examples of primary immunodeficiency diseases which are associated with a high incidence of cancer development are the Wiskott-Aldrich syndrome and the common variable ("late-onset") primary immunodeficiency.

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6

Tryptophan Metabolism: Cancer of the Bladder; the Carcinoid Syndrome

I. Introduction

Because of convenience from a biochemical point of view, we shall discuss in this chapter two greatly different types of neoplasms: cancer of the bladder, and the carcinoid syndrome. The basis for this common discussion is that the metabolism of the amino acid, tryptophan, has been greatly involved in considerations of the biochemical aspects of both of these neoplasms.

Tryptophan cannot be synthesized by the animal organism. It is therefore, one of the essential amino acids and must be supplied in the food. Two main pathways of tryptophan metabolism exist in normal mammalian tissues. The first involves the breakage of the indole ring with the formation of *N*-formylkynurenine and thence, through various minor pathways, of quinaldic, 8-hydroxyquinaldic, glutaric, and nicotinic acids. The second major pathway consists of the conversion to indoleacetic acid or through preliminary hydroxylation to 5-hydroxytryptophan and thence to 5-hydroxyindoleacetic acid. These steps will be shown in detail later.

II. Cancer of the Bladder

A. Introduction

It has been estimated that 20,800 new cases of bladder cancer will occur in 1973, and that 9,200 will die of this neoplasm during the same year (Silverberg and Holleb, 1973). The incidence in males is about 2–3 times as high as in females. Although, as in many other types of neoplasia, causative factors in cancer of the bladder are largely unknown, it has been appreciated for many years that chronic industrial exposure to certain dye intermediates constitutes a high risk of carcinoma of the bladder. There are many reports in the literature of Germany, England, Russia, and the United States concerning the high incidence of cancer in persons connected with the dye industries in these countries (Ackerman and del Regato, 1970).

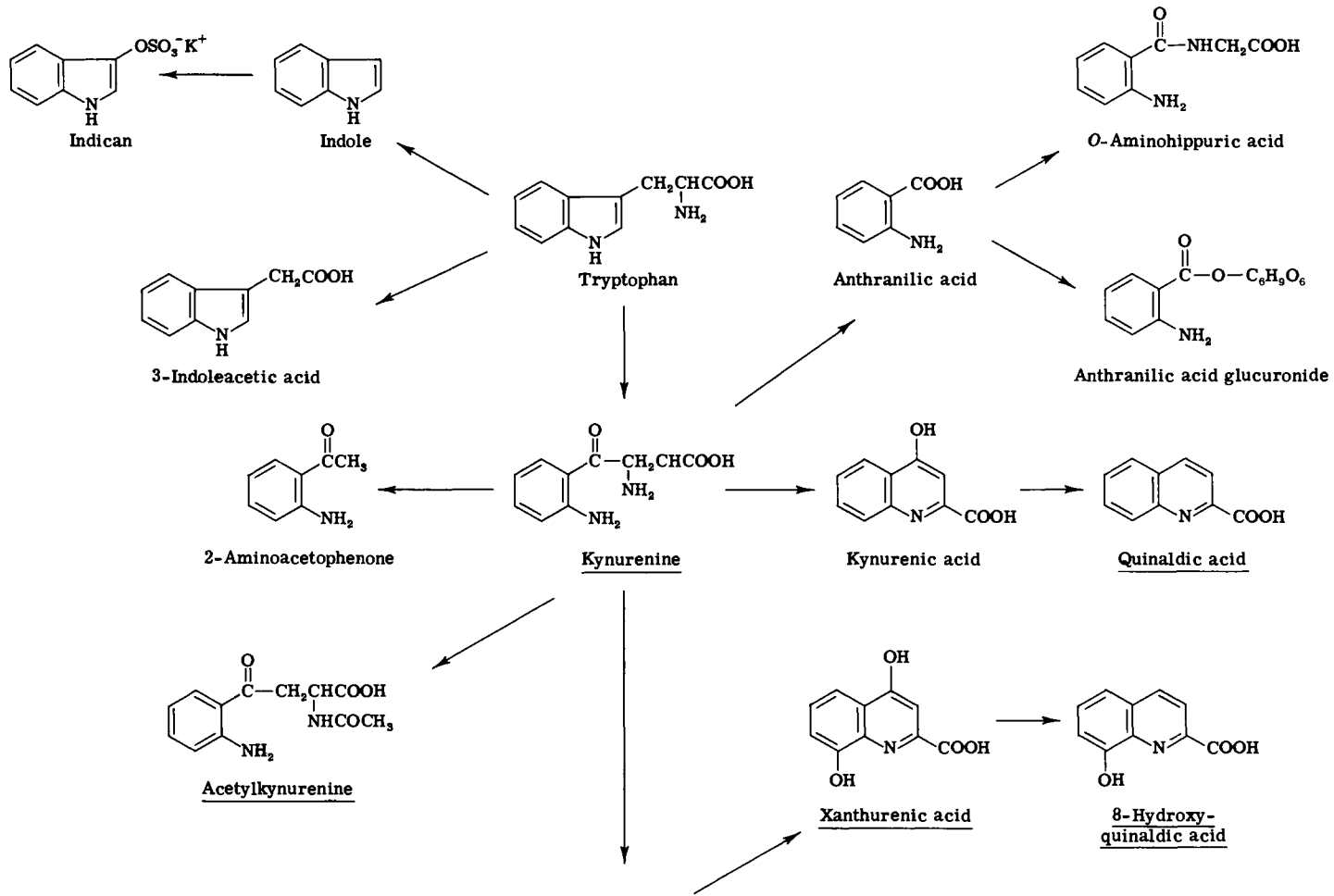
Tumors of the bladder arise from the transitional epithelium, and as they grow to form well-differentiated tumors they become supported by vascularized connective tissue stroma. They have been classified into various stages depending upon their freedom of movement, extent of induration of the bladder wall, and extension into neighboring organs such as the vagina or prostate. The most common initial symptoms are hematuria and frequency of urination; dysuria and pain unrelated to urination occur in decreasing incidence.

The possibility that cancer of the bladder in man might be associated with abnormal metabolism of tryptophan was first suggested by Price and his associates (Brown *et al.*, 1955; Price *et al.*, 1965a) and was based on early observations by Dunning *et al.* (1950) that carcinoma of the bladder developed in rats when the feeding of 2-acetylaminofluorene was supplemented with DL-tryptophan. Price, Brown, and their associates (Price, 1958; Brown *et al.*, 1960) pointed out that the metabolites of tryptophan might resemble aromatic amine structures that had been implicated in the genesis of animal and human bladder cancer.

B. Tryptophan Metabolism

As was indicated above, one of the two major pathways of tryptophan metabolism consists of the scission of the indole ring and the subsequent reactions, as shown in Fig. 6-1.

The daily urinary excretion of these metabolites in normal individuals


Indican
Indole
Tryptophan
O-Aminohippuric acid
Anthranilic acid
3-Indoleacetic acid
Anthranilic acid glucuronide
2-Aminoacetophenone
Kynurenine
Kynurenic acid
Quinaldic acid
Acetylkynurenine
Xanthurenic acid
8-Hydroxy-quinaldic acid

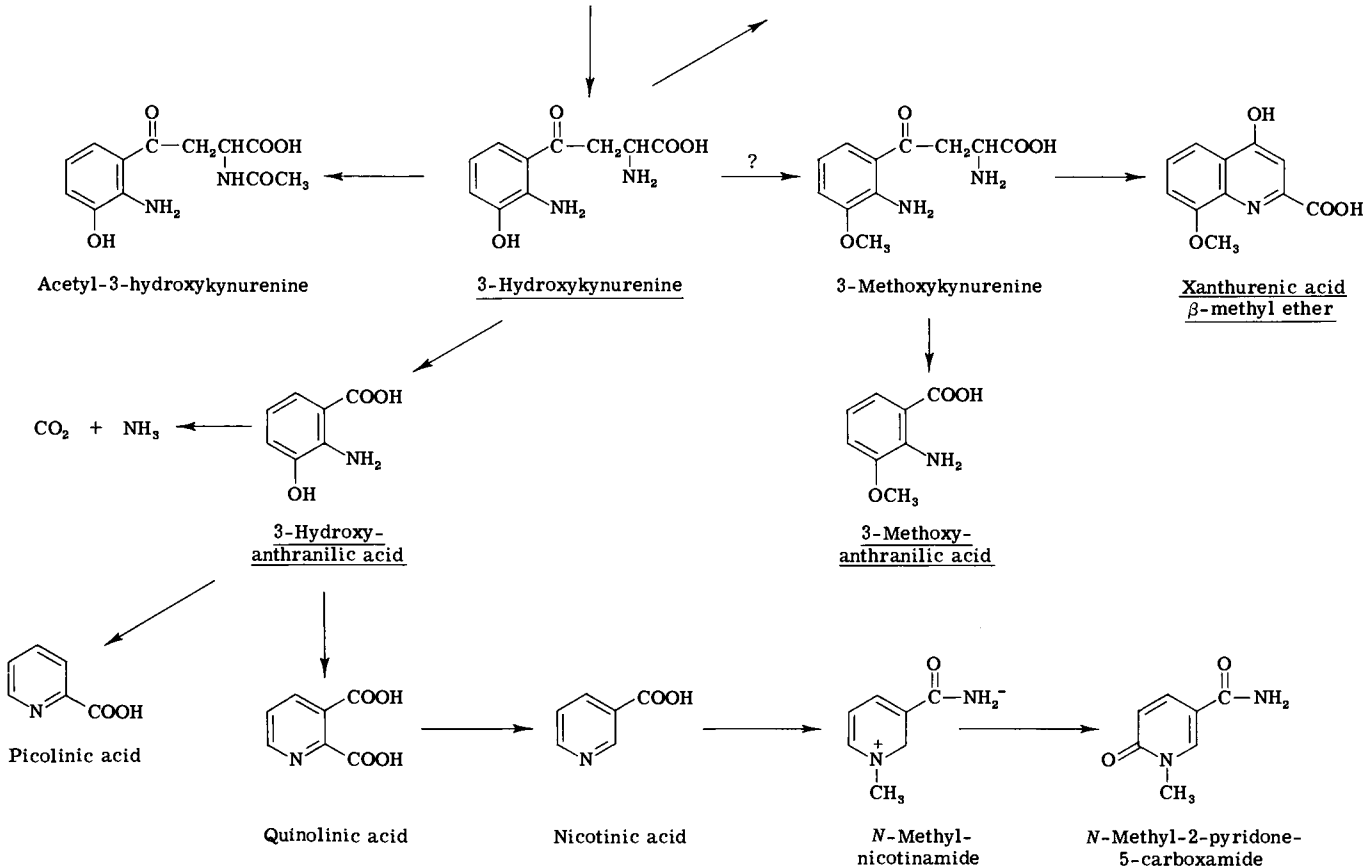


Fig. 6-1 Metabolic scheme of the conversion of tryptophan to niacin, illustrating structures of metabolites tested as potential bladder carcinogens. Compounds underlined have been shown to be carcinogenic for the mouse bladder. From Yoshida *et al.* (1970). Reproduced by permission of J. B. Lippincott Company.

has been studied by several groups of investigators (Boyland and Williams, 1956; Tompsett, 1959; Price, 1958; Musajo and Benassi, 1964). As will be noted later, differences exist between the values obtained by these groups and are attributable to differences in methodology and specificity of the procedure employed (Musajo and Benassi, 1964). In evaluating the effects of various physiological factors or of disease, it is advisable to compare the normal and abnormal values obtained by the same investigator.

Table 6-1 shows that the excretions of various tryptophan metabolites are higher in patients with cancer of the bladder than in normal controls. For example, Benassi *et al.* (1963) presented substantial data on the excretion of various tryptophan metabolites in a series of patients with bladder cancer and other urological diseases. These data for 26 patients with cancer of the bladder permit the calculation of a mean value of 90.9 ± 10.8 (SE) mg for the daily urinary excretion of kynurenine and of 20.9 ± 1.3 (SE) mg for the daily urinary excretion of 3-hydroxykynurenine. These are substantially higher than members of the same laboratory obtained for normal individuals (Musajo and Benassi, 1964).

But several groups of investigators have stressed that a substantial proportion of patients with cancer of the bladder do not show any abnormal excretion of any of the major metabolites of tryptophan. Benassi *et al.* (1963) found that the daily urinary excretion of one or more of the following metabolites or combinaton—kynurenine, kynurenine and 3-hydroxykynurenine, 3-hydroxyanthanilic acid, kynurenine + 3-hydroxyanthranilic—was abnormal in only 60 of 201 cases with carcinoma of the bladder. Although standard errors are not readily available for the data obtained by Brown *et al.* (1960), this point is also fairly well illustrated by the mean values recorded in Table 6-2. Of 41 patients with cancer of the bladder, 20 had abnormal metabolism and 21 had normal metabolism. It may be seen that there is no essential difference between the latter group and the control subjects with respect to spontaneous urinary excretion of any of the metabolites.

The loading test, that is, the assay of metabolites after the administration of a stated dose of L-tryptophan does not elicit any differences between the normal controls and those patients with bladder cancer and normal metabolism, but it does accentuate the differences in the group of patients with abnormal metabolism (Table 6-2). For example, the average spontaneous daily excretions of kynurenine in the normal subjects and in the cancer patients with normal metabolism were essentially the same, 12 and 16 μmoles, respectively. After a loading dose of 2 gm L-tryptophan, the daily excretions were raised to 41 and 38 μmoles, again essentially the same level. In the patients with bladder

TABLE 6-1

Spontaneous Excretion of Metabolites of Kynurenine Pathway in Cancer of the Bladder

Metabolites	Boyland and Williams (1956)		Brown *et al.* (1960)		Musajo and Benassi (1964)	Benassi *et al.* (1963)
	10 controls (mg/day)	10 patients (mg/day)	30 controls (μmole/day)	20 patients[a] (μmole/day)	20 controls (mg/day)	26 patients (mg/day)
Anthranilic acid	19 (9–36)	89 (51–142)	—	—	—	—
3-Hydroxyanthranilic acid	31 (13–50)	150 (62–282)	—	—	—	—
3-Hydroxyanthranilic acid-*o*-sulfate	14 (8–19)	13 (3.4–22)	—	—	—	—
Kynurenine	16 (5–19)	69 (34–93)	12	31	1.1	90.9 ± 10.8[b]
3-Hydroxykynurenine	3 (6–8)	17 (7–31)	26	39	0.49	20.9 ± 1.3[b]
3-Hydroxykynurenine sulfate	8 (5–14)	3 (0–10)	—	—	—	—
Kynurenic acid	—	—	11	10	—	—
Acetyl kynurenine	—	—	12	20	—	—
Xanthurenic acid	—	—	10	8	—	—

[a] With abnormal metabolism (see text).

[b] Standard error of mean.

TABLE 6-2

Urinary Excretion of Tryotophan Metabolites after Loading Dose in Normals and In Group of Patients with Cancer of the Bladder[a,b]

Metabolites	Normal controls		Patients with cancer of the bladder and			
			Normal metabolism		Abnormal metabolism	
	Basal	+ Tryptophan	Basal	+ Tryptophan	Basal	+ Tryptophan
Kynurenine	12	41	16	38	31	198
Kynurenic acid	11	62	10	44	10	110
Acetylkynurenine	12	19	12	19	20	57
3-Hydroxykynurenine	26	57	26	54	39	151
Xanthurenic acid	10	45	9	26	8	52
Anthranilic acid glucuronide	5	8	4	7	7	10
o-Aminohippuric acid	24	46	19	38	26	61
N-methyl-2-pyridone-5-carboxamide	71	134	80	117	81	116
Kynurenine + kynurenic acid + acetylkynurenine	35	122	38	102	60	366

[a] Data of Brown *et al.* (1960).

[b] Thirty controls; 21 patients with cancer of the bladder and normal metabolism; 20 patients with cancer of the bladder and abnormal metabolism. In micromoles per day. Loading dose was 2 gm L-tryptophan.

cancer and abnormal metabolism, the spontaneous daily excretion was 31 μmoles, substantially higher than that in the other two groups, and a loading dose of 2 gm L-tryptophan raised the excretion to a very high level, 198 μmoles. Essentially the same effects were observed with the other metabolites of tryptophan as shown in Table 6-2 and have been confirmed by Benassi *et al.* (1963), using a much larger loading dose of L-tryptophan, namely, 490 μmoles (100 mg)/kg. The sum of the metabolites, expressed as a fraction of the administered tryptophan, was 9.89% for the bladder tumor patients and significantly higher than the value, 6.67% for the normal controls.

Evidence has been presented to support the hypothesis that tryptophan metabolites may play a role in human bladder carcinogenesis. It has already been noted that induction of bladder tumors has been accomplished by feeding rats 2-acetylaminofluorene in combination with tryptophan (Dunning *et al.*, 1950) and by implanting pellets of 3-hydroxyanthranilic acid in the bladder of mice (Boyland and Watson, 1956). Yoshida *et al.* (1970) studied tryptophan metabolites in 38 patients with low stage bladder tumors. One or more basal 24-hour urine specimens were collected; the patient was given an oral dose of 2.0 gm L-tryptophan, and the urine was collected for several days thereafter. The tryptophan metabolism was deemed abnormal if, after an oral loading dose of 2.0 gm L-tryptophan, the excretion of two or more of the urinary metabolites was greater by two standard deviations than the mean values for the corresponding metabolites in healthy control subjects (Price *et al.*, 1965b).

In accordance with this criterion, 20 of the 38 patients had normal tryptophan metabolism, and the remaining 18 had abnormal metabolism. The patients were all followed up by periodic cystoscopic examination at 3-month intervals for the first year, and at 6-month intervals for the lifetime of the patient or until detection of recurrence. Diagnoses of heterotopic recurrence were based on the presence of a tumor at a site different from that of the primary lesion. All 18 patients with abnormal tryptophan metabolism had one or more recurrences within 5 years. Among the 20 patients with normal tryptophan metabolism, only 12 patients had recurrences within this period. The distributions may be shown to be significantly different ($\chi^2 = 9.12$).

C. Tryptophan Metabolism in Other Types of Cancer

Suggestive as the preceding results are that tryptophan metabolites may play a role in heterotopic recurrence of bladder tumors in patients

with abnormal tryptophan metabolism and, by implication, in the genesis of the primary tumor, the question arises whether this metabolism is specific for bladder cancer, or whether it prevails in patients with other types of cancer and, indeed, in patients with nonneoplastic diseases. Employing as criterion of abnormal tryptophan metabolism high urinary excretions of one or more of a group of metabolites, Musajo and Benassi (1964) obtained the following incidences of increased excretion of tryptophan metabolites: 30% of 201 patients with bladder tumors, 49% of 55 patients with extrabladder tumors, 59% of 32 patients with kidney tumors, and 15% of 112 patients with nonneoplastic urological diseases.

The extents of these increases are illustrated in Table 6-3. Rose (1967) studied the excretion of tryptophan metabolites in patients with carcinoma of the breast who had been treated by mastectomy alone and by mastectomy and oophrectomy. The loading dose was 5 gm or 24,500 μmoles of L-tryptophan which corresponds to about 450 μmoles/kg. The control group consisted of 12 female members of the hospital staff and 3 hospital patients without evidence of malignancy, hepatic or renal disease. The mean values for the urinary 8-hour excretion of these metabolites in the patients with carcinoma of the breast who had been mastec-

TABLE 6-3

Urinary Excretion of Tryptophan Metabolites in Patients with Cancer after Loading Dose of Tryptophan

	Carcinoma of breast (Rose, 1967)[a]		Hodgkin's disease (Crepaldi and Parpajola, 1964)[c]	
Metabolite	Controls $N = 15$ Mean ± SE μmole/8 hr	Mastectomized patients $N = 20$ Mean ± SE μmole/8 hr	Controls $N = 5$ Mean μmole/24 hr	Patients $N = 8$ Mean μmole/24 hr
Kynurenine	—	—	210	1165
3-Hydroxyurenine	360 ± 30	517 ± 44[b]	34	284
Kynurenic acid	—	—	176	258
Xanthurenic acid	178 ± 19	389 ± 58[b]	41	182
3-Hydroxyanthranilic acid	185 ± 24	404 ± 70[b]	89	113

[a] Mean values and standard errors were calculated from original data of Rose (1967). The loading dose was 24,500 μmoles (5 gm) or 450 μmoles/kg.

[b] Significantly different from control values at $p < 0.01$.

[c] The load in study of Hodgkin's disease was 245 μmoles/kg.

tomized were significantly higher than those of the normal subjects (Table 6-3).

The excretion of tryptophan metabolites in Hodgkin's disease is also shown in Table 6-3. It may be noted that the control values, presumably for subjects without clinically recognizable disease, are considerably lower in this study by Crepaldi and Parpajola (1964) than the values for the normal subjects in Rose's study (1967). The loading dose is about one-half that in Rose's study, but the collection period was much longer. It is difficult to explain this discrepancy on any other basis than a methodological one. However, with respect to the control values in the same study, the excretions of tryptophan metabolites in the patients with Hodgkin's disease are greatly increased.

In a more recent study (Chabner *et al.*, 1970), the daily urinary excretions in μmoles $\pm$ SD for 9 female normal subjects after a 2-gm loading dose were kynurenine, 33.8 ± 9.7; 3-hydroxykynurenine, 34.5 ± 14.1; and xanthurenic acid, 33.2 ± 20.4. The corresponding values for 9 male normal subjects were 67.0 ± 35.5, 42.4 ± 28.9, and 37.1 ± 9.6. Thus, except for kynurenine, these values were of the same order of magnitude as those obtained by Crepaldi and Parpajola (1964). Chabner *et al.* (1970) found that about 60–70% of 21 patients with Hodgkin's disease excreted increased quantities of at least one of the three metabolites listed above. In some of the patients with advanced disease, the excretion of kynurenine and hydroxykynurenine were greatly elevated, ranging between about 400 and 900 μmoles/day after the 2-gm loading dose. Plasma levels of pyridoxal phosphate were depressed in 8 of 14 untreated patients tested. Twelve patients in complete remission after chemotherapy all had normal plasma levels of pyridoxal phosphate. Pyridoxine was administered to 3 patients with far advanced Hodgkin's disease, and the plasma pyridoxal phosphate rose from low or normal levels to supranormal levels.

D. Tryptophan Metabolism in Nonneoplastic Disease

We have already indicated (Section II, C) that increased excretion of tryptophan metabolites may occur in nonneoplastic urological disease. But several other conditions and diseases bear this characteristic. Thus, in their 1964 review, Musajo and Benassi listed the following conditions among others as having been reported to have high excretions of one or more tryptophan metabolites: pregnancy, old age, schizophrenia, infectious hepatitis, diabetes, and rheumatoid arthritis. However, there have been conflicting reports for some of these conditions. For example, Wiseman *et al.* (1958) found no difference between diabetic patients and

normal individuals in the excretion of tryptophan metabolites during a basal period, except for a lower level of kynurenine. After ingestion of 4 gm of L-tryptophan, the mean values in a group of 12 diabetic patients for the incremental excretions of tryptophan, kynurenine, and anthranilic and xanthurenic acids were less than the mean values for the incremental excretions of the corresponding metabolites in a group of 6 control subjects.

It may be appreciated from the preceding discussion that the finding of deranged tryptophan metabolism in cancer of the bladder, originally considered to be specific for this condition, has been generally observed to hold for other varieties of neoplastic disease and, indeed, for other diseases as well. Altman and Greengard (1966) reported that the administration of hydrocortisone in man caused a two- to fourfold increase in the level of liver tryptophan pyrrolase in needle biopsy specimens and an increased urinary excretion of kynurenine. These findings raised the possibility that abnormal tryptophan metabolism observed in a variety of diseases is the result of a stress-induced increase in tryptophan pyrrolase in all of these diseases. Humans on pyridoxine (vitamin B_6)-deficient diet or those fed deoxypyridine have been found to excrete abnormally large amounts of xanthurenic acid and other tryptophan metabolites (Greenberg *et al.*, 1949; Glazer *et al.*, 1951). It is possible that subclinical deficiency of this vitamin in several disease states, and not the disease *per se*, may contribute to increased excretion of tryptophan metabolites.

Yet it may be premature to conclude that the increased excretion of tryptophan metabolites and the character of the pattern of this increased excretion are nonspecific for cancer of the bladder. The tryptophan-niacin pathway is extraorinarily complex and it is possible that a variety of diseases affect it, each in its own way, at a specific point or at a specific constellation of points. This possibility is revealed by the discovery of several hereditary disorders (Baron *et al.*, 1956; Milne *et al.*, 1960; Knapp, 1960; Komrower *et al.*, 1964).

Clinical investigation in the field of tryptophan metabolism has been burdened by changing methodology. Although the importance of tryptophan intake with regard to the level of excretion of its metabolites has been recognized in the loading test, the level of the intake in the ordinary diet has not usually been evaluated or regulated. Quantitative methods for assaying the amounts of metabolites in the urine have been changing during the past decade or two, and it was only in 1965 that a systematic scheme for such assays was submitted (Price *et al.*, 1965b). It is possible that further studies of the tryptophan-nicotinic acid pathway may reveal differential features between cancer and other diseases.

III. Carcinoid Tumors and the Carcinoid Syndrome

A. Introduction

Originally and classically, the symptoms of carcinoid tumors were associated with their presence in the gastrointestinal tract. The tumors are situated most frequently near or in the appendix. The lesions are usually less than 3 cm in diameter, yellow or yellowish gray in color, and moderately firm. Metastases are associated most frequently with primary tumors of the ileum, and then occur in only about 50% of the cases. The most common sites of metastases are liver, mesentery, and the abdominal, pelvic, or thoracic lymph nodes. The cells of the tumor have large ovoid nuclei, are arranged in sheets, strands, and clusters, tend to be palisaded at the periphery, and extend into the muscularis. The cells arise presumably from the silver-positive (argentaffin) Kulchitshy's cells situated in the bases of the crypts of Lieberkhun and, for this reason, are frequently known as argentaffinomas (Sjoerdsma *et al.*, 1956; Pernow and Waldenström, 1957; Bodansky, 1963).

Carcinoid tumors can occur wherever Kulchitsky's or argentaffin cells are located and, hence, can arise outside the small intestine, including the bronchus (Williams and Azzopardi, 1960; Melmon *et al.*, 1965), the ovary (Pernow and Waldenström, 1957; Torvik, 1960), the stomach (Oates and Sjoerdsma, 1962), the biliary tract (Rosenbaum *et al.*, 1953), and the pancreas (Murray *et al.*, 1961; Gordon *et al.*, 1971). According to the series of Linnel and Månsson (1966), bronchial carcinoids were the only type found outside the gastrointestinal tract, and constituted 77% of the total number of 58 cases of carcinoids found at autopsy in a 4-year period in a city with 230,000 inhabitants.

The World Health Organization (1970) monograph, "Mortality from Malignant Neoplasms," lists the frequency according to site and, therefore, does not contain the mortality rate from the carcinoid syndrome. Linell and Månsson (1966) have reviewed the frequency of gastrointestinal carcinoid in various large autopsy series reported in the literature, and found it to range from 0.14 to 0.65%. Kuehn *et al.* (1973) reported a total of 550 carcinoid tumor cases registered in the Connecticut Tumor Registry. Hajdu and Myers (1973) noted that 204 cases had been diagnosed at Memorial Hospital for Cancer and Allied Diseases during the 20-year period from 1950 to 1969. The incidence or frequency of cases diagnosed during a defined period has been estimated at 1 in 100,000 population per year, at least for the town of Malmö (Linell and Månsson, 1966).

B. Correlation of Clinical Symptoms and Tryptophan Metabolism

1. Introduction

In 1952, Biörck and his associates noted at autopsy a malignant carcinoid of the jejunum in a 19-year-old boy with congenital pulmonary stenosis and tricuspid insufficiency. The patient had died following angiocardiography. Two years later, these investigators observed several other cases, realized that this association was not accidental, and suggested that it was a new syndrome characterized by flushes, cyanosis, chronic diarrhea, respiratory distress, and valvular disease of the heart. It was later found that the symptomatology was associated with the production of metabolites through the tryptophan-5-hydroxyindoleacetic pathway (Thorson *et al.*, 1954; Pernow and Waldenström, 1957; Sjoerdsma *et al.*, 1956).

Since these early reports, the concept of the syndrome has broadened greatly. 5-Hydroxytryptamine, originally found in the carcinoid and considered to be chiefly a vasoconstrictor, has been shown to have several functions. As has just been noted, carcinoid tumors have been found at other sites. Variant syndromes have been revealed which secrete large amounts of histamine and 5-hydroxytryptophan (5-HTP) rather than 5-hydroxytryptamine (5-HT or serotonin) or in which there is no elevated excretion of 5-hydroxyindoleacetic acid (5-HIAA). Indeed, the term "carcinoid spectrum" has been proposed to designate this variability (Sjoerdsma and Melmon, 1964).

2. The Tryptophan-5-Hydroxyindoleacetic Acid Metabolic Pathway

The chief sequence of reactions occurs after the hydroxylation of tryptophan and is pictured in Fig. 6-2. Another parallel, but less important, pathway involves the decarboxylation of tryptophan to form tryptamine. This is acted upon by monoamine oxidase to form indoleacetaldehyde, and the latter is converted to indoleacetic acid by aldehyde oxidase. Tryptophan can also be oxidatively deaminated to form indolepyruvic acid.

3. Tryptophan Metabolites in Tissues

As the reader will note from Fig. 6-2, the formation of 5-HT (serotonin) from tryptophan occurs in 2 steps: the hydroxylation of tryptophan and its subsequent decarboxylation. The enzymes, tryptophan 5-

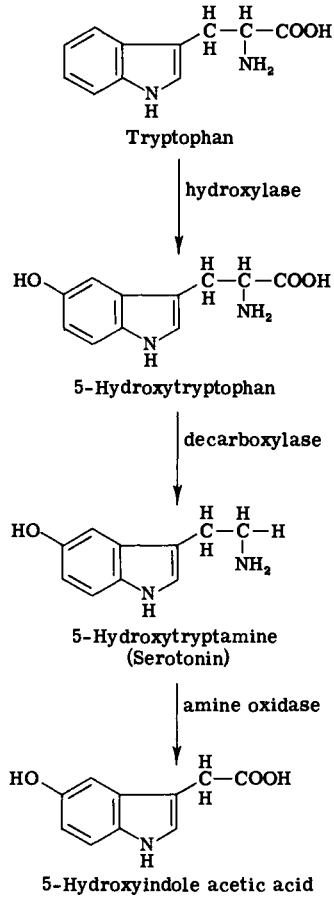


Fig. 6-2 The 5-hydroxyindole pathway of tryptophan metabolism.

hydroxylase and the aromatic L-amino acid decarboxylase, that mediate these reactions are present in carcinoid tumors as well as in normal tissues. The 5-HT that is formed in the tumor is probably stored in the argentaffin granules (Grahame-Smith, 1968). The precise mechanism by which it is released into the circulation from these granules is not yet known but, once released, it is quickly bound to platelets (Zucker *et al.*, 1954; Stacey, 1961). Accordingly, the whole blood reveals the concentration of 5-HT in the circulation, whereas plasma centrifuged from the cellular elements may not do so. During the collection of serum, the platelets may be lysed and the 5-HT liberated. Physiologically, 5-HT is carried by the platelets in the circulation, but the mechanism of the release

of 5-HT from the platelets at various tissues where it exerts its effects is not yet known.

The presence of large amounts of 5-HT (2.5 mg/gm wet weight) in carcinoid tumors was first demonstrated by Lembeck (1953). Pernow and Waldenström (1957) found values, ranging from 15 to 432 μg/gm wet weight of carcinoid tumor, in liver and mesenteric node metastases from patients with increased urinary excretion of 5-HT and 5-HIAA. Concentrations of 5-HT from the ileum adjacent to the site of the tumor, or for kidney, brain, or myocardium range up to only about 4 μg/gm of tissue (Grahame-Smith, 1964).

It has been estimated that in the normal individual about 1–3% of dietary tryptophan is metabolized through the pathway indicated in Fig. 6-2 (Grahame-Smith, 1968; White *et al.*, 1968). The urinary excretion of 5-hydroxyindoles remains constant over a wide range of tryptophan intake. In the carcinoid patients, on the other hand, the excretion of these substances increases with the amount of tryptophan in the diet, and the tryptophan-hydroxyindole pathway is favored. Thus, in one case studied in some detail by Sjoerdsma *et al.* (1956), at a daily intake of 500 mg of tryptophan as much as 60% was converted to 5-hydroxyindoles.

4. Tryptophan-Hydroxyindoleacetic Acid Metabolites in Blood and Urine

The intensity of the tryptophan-hydroxyindole pathway in carcinoid tissue is reflected in the blood and urine. A convenient chemical method of assay for urinary 5-HIAA was made available by Udenfriend *et al.* (1955) and the biological method for 5-hydroxytryptamine (5-HT) was soon replaced by a chemical method, also proposed by Udenfriend and his associates (1955).

The distinctive biochemical findings in the carcinoid syndrome are an elevated level of 5-HT in the blood and an increased urinary excretion of 5-HIAA. Sjoerdsma *et al.* (1956) reported a range of 0.1–0.3 μg 5-HT per ml blood in a series of 40 normal individuals. Pernow and Waldenström (1957) recorded a normal range of 0.03–0.2 μg/ml serum. As we have already indicated, most, if not all, of the circulating 5-HT is bound to and stored by the platelets (Zucker *et al.*, 1954). The process of clotting of the blood to obtain serum causes the release of 5-HT from the platelets. Table 6-4 shows that of 13 nonoperated patients and in operated cases in which the carcinoid tumor was not completely resectable, 10 had elevated blood serum levels of 5-HT, ranging from 0.25 to 5.2 μg/ml.

TABLE 6-4

Blood Serum 5-HT and 24-Hour Urinary Excretions of 5-HIAA in Patients with Carcinoid Tumor

	Blood serum 5-HT in μg/ml					Urinary excretion of 5-HIAA in mg/24 hr						
Investigator	No. of cases	0.03–0.2[a]	0.21–0.5	0.51–1.0	1.1–5.2	No. of cases	2–10[a]	11–50	51–100	101–200	201–452	453–1270
Pernow and Waldenström (1957)	13[b,c]	3	3	3	4	21[b,c]	1	10	1	6	3	—
Roberts and Sjoerdsma (1964)	—	—	—	—	—	15[b,d]	0	1	2	2	5	5

[a] Normal range.
[b] These included nonoperated cases and cases in which the carcinoid tumor was not completely resectable.
[c] The highest value for each individual was employed.
[d] Cases of carcinoid heart disease.

The urinary excretion of 5-HT as well as of 5-HIAA is elevated in patients with carcinoid tumor. The normal urinary excretion of 5-HT is less than 200 μg per 24 hours. Ten of fourteen cases in whom this determination was done showed elevated urinary excretions ranging up to about 4620 μg per 24 hours. The normal excretion of 5-HIAA is 2–10 mg per 24 hours. Table 6-4 shows that the urinary excretion of this substance was found to be elevated in all but one of these patients. The excretion ranged up to 452 mg per 24 hours. In a subsequent series of 15 patients with carcinoid heart disease studied by Roberts and Sjoerdsma (1964), all the patients showed elevated excretions of 5-HIAA, ranging from 17 to 1270 mg per 24 hours (Table 6-4). Of 10 cases studied by Pernow and Waldenström (1957), the urinary excretion of histamine was greater than normal (6–19 μg per 24 hours) in 8 patients; the excretions ranged from 22 to 6800 μg per 24 hours. The significance of urinary excretion of histamine will be discussed later. There were 11 cases in which the tumor had probably been completely removed. The urinary excretion of 5-HT was normal in 4 of the patients in whom this determination was done. The urinary excretion of 5-HIAA was determined in all 11 cases and was normal in 6. The excretions were only slightly elevated in the remaining 5, ranging from 12 to 20 μg/day.

The ingestion of certain drugs and foods may yield spurious results. Bananas, tomatoes, plantain, and possibly other fruits or vegetables contain 5-HT and its precursors, and the feeding of these materials leads to excretions of 5-HIAA above the normal range (Bodansky, 1963; Schwartz, 1973). Pedersen *et al.* (1970) have reported that the ingestion of glyceryl guaicolate, a widely used expectorant, leads to excretion of substances which yield presumably high values, about 100–200 mg per 24 hours for 5-HIAA.

To follow more easily the results that we are discussing, it may be well to note again that 5-HT is the abbreviation for 5-hydroxytryptamine (serotonin), 5-HTP for 5-hydroxytryptophan, and 5-HIAA for 5-hydroxyindoleacetic acid.

5. Variants of the Carcinoid Syndrome

As has been pointed out earlier, carcinoid tumors producing the carcinoid syndrome most commonly arise in the gastrointestinal tract, but may also be found in other organs (Grahame-Smith, 1968). The general pattern of the syndrome includes flushing, diarrhea, wheezing, and cardiac disease and the biochemical findings that we have been discussing.

However, there are departures from the usual pattern. Davis and

Rosenberg (1961) reported a case of primary carcinoid of the ileum with hepatic metastases and attacks of flushing over a 3-year period. During an 8-month period, the urinary excretion of 5-HIAA fluctuated between 0.5 and 8.2 mg per 24 hours, amounts well within the normal range. The urinary tryptophan excretion at various times during this period varied between 58 and 178 mg per 24 hours, amounts either within the normal range (69 ± 25 mg/day) or slightly elevated (von Heilmeyer and Clotten, 1958). The serum HT levels fluctuated between 0.6 and 1.2 μg/ml during this period, decreasing to normal levels only immediately after treatment with reserpine. However, studies following the oral ingestion of [3-^{14}C]5-hydroxytryptamine did not indicate any significant deviation from the usual pathway of 5-HT (serotonin) metabolism. This suggested that, in certain cases of carcinoid tumor, the production of 5-HT may be small and its elevation in the serum may be demonstrated before excessive amounts of 5-HIAA are excreted in the urine.

Sandler and Snow (1958) reported a male patient who had flushing attacks precipitated by food and particularly by alcohol. The skin had patchy, bright red areas, different in intensity and localization from the usual flush, and could be abated by the administration of antihistaminic drugs. These symptoms had been present for more than 20 years. A hard, enlarged liver was found at laparatomy to be full of metastases from a small primary tumor in the stomach. Histologically, the tumor tissue resembled that of a carcinoid tumor, but no typical argentaffin granules were found. The 24-hour urinary excretion of 5-HIAA was increased moderately to levels ranging from 43 to 59 mg per 24 hours, and the excretion of 5-HT greatly raised to levels ranging from 18 to 24 mg per 24 hours. This was in general agreement with findings in other cases of carcinoid. However, 5-HTP, which is usually absent from the urine of carcinoid patients, amounted to 34–49 mg per 24 hours, and constituted 30–44% of the total hydroxyindoles.

The clinical and biochemical features of bronchial carcinoid may be illustrated by 1 of the 3 cases reported by Melmon *et al.* in 1965. The patient, a 45-year-old man, had remarkably severe and prolonged flashes, recurrent at 3–7 month intervals during the 5 years prior to his last admission. These attacks were associated with symptoms such as severe anxiety, occasional disorientation, fever, nausea and vomiting, profuse watery diarrhea, and wheezing. Early in his course, 5-HIAA excretion ranged from 200 to 400 mg/day but rose to levels of 600 to 900 mg/day during flushing attacks. Later in his course, the 5-HIAA excretions fluctuated between 480 and 580 mg/day and rose to values of over 1000 mg/day during a flushing attack. No determinations of blood 5-HT ap-

pear to have been performed. Chromatography revealed no other urinary indoles and histamine excretion was normal. At autopsy, carcinoid metastases were found in the gallbladder, liver, heart, muscle, pancreas, adrenal glands, bones, lungs, kidneys, and abdominal and thoracic lymph nodes.

In addition to the 3 cases they reported, Melmon *et al.* (1965) reviewed 12 others which had been described in the literature. Bronchial carcinoids appear to produce some of the most striking clinical variants, and the following have been described: (a) excess serotonin production in the absence of an associated clinical syndrome; (b) secretion of 5-HTP as well as 5-HT; (c) high excretion of 5-HIAA with normal blood levels; (d) predominance of left-sided cardiac lesions; (e) more frequent association with other endocrine disorders such as Cushing's syndrome; (f) frequent metastases to bone; and (g) flushing attacks which are more prolonged and severe than in the usual carcinoid syndrome and are associated frequently with temperature elevations, anxiety and tremulousness, preorbital and facial edema, increased lacrimation and salivation, rhinorrhea, diaphoresis, explosive diarrhea, nausea and vomiting, hypotension and oliguria.

Some other instances of variants of the carcinoid syndrome may be briefly described. Kowlessar *et al.* (1959) reported a severe malabsorption syndrome that was associated with ileal carcinoid and extensive metastases to the liver, typical symptoms of flushing and diarrhea, and high excretions, 384 and 635 mg per 24 hours, of 5-HIAA. Moertel *et al.* (1965) presented a case of a thyroid carcinoma and reviewed 7 other carcinomas in the literature, including 3 of the pancreas, 3 oat-cell carcinomas of the lung, and an anaplastic carcinoma which produced the symptoms of the carcinoid syndrome. Flushing and frequently diarrhea and asthma were the outstanding clinical symptoms. The urinary excretion of 5-HIAA was elevated in all 8 cases, that of 5-HT in 2 of the 4 cases in which the determination was done. 5-Hydroxytryptophan was elevated in 1 of the 3 cases in which the assay was carried out. None of the tumors presented a histological picture of carcinoid but, of 6 tumors which were analyzed for 5-HT, 3 showed elevated levels. Moertel's own case (Moertel *et al.*, 1965) showed values for 5-HT of 31 and 63 μg/gm for cutaneous and hepatic metastases, respectively, but none was detectable in uninvolved skin. The anaplastic carcinoma of unknown origin studied by Smith *et al.* (1957) had a sensationally high content of 4000 μg/gm of tissue. It will be recalled that values for normal tissue range up to only about 4 μg/gm (Kirkland *et al.*, 1959; Grahame-Smith, 1968). Of interest was the finding that 3 of the 8 patients had had other evidences of endocrine hyperfunction.

6. Role of Kinins and Prostaglandins in the Carcinoid Syndrome

In 1964, Oates *et al.* submitted evidence indicating that a kinin peptide is released into the circulation during the flushes of patients with carcinoid syndrome. In general, the kinins have a common nonapeptide sequence and differ from each other in having additional N- or C-terminal amino acid residues. The kinins of natural origin have certain pharmacological properties in common, such as increasing capillary permeability, producing pain when applied to a blister base on human skin, having a hypotensive effect, and producing contraction and, in some instances, relaxation of isolated smooth muscle preparations (Schachtor, 1969). The kinins are released from certain inactive proteins or kininogens by the action of enzymes known as kallikreins or kininogenases. The sequence of the reactions involved in this release is shown in Fig. 6-3.

Oates *et al.* (1964, 1966) analyzed hepatic vein blood for kinin activity by preparing a suitable extract which was assayed on an estrus rat uterus preparation. The injection of epinephrine produced no rise in kinin levels of noncarcinoid subjects but, in 9 patients with carcinoids, the kinin activity rose from normal values, less than 10 μg bradykinin equivalents per 100 ml, to levels ranging from 15 to 120 μg per 100 ml. In patients with the carcinoid syndrome, the intravenous injection of 1 μg epinephrine or 100 μg synthetic bradykinin produced within about 4 to 8 minutes flushing, a drop in blood pressure, and an increase in the rate of respiration. Kallikrein (kininogenase) activity

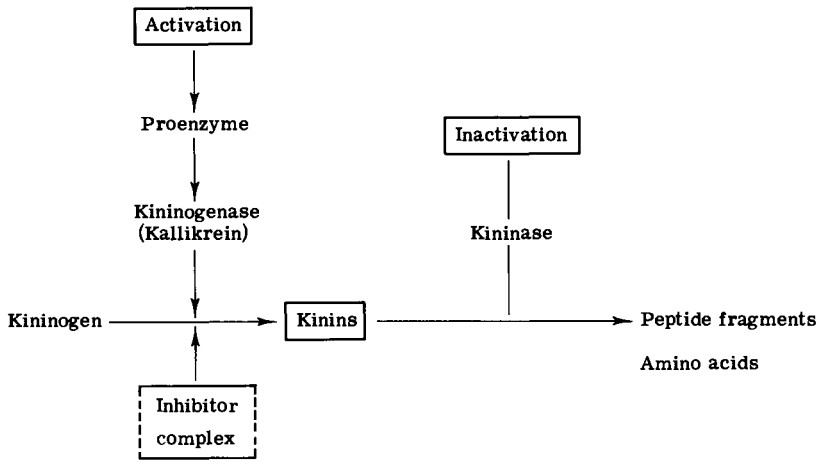


Fig. 6-3 Endogenous kinin system. After Hamberg (1969). Reproduced by permission of Universitetsforlaget, Oslo.

could not be demonstrated in liver tissue from patients without carcinoid tumors, but in 6 patients with this syndrome and hepatic metastases the kallikrein enzyme activity ranged from 20 to 500 Frey unit/gm of tumor dialysate (Oates *et al.*, 1964). The observation that bradykinin-like activity is released during the flushing phase of carcinoid disease has been confirmed by other investigations (Zeitlin and Smith, 1966), but such release does not occur in all cases (Oates *et al.*, 1966; Grahame-Smith, 1970).

By means of gradient elution and paper chromatography, electrophoresis, enzyme activation, and pharmacological studies, it was concluded that the carcinoid kinin was bradykinin (Oates *et al.*, 1966). This is a nonapeptide with the following sequence of amino acids: Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (Schachtor, 1969; Hamberg, 1969). It may be considered that carcinoid tumors release the kinin-forming enzyme, kallikrein, in response to stimuli such as epinephrine. This enzyme splits lysylbradykinin from a protein substrate in plasma, and the lysylbradykinin is then converted rapidly to bradykinin. As has been pointed out, however, the details of the mechanisms by which bradykinin is elevated and causes flushing require further study (Grahame-Smith, 1970).

Prostaglandins are C_{20} fatty acids containing a five-membered ring. A number of prostaglandins have been identified and differ from one another with respect to the number and position of double bonds and hydroxyl group substituents. For example, the structure of prostaglandin E is indicated by the designation, 11α,15α-dihydroxy-9-keto-13 *trans*-prostenoic acid. The biological effects of the prostaglandins include a lowering of blood pressure and a stimulation of a variety of smooth muscle organs (Horton, 1969). Prostglandin F_2 has been reported to be present in a bronchial carcinoid, and an unidentified hydroxy fatty acid, possibly a prostaglandin, has been found in 2 ileal carcinoids (Sandler *et al.*, 1968). The relationship of the prostaglandins to the clinical symptoms of the carcinoid syndrome remains to be determined.

7. Relationship of Biochemical Findings to Clinical Symptoms in the Carcinoid Syndrome

We have already indicated the possible biochemical basis for the phenomenon of flushing in the carcinoid syndrome. Some of the other clinical phenomena are not so easily explained, although the use of various therapeutic agents has contributed some information. With regard to diarrhea, it has been reported that some improvement was achieved with the use of 1-methyl-D-lysergic acid butanolamide tartrate (Peart and Robertson, 1961) or with *p*-chloro-DL-phenylalanine (Engelman *et*

al., 1967). The former is a potent *in vitro* serotonin inhibitor, and one of the various functions of serotonin (5-HT) is to mediate gastrointestinal motility. The association of this function and the therapeutic effect achieved in the few reported cases does not warrant the conclusion as to causality. Peart and Robertson (1961) did not supply any metabolic data showing any effect on the blood 5-HT or urinary excretion of 5-HIAA.

Administration of *p*-chlorophenylalanine in oral doses up to 4.0 gm/day to 5 patients with the carcinoid syndrome reduced urinary excretion of 5-HIAA by 72–88%. Of these 5 cases, one had a gastric carcinoid tumor, a second had a bronchial carcinoid, and the remaining 3 had ileal carcinoids (Engelmann *et al.*, 1967). Some evidence of the mechanisms involved could be gleaned from the study of the urinary excretions of 5-HIAA, 5-HT, and total hydroxyindoles in the patients with gastric carcinoid. All these decreased with the institution of *p*-chlorophenylalanine therapy. This indicates that inhibition of 5-hydroxyindole formation might occur at the tryptophan hydroxylase step, i.e., the conversion of tryptophan to 5-hydroxytryptophan (Fig. 6-2). Four of the five patients treated had relief of their gastrointestinal symptoms, but the flushing episodes did not appear to be affected.

The administration of α-methyl-3,4-dihydroxy-DL-phenylalanine (α-methyldopa) to patients with carcinoid syndrome decreased the excretion of urinary 5-HIAA and simultaneously increased that of 5-HTP (Sjoerdsma *et al.*, 1960). This indicated that α-methyldopa blocked the decarboxylation step, or the conversion of 5-HTP to 5-HT. However, the actual use of α-methyldopa did not appear to alter significantly the clinical symptomatology while, at the same time, it produced undesirable side effects such as extreme weakness, prostration, lethargy, dizziness, and increases in diarrhea (Collini, 1961).

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7

Amino Acid Metabolism and Tumors of the Neural Crest

I. Introduction

Early in embryonic life, at about 4–5 weeks after fertilization and at the time of fusion of the neural folds to produce the neural tube, some of the cells at the site of fusion are left behind and remain dorsolateral to, and outside of, the tube. These cells constitute the neural crest and give rise to different neurons in the dorsal root ganglia and ganglia of cranial nerves, to the multiple neurons of the autonomic ganglia and to cells of the parganglia, called "chromaffin cells" (Moore, 1973). Although there have been several types of classification of and terminologies for these cells, those of Käser (1966) and of Gjessing (1968) appear to be most useful for our purposes and are shown in Fig. 7-1. The sympathogonia is a small lymphocytelike cell with a dense, chromatin-rich, spherical or pyriform nucleus and a small rim of clear, poorly staining cytoplasm. This cell can differentiate along, first, the chromaffinic line which gives rise to the pheochromoblast and the pheochromocyte and, second, along the neurogenic line which gives rise to the sympathoblast (neuroblast) and sympathetic ganglion cell.

As may be seen from Fig. 7-1, each type of cell, from the primitive sympathogonia to the more mature sympathetic ganglion cell, can give rise to its own type of tumor. The sympathogonia may also give rise

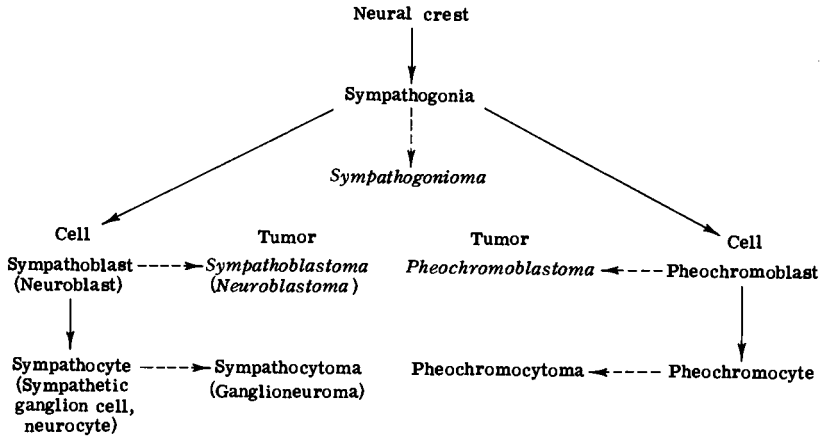


Fig. 7-1 Classification and terminology of neural crest tumors, based on Käser (1966), Willis (1967), and Gjessing (1968). Italicized designations represent malignant tumors.

to the pheochromocytes or mature chromaffin cells, so named because of intracellular granules which yield a brown color on treatment with chromic salts. We shall consider later in this chapter the incidence and clinical characteristics of each type of tumor.

Although the amino acids, phenylalanine and tryosine, follow several metabolic pathways, the one that is of relevance to our present subject is the conversion of these two amino acids to the catecholamines, epinephrine and norepinephrine, and their derivatives. The synthesis of these compounds takes place in the adrenergic neurons in the central nervous system and in the sympathetic ganglion cells or sympathocytes as well as in the pheochromocytes or chromaffin cells (Gjessing, 1968).

II. The Normal Tyrosine-Epinephrine, -Norepinephrine Metabolic Pathway

A. Biosythesis of Epinephrine and Norepinephrine

The sequence of reactions involved in the synthesis of norepinephrine and epinephrine from tyrosine (Fig. 7-2) has been formulated on the basis of *in vivo* and *in vitro* studies in animal studies, and has been demonstrated to occur in brain (Udenfriend and Zaltzman-Nirenberg, 1963), chromaffin cells which are mostly in the adrenal medulla in man

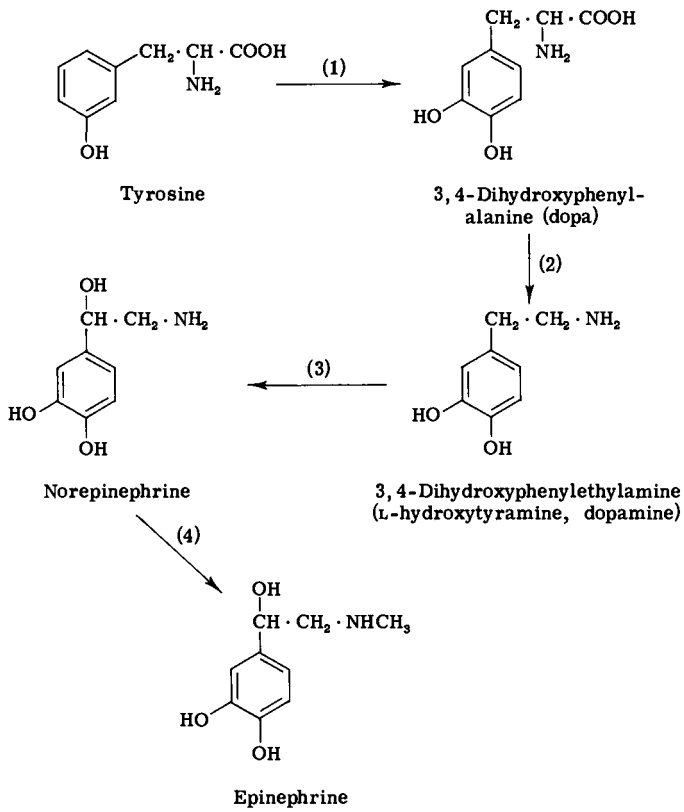


Fig. 7-2 Biosynthesis of catecholamines in brain, chromaffin tissue, and sympathetic nerve endings. Mediating enzymes are as follows: (1) tyrosine hydroxylase, (2) aromatic L-amino acid decarboxylase, (3) dopamine β-oxidase, and (4) phenylethanolamine *N*-methyltransferase.

(Udenfriend and Wyngaarden, 1956), and in the sympathetic nerve endings which are present in almost all tissues (Musacchio and Goldstein, 1963). Indeed, many of the values recorded for epinephrine and norepinehprine in tissues are due to the degree of sympathetic nerve innervation of these tissues (Wurtman, 1965a). There are also alternate pathways of norepinephrine biosynthesis. For example, tyrosine may be decarboxylated directly to tyramine, and the latter then acted on successively by dopamine β-oxidase to form octapine and by a catechol-forming enzyme to form norepinephrine (Wurtman, 1965a).

The details of catecholamine biosynthesis have been summarized by Wurtman (1965a) and by Axelrod (1962; 1973). Tyrosine, present in a concentration of 1.0 to 1.5 mg per 100 ml in the bloodstream.

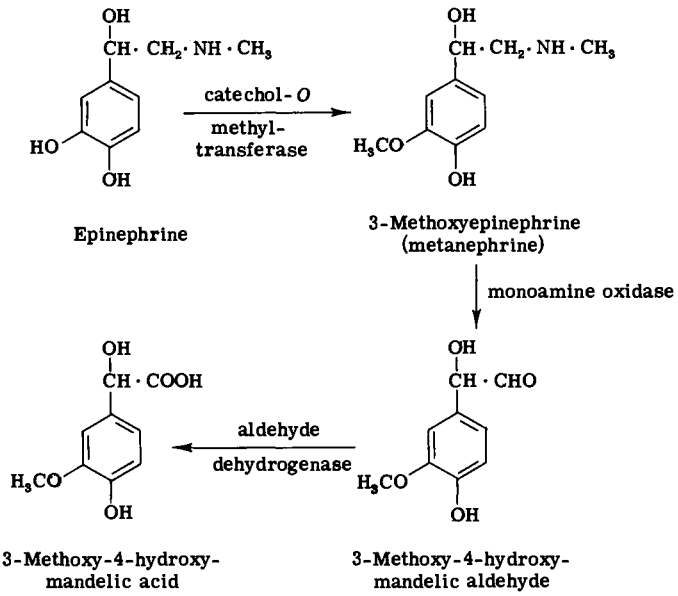
is transported actively into the sympathetic nerve endings or chromaffin cells where it is hydroxylated enzymically to dopa, as shown in Fig. 7-2. This enzyme, tyrosine hydroxylase, requires tetrahydroperidines as a cofactor, and Fe^{2+} and O_2 to facilitate maximum activity. Catecholamines inhibit the hydroxylase and may thus act to regulate the stream of biosynthesis. Again, as shown in Fig. 7-2, dopa is decarboxylated enzymically to dopamine; this is a rapid reaction. The resulting dopamine is localized in the specific storage vesicles in nerve terminals or in the chromaffin granules of the adrenal medulla and is hydroxylated by the enzyme, dopamine-β-hydroxylase, to form norepinephrine. In the adrenal gland, an additional biosynthetic step occurs. Norepinephrine is *N*-methylated by phenylethanolamine-*N*-methyltransferase to epinephrine which then moves to the cytoplasm, where it is *N*-methylated by phenylethanolamine-*N*-methyltransferase to form L-epinephrine, which then migrates back to the chromaffin granule for storage (Axelrod, 1962; Wurtman, 1965a). In mammals, this transferase is present chiefly in the adrenal medulla, and measurable activity is absent from other tissues, such as the small intestine, kidney, liver, salivary gland, pancreas, whole brain, and uterus (Axelrod, 1962). The methyl donor acting in conjunction with the transferase is S-adenosylmethionine and is formed by the interaction of L-methionine and ATP in the presence of an activating enzyme and Mg^{2+} (Kirschner and Goodall, 1957).

Before we discuss the further metabolic fate of epinephrine and norepinephrine, it is appropriate to note briefly that these substances are localized in highly specialized subcellular particles, the "granules" within adrenergic nerves (von Euler and Hillarp, 1956) and chromaffin cells (Blaschko and Welch, 1953). Upon electron microscopy, they show a dense core which contains the norepinephrine and an outer limiting membrane (Wurtman, 1965a). Chromaffin granules range in size from 500 to 4000 Å in diameter, whereas granules in sympathetic nerve endings are generally smaller, ranging from 400 to 500 Å in diameter. In comparison with the conventionally defined subcellular particles, chromaffin granules are heavier than mitochondria, brain granules centrifuge down between mitochondria and microsomes, and sympathetic nerve granules have about the same density as microsomes. Some of the mechanisms that govern the binding of norepinephrine to these granules and release from them have been summarized by Wurtman (1965a).

B. Metabolism of Epinephrine and Norepinephrine

Figure 7-3 shows the major pathways in the metabolism of norepinephrine. We have already seen that norepinephrine is methylated to epineph-

rine. The first two steps in the metabolism of epinephrine are as follows:



From 3-methoxy-4-hydroxymandelic aldehyde on, the metabolic steps are the same as described for the metabolism of norepinephrine.

As may be seen, the enzyme most responsible for the destruction of the circulating catecholamines is catechol-*O*-methyltransferase, first purified by Axelrod and Tomchick in 1958. Its activity requires the conjoint presence of Mg^{2+}, ATP, and methionine or, preferably, *S*-adenosylmethionine which forms under these conditions. The enzyme catalyzes *O*-methylation at the *meta* or 3 position of many substances having a catechol nucleus—norepinephrine, dopamine, 3,4-dihydroxyphenylanine, 3,4-dihydroxymandelic acid, and synthetic catechols. Monophenols are not *O*-methylated, and there is no stereospecificity toward the D or L isomers of epinephrine or related substances. The enzyme is distributed in the tissues of several species, including man. In the rat, it is present in high concentrations in the liver and kidney, in somewhat lesser concentrations in the lung, spleen, small intestine, brain, and heart and in negligible amounts in skeletal muscle (Axelrod and Tomchick, 1958; Wurtman, 1965b).

The sequence of metabolites that constitute the direct pathway in the biosynthesis and metabolism of norepinephrine and epinephrine has been derived from *in vitro* and *in vivo* animal studies as well as from a study of urinary metabolites in man, either with or without the adjunct of intravenously injected labeled epinephrine or norepinephrine. Some

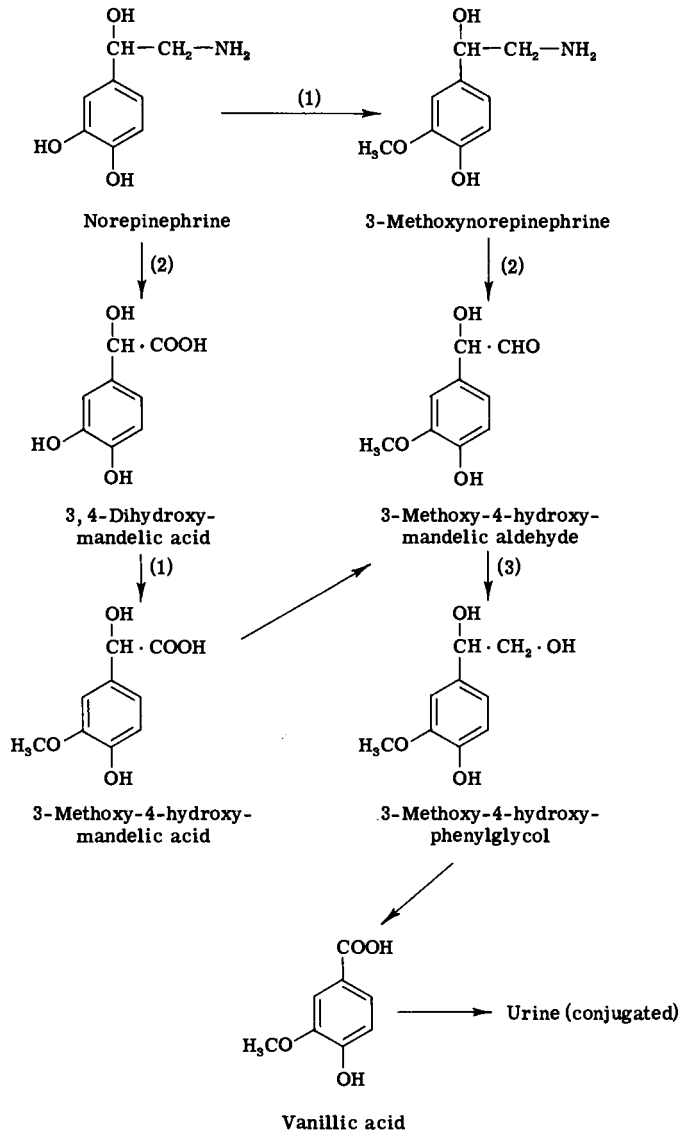


Fig. 7-3 Major pathways of metabolism of norepinephrine. Enzymes catalyzing steps are indicated at arrows: (1) catechol *O*-methyltransferase, (2) monoamine oxidase, and (3) aldehyde reductase.

of the metabolites in the side reactions are excreted in sufficient quantities to be detectable in normal individuals, but others are only apparent in disease. These ancillary metabolic sequences are listed in Fig. 7-4.

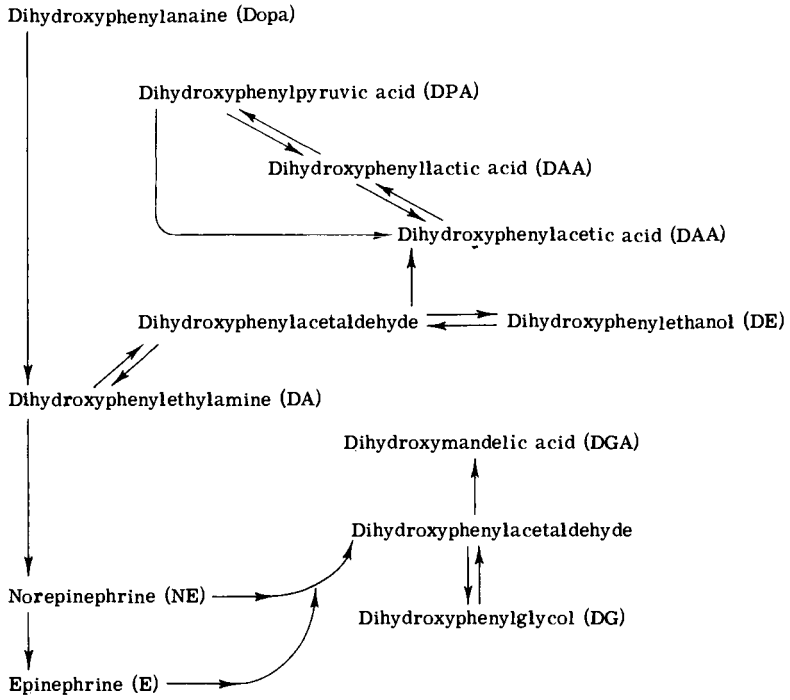


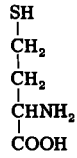
Fig. 7-4 Side reactions in biosynthesis and metabolism of norepinephrine and epinephrine. Based on data of Gjessing (1968).

The terminology of the various compounds listed in Figs. 7-2, 7-3, and 7-4 has not been consistent in the literature. For example, 3-methoxy-4-hydroxymandelic acid has also been designated as "vanillylmandelic acid" (VMA). Gjessing (1968) has suggested a new terminology for the group of compounds where the configuration dihydroxyphenyl would be termed "dopyl" and the configuration 3-methoxy-4-hydroxyphenyl would be designated as "vanyl." This terminology has not been widely accepted and, in the following discussions, we shall usually employ the older, more conventional terminology.

III. Methionine Metabolism

We have already noted that methionine interacts with ATP in the presence of an activating enzyme, methyl adenosyltransferase, to form S-adenosylmethionine, which plays a role in many methylation reactions, including those that we have just discussed as involved in the metabolism

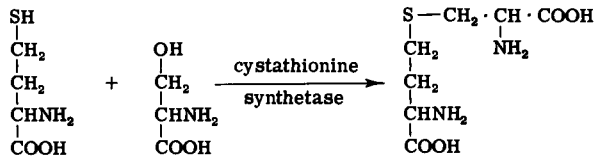
of the catecholamines. Transfer of the methyl group from S-adenosylmethionine results in the formation of S-adenosyl-L-honocysteine, which is then cleaved to adenosine and homocysteine.



Homocysteine

But S-adenoxylmethionine can also be hydrolyzed nonenzymically to form homoserine, in which an OH group replaces the SH group of homocysteine.

Homocysteine condenses with serine in a reaction catalyzed by *cystathionine synthetase*, a pyridoxal phosphate-requiring enzyme, to form cystathionine:



Homocysteine Serine Cystathionine

We shall presently describe the extent to which homoserine and cystathionine are excreted in increased amounts in the urine of patients with neural crest tumors.

IV. Biochemistry of Pheochromocytoma and Pheochromoblastoma

A. Introduction

As we indicated at the beginning of this chapter and in Fig. 7-1, the pheochromocytoma is a catecholamine-producing tumor arising from chromaffin cells of the sympatho-adrenal system. Pheochromoblastomas, or malignant pheochromocytomas, reported to constitute about 8–16% of all pheochromocytomas, are considered to arise from the more primitive cell, the pheochromoblast. Approximately 85% of benign and malignant pheochromocytomas originate in the adrenal glands, another 10% in other abdominal and pelvic regions, and the remainder in the paravertebral area of the thorax and neck (Straus and Wurm, 1960; Gjessing, 1968).

The incidence of reported cases of pheochromocytoma has varied widely. Straus and Wurm (1960) collected a number of reports which indicated that 800–1000 deaths per year were caused by pheochromocytoma. In contrast, MacKeith's (1944) review of the literature yielded a total of 152 cases. In 1950, Smithwick and his colleagues collected 270 cases from the literature. Some 10 years later, a search of the literature revealed the total reported number of cases to be 537 (Sipple, 1961). The data of Roth and Kvale (1956) indicated that the incidence of deaths is 0.053% of all deaths in the United States related to hypertension and, since there are approximately 85,000 hypertensive deaths per year, pheochromocytoma would be responsible for about 50 deaths per year, a figure more recently confirmed to hold for England and Wales (Ross, 1972). Pheochromocytoma may also occur as a familial phenomenon, and 105 such cases had been reported by 1971 (Funyu *et al.*, 1973).

The clinical manifestations of pheochromocytoma are due almost entirely to an excessive elaboration of epinephrine and norepinephrine. There appear to be 2 types of clinical syndromes—one characterized by intermittent or paroxysmal hypertension and the other by persistent but somewhat fluctuant hypertension. The first type occurs in about 35–50% of cases, and its episodes are ushered in by the sudden appearance of such symptoms as anxiety, palpitation, tremulousness, throbbing headache, visual blurring, excessive perspiration, nausea, and occasionally vomiting and retrosternal chest pain. In severe and prolonged episodes, a shocklike state may set in, and death may occur from pulmonary edema, ventricular fibrillation, or cerebral hemorrhage (Sjoerdsma, 1967). Thomas *et al.* (1966) have tabulated the frequencies of symptoms as percentages in 100 patients: headache, 80; perspiration, 71; palpitation, 64; pallor, 42; nausea (with or without vomiting), 42; tremor or trembling, 31; weakness or exhaustion, 28; nervousness or anxiety, 22; and epigastric pain, 22. The incidence of other symptoms, such as chest pain, dyspnea or flushing, was less than 20%.

B. Concentrations of Catecholamines and their Metabolites in Pheochromocytoma

The weight of both adrenals in man is 12–15 gm, and the concentration of both epinephrine and norepinephrine in the normal adrenal ranges from 0.27 to 1.0 mg/gm of tissue (Hermann and Mornex, 1964). In general, the weights of pheochromocytomas are greater than those of normal adrenals, and the concentrations of the catecholamines are higher. For example, in the earlier study of Goldenberg *et al.* (1950) on 10

cases, the concentration of epinephrine ranged from 0.03 to 8.2 mg/gm of tumor, and 7 of the 10 tumors had concentrations higher than 2 mg/gm. Similarly, the concentration of norepinephrine ranged from 1.0 to 7.0 mg/gm of tumor. The weights of the tumors reported by Goldenberg *et al.* (1950) ranged from 21 to 567 gm, so that the total amount of the two catecholamines present in the pheochromocytoma could be vastly greater than in the normal adrenal gland. For example, in the largest tumor, the total amounts of epinephine and norepinephrine were 3345 and 1418 mg, respectively.

This early report has been amply substantiated by later work. Straus and Wurm's review of the literature up to 1960 showed tumor weights ranging from 1.4 to 430 gm, and concentrations of epinephrine ranging from 0 to 8.7 mg/gm of tissue and of norepinephrine ranging from 0.11 to 10.6 mg/gm. More recent reports (Herman and Mornex, 1964; Robinson, 1963; Itoh and Ohmori, 1973) indicate that most pheochromocytomas contain 0.5–10 mg catecholamines (essentially the sum of epinephrine and norepinephrine) per gm of tissue.

The ratio of epinephrine to norepinephrine varies greatly from tumor to tumor, but usually norepinephrine is present in greater amounts. Indeed, some tumors contain only epineprine, and some only norepinephrine (Straus and Wurm, 1960; Hermann and Mornex, 1964). Other metabolites of the catecholamines may also be present. Employing paper chromatography, Sjoerdsma *et al.* (1959) showed that the 3-methoxy derivative of norepinephrine (normetanephrine) was present in each of four pheochromocytomas studied. In a similar fashion, the presence of metanephrine and 3-methoxy-4-hydroxyphenylglycol was demonstrated, and the concentration of the latter was estimated to be 5–10 μg/gm of tissue (Kopin and Axelrod, 1960). The sum of the concentrations of normetanephrine and metanephrine ranged from 1.54 to 2.30 μg/gm of tissue in four pheochromocytomas. 3-Methoxy-4-hydroxymandelic acid (vanillylmandelic acid, VMA) has also been found in a concentration of 0.57 μg/gm of tissue in a case of pheochromocytoma (Page and Jacoby, 1964). In a series of tumors from 11 patients, the concentration of dopamine (DA) ranged from 3 to 389 μg/gm tissue (Ito and Ohmori, 1973).

The secretion of catecholamines from the adrenal gland and sympathetic nervous system into the circulation is of interest. As we have indicated earlier in this chapter (Section II,A), norepinephrine is synthesized in sympathetic nerve endings and stored in the granulated vesicle of the sympathetic nerve ending. In chromaffin cells (pheochromocytes) of the adrenal gland, the norepinephrine migrates from the chromaffin granule to the cytoplasm and is there methylated by the enzyme, phenyl-

ethylamine *N*-methyltransferase, to epinephrine, which then migrates back to the chromaffin granule for storage (Wurtman, 1965a). The normal mammalian adrenal is innervated by preganglionic nerve fibers, and liberation of acetylcholine at the nerve endings, which are in close contact with the pheochromocytes, leads to the discharge of catecholamines into the extracellular space and thence into the adrenal veins and the posterior vena cava (Gjessing, 1968).

C. *Catecholamines in Blood of Patients with Pheochromocytoma*

The procedure for the determination of epinephrine and norepinephrine in the plasma is not simple and has been modified several times to attain greater specificity. Table 7-1 shows the normal values obtained by several groups of investigators. It may be seen that the more recent series (Weil-Malherbe and Bone, 1958; Vendsalu, 1960) show lower values, possibly as the results of increasing specificity of method. Platelets also contain small amounts of epinephrine and norepinephrine, and good centrifugation of plasma is necessary to eliminate the moiety of catecholamine resulting from this blood element (Weil-Malherbe and Bone, 1958). Vendsalu (1960) demonstrated that work increased the plasma levels of epinephrine and norepinephrine.

It is apparent that the results for the plasma levels of catecholamines obtained in pheochromocytoma should, in general, be compared, with the normal levels obtained by the particular groups of investigators. The upper limit of normal can be considered as the mean plus two standard deviations. Manger *et al.* (1954) found the plasma epinephrine was elevated in 5 of 8 patients with pheochromocytoma, and the highest value was 3.0 μg/liter. All 8 patients had elevated plasma norepinephrine

TABLE 7-1

Concentrations of Catecholamines in Human Plasma of Normal Persons at Rest

Reference	No. of persons	Epinephrine (μg/liter)			Norepinephrine (μg/liter)	
		Mean	SD	Range	Mean	SD
Manger *et al.* (1954)	11	0.14	±0.2	—	3.96	±1.7
Roth *et al.* (1960)	490	2.5	—	0.75–5.5	—	—
Weil-Malherbe and Bone (1958)	85	1.11	±0.056	—	3.76	±0.115
Vendsalu (1960)	21	0.08	±0.02	—	0.31	±0.02

levels, and the highest value was 23.0 μg/liter. These results have been amply confirmed in subsequent reports. In their 1960 review, Straus and Wurm listed values for the total plasma catecholamines (epinephrine plus norepinephrine) that were 10- to 100-fold the normal levels. Sjoerdsma *et al.* (1959) obtained a value as high as 69.5 μg epinephrine per liter, and Ross and Turnbull (1955) obtained values of 227.1, 6.9 and 220.4 μg/liter for plasma norepinephrine. Recent studies continue to affirm these findings. For example, Geffen *et al.* (1973) reported a mean plasma catecholamine concentration of 5.75 ± 1.39 (SE) μg/liter and a range of approximately 2–200 μg/liter in a series of 11 patients with pheochromocytomas, as compared with a mean value of 0.34 ± 0.04 (SE) μg/liter in a series of 30 control patients with essential hypertension. There was no overlap between the values in the two groups.

D. Urinary Excretion of Catecholamines and Their Metabolites in Patients with Pheochromocytoma

Because of the small amounts of epinephrine and norepinephrine metabolites, it was difficult to obtain direct quantitative estimates of the pattern of excretory products. By using a slow intravenous infusion of [2-^{14}C]DL-epinephrine over a period of 1 hour into each of 6 normal healthy males, Goodall (1959) found that the ^{14}C-labeled compounds were excreted at the rate of about 10% per hour of the injected dose for the first 4 hours, then more slowly until a total of about 73% was reached for the first 24 hours. Separation of the urinary metabolic products was attained by means of a combination of ion exchange and paper chromatography. The following compounds were identifiable as fractions of total excreted radioactivity and their proportions determined: epinephrine, 4%; metanephrine, 5%; conjugated metanephrine, 42%; 3-methoxy-4-hydroxymandelic acid, 27% and 3,4-dihydroxymandelic acid, 12%. The remaining 10% was not identifiable.

Similar studies on norepinephrine showed a recovery of 67% of the infused dose (Goodall, 1959). Again, the following compounds and their amounts were identifiable as fractions of the total 24-hour urinary radioactivity: norepinephrine. 4%; normetanephrine, 3%; conjugated normetanephrine, 19%; 3-methoxy-4-hydroxymandelic acid, 32%; and dihydroxymandelic acid and some unknown components, 40%.

Table 7-2 is a summary by Bodansky (1965) of various studies in the literature on the distribution of urinary excretion of catecholamines and 3-methoxy-4-hydroxymandelic acid by normal individuals and patients with pheochromocytoma. It may be seen that approximately 90–

TABLE 7-2

Urinary Excretion of Catecholamines and 3-Methoxy-4-hydroxymandelic Acid in Normal Persons and in Patients with Pheochromocytoma[a]

Group and reference	Percent of cases having daily excretion, in mg/day, of catecholamines				
	0.00–0.20	0.21–0.50	0.51–2.00	2.01–5.00	>5.0
Normals					
Straus and Wurm (1960)	92	8	0	0	0
Crout and Sjoerdsma (1964)	100	0	0	0	0
Pheochromocytoma					
Straus and Wurm (1960)	12	4	59	25	0
Crout and Sjoerdsma (1964)	4	8	46	38	4

	3-Methoxy-4-hydroxymandelic acid					
	0–6.0	6.1–12.0	12.1–20	21–50	51–300	>300
Normals, 20 persons	95	5	0	0	0	0
Pheochromocytoma, 24 patients	0	13	25	42	12	8

[a] Distribution calculated by Bodansky (1965) from survey of 24 studies by Straus and Wurm (1960) and from data of Crout and Sjoerdsma (1964) on 24 normal persons and 24 patients with pheochromocytoma. Reproduced by permission of J. B. Lippincott Company.

100% of normal individuals, and about 0–10% of patients with pheochromocytoma, excrete less than 0.20 mg of catecholamines and less than 6.0 mg of 3-methoxy-4-hydroxymandelic acid per day. Conversely, no normal individuals excrete more than 0.5 mg catecholamines or more than 12 mg 3-methoxy-4-hydroxymandelic acid per day, whereas approximately 75–85% of patients with pheochromocytoma excrete more than these amounts.

The determination of urinary catecholamines and of 3-methoxy-4-hydroxymandelic acid has proved to be of substantial aid in the diagnosis of pheochromocytoma or in following the progress of such patients. For example, von Euler and Ström (1957) reported the case of a 26-year-old female who excreted 418–1600 μg norepinephrine per day when tested at several times during a 3-month period. The patient was operated on, and a pheochromocytoma at the left renal hilus was removed. The day after operation, the 24-hour norepinephrine excretion decreased precipitously to 162 μg, and during the next 21 months fluctuated at essentially normal levels between 30 and 140 μg. The urinary epinephrine

excretion was not elevated preoperatively, and was 21 μg on the first postoperative day, then ranged between 0 and 11 μg/day during the next 21-month period. Other cases showing decrease of urinary excretion of catecholamines in patients following removal of tumor have been reported (Bell *et al.*, 1962).

Crout and Sjoerdsma (1964) have expressed the daily production of catecholamines in patients with pheochromocytoma by subtracting the daily excretion of normal individuals from that of the patients. The value for the production divided by the catecholamine content of the tumor represented the turnover rate. In general, and in spite of considerable variation, high or moderate turnover rates, 50–100%, were characteristic of the smaller tumors, whereas larger tumors, 100–830 gm in weight, were characterized by low turnover rates, usually less than 10%. Crout and Sjoerdsma (1964) suggested the concept that one or more of the factors—intracellular binding of catecholamines, regulation of catecholamine synthesis, and activity of catechol-degrading enzymes—may be abnormal to varying extents in the pheochromocytoma cell.

In spite of the wide use of urinary catecholamines and 3-methoxy-4-hydroxymandelic acid as diagnostic procedures, a report by Sheps *et al.* (1966) claims a higher efficiency for the determination of plasma catecholamines. Of 28 cases of pheochromocytoma, 1 case had a normal plasma catecholamine value, and only 2 of 101 nontumor patients were above the normal plasma level. In contrast, 25% of the tumor patients had normal urinary catecholamine excretions, and 20% of 201 nontumor patients had abnormal excretions. These values for urinary catecholamine excretion are not in agreement with other reports in the literature listed in Table 7-2 which show a much sharper differentiation between patients with pheochromocytoma and control individuals. The statement by Sheps *et al.* (1966) that urinary 3-methoxy-4-hydroxymandelic acid excretions were normal in 21% of the tumor cases is also in disagreement with the results in Table 7-2.

E. Catecholamines and Their Metabolites in Patients with Pheochromoblastoma

1. Catecholamines in Tissue of Pheochromoblastoma

It will be recalled that "pheochromoblastoma" arises from the pheochromoblast, a cell more primitive than the pheochromocyte (Fig. 7-1). The proportion of pheochromocytomas which have been designated as malignant and, therefore, merit the term "pheochromoblastoma" has been

variously estimated as 8–16% (Straus and Wurm, 1960). This variability reflects the inadequacy of data needed to establish malignancy in reported cases (Gjessing, 1968). Microscopically, the structure of the primary pheochromoblastoma does not appear to be different from benign pheochromocytoma (Kennedy *et al.*, 1961; Robinson *et al.*, 1964), and it is usually the presence of metastases that is of importance in distinguishing between the two.

Nor is there any clear evidence that pheochromoblastoma tissue has a different pattern of catecholamines from that of benign pheochromocytoma. Weil-Malherbe (1956) and McMillan (1956) reported the presence of dopa or dopamine in the primary pheochromoblastoma, but Kennedy *et al.* (1961) were unable to detect any in the primary tumor or in liver metastases. However, as has already been pointed out, Itoh and Ohmori (1973) have quite recently found that the content of dihydroxyphenylethylamine (dopamine) in a series of 11 pheochromocytomas ranged from 3 to 389 μg/gm of tissue. Metastases in organs not normally containing chromaffin cells have about 1–2 mg of catecholamine per gm of tissue, of which 90–100% is norepinephrine (Davis *et al.*, 1955; Kennedy *et al.*, 1961).

2. Plasma and Urinary Catecholamines

There are practically no data on the levels of plasma catecholamines in patients with malignant pheochromocytoma, but there are a few instances of altered pattern of urinary excretion of catecholamines and their metabolites. For example, of 36 pheochromocytoma patients studied by Robinson *et al.* (1964), only one excreted appreciably abnormal amounts of vanylalanine, 3-methoxytyramine, and homovanillic (vanylacetic) acid. Removal of an adrenal tumor weighing 900 gm led to the decrease of these metabolites to normal levels of excretion. Within 6–12 months, the excretion of these metabolites increased above the normal range, and attained preoperative levels about 16–17 months postoperatively, just before death. These rises were associated with the recurrence of clinical symptoms characteristic of pheochromocytoma. Radiographs of the skeleton revealed osteolytic lesions in the left lower scapula and the right scapula. At autopsy, the liver was found to be greatly enlarged, and metastatic deposits were found in the liver, skeleton, lymph nodes, and at the site of the removed adrenal gland.

Several other examples of altered pattern of urinary catecholamine excretions in malignant pheochromocytoma may be noted. In a patient with a pheochromocytoma that was removed but who was later found to have metastases, the daily excretion of homovanillic acid (HVA)

ranged from 13 to 41 mg per 24 hours, as compared with a normal range of 8.23 ± 2.96 (SD) mg per 24 hours (Sankoff and Sourkes, 1963). Large amounts of *N*-acetylated dopamine in urine were reported by Karlson *et al.* (1963). Von Studnitz (1965) found hypercystathionuria and hypomethionuria in a case of pheochromoblastoma with metastases. These changes are not present in pheochromocytoma, although, as we shall note later, they are also characteristic of sympathoblastoma (Gjessing, 1963b). In general, it may be seen that not enough malignant pheochromocytomas have been studied to offer any distinctive criterion for distinguishing them from the benign tumors.

V. Biochemistry of Sympathocytoma (Ganglioneuroma) and Sympathoblastoma (Neuroblastoma)

A. Introduction

As was pointed out earlier in this chapter (see also Fig. 7-1), the primitive sympathetic cells (sympathogonia) give rise successively to sympathoblasts and sympathetic ganglion cells. The sympathoblasts may develop into the malignant neuroblastoma (sympathoblastoma) and the sympathetic ganglion cells into the benign ganglioneuroma (sympathocytoma).

The benign sympathocytoma is found in the brain, peripheral ganglia, and adrenal medulla. It develops from and consists of mature ganglion nerve cells and nerve fibers, medullated or nonmedullated. The corresponding malignant sympathoblastomas (neuroblastoma) are usually small but may reach a size of 10 cm in diameter, becoming hemorrhagic and necrotic and invading veins. Microscopically, the neuroblastoma consists of large numbers of cells with narrow rims of cytoplasm. They resemble, but are slightly larger than, lymphocytes. Grouping in rosette form is common (Ackerman and del Regato, 1970). Willis (1967) has noted that the distinction between the benign and malignant tumors is not always sharp, and intermediate gradations of structure may occur. These are usually classified as ganglioneuroblastomas. In the following discussion, we shall use the terms "ganglioneuroma" to designate the benign sympathetic nervous system tumor and "neuroblastoma" to indicate the malignant type.

The vast majority of tumors of the sympathetic nervous system consists of neuroblastomas, and the benign ganglioneuroma is relatively rare

(Dargeon, 1960). Indeed, neuroblastoma is one of the most frequently observed solid tissue cancers of childhood. During the 5-year period between 1960 and 1964, 1535 children in the United States are known to have died as a direct result of this neoplasm (Miller *et al.*, 1968). The tumor may arise at many different sites, most frequently at the adrenal medulla, but the ganglia in the cervical, thoracic, retroperitoneal, pelvic regions, the aortic bodies and the celiac ganglia may also be the sites of origin. Growth may be slow or rapid. Although, as we shall see presently, the majority of patients with ganglioneuroma or neuroblastoma have elevated levels of catecholamines or their metabolic derivatives in the urine, only a few have such clinical signs of functional activity as hypertension, tachycardia, sweating, and pallor. Diarrhea associated with hypokalemia and symptoms such as failure to thrive and abdominal distention may occur more commonly in ganglioneuroblastoma or ganglioneuroma than in neuroblastoma (Voorhess, 1972).

B. Catecholamines and Other Metabolites in Ganglioneuroma and Neuroblastoma Tissue

In 1957, Mason *et al.* reported a 5-month-old infant with an intrathoracic tumor which proved to be a neuroblastoma. It contained, by bioassay, 0.14 mg of pressor amines per gm of tumor, 48% of which was norepinephrine. Table 7-3 lists available catecholamine analyses of tissue from ganglioneuromas and neuroblastomas. Although there are not many of these analyses and they show wide variation, there do not appear to be any differences with respect to the amounts of norepinephrine, epinephrine, and dopamine in the various types of tumor. With the exception of Gjessing's assays (1964), those of the other investigators (Käser, 1966; Greer *et al.*, 1965; Greenberg and Gardner, 1960) are in fairly good agreement. The concentrations of norepinephrine range from 0.01 to 8.6 μg/gm of tumor tissue, those of epinephrine from 0.0 to about 1 μg/gm, and those of dopamine from 0.04 to about 2 μg/gm. As can be seen from Table 7-3, Gjessing's results (1964) are about 10- to 20-fold higher.

The concentrations of catecholamines in these tumors are much lower, indeed, about one-hundredth to one-thousandth the amounts in pheochromocytoma or pheochromoblastoma which, it will be recalled (Section IV,B), contain about 0.5 to 10 mg catecholamines per gm of tissue. This difference is even more strikingly illustrated in studies with analysis on pheochromocytoma and neuroblastoma performed by the same method. For example, Käser (1966) obtained concentrations of 6315, 2245, and 8424 μg of norepinephrine per gm of tissue in 3 pheochromocy-

TABLE 7-3

Concentrations of Catecholamines in Neuroblastoma and Ganglioneuroma Tissue

Investigator	Catecholamines in μg/gm wet weight		
	Norepinephrine	Epinephrine	Dopamine
Neuroblastoma (sympathoblastoma)			
Gjessing (1964)	25	78	40
	35	10	20
	6	10	6
Greer *et al.* (1965)	2.4	0.39	0.02
	0.68	1.12	2.2
	1.9	0.05	0.30
	0.07	1.09	—
	0.01	0.01	—
Käser (1966)	1.13	0.03	0.20
	trace	0	0.04
	4.05	0	0.64
	0.21	0.07	0.95
Ganglioneuroblastoma			
Käser (1966)	2.98	0	1.05
Ganglioneuroma (sympathocytoma)			
Greenberg and Gardner (1960)	8.6	0.074	2.09

tomas, as compared with ranges of 0.0–4.05 μg/gm in 4 neuroblastomas. The concentrations of epinephrine in the pheochromocytomas were 5748, 76, and 6200 μg/gm of tissue, as compared with values of 0.0–0.7 μg/gm of tissue for the neuroblastomas. Similarly, the concentrations of dopamine were 9.4–16.3 μg/gm for the pheochromocytomas, substantially higher than the range of 0.04–0.95 μg/gm for the neuroblastomas.

We noted earlier in this chapter that homocysteine may arise from adenosylmethionine since the latter compound acts as a methyl donor (Section III). Homocysteine condenses with serine to form cystathionine in a reaction catalyzed by cystathionine synthetase. Since methylation is active in the tumors arising from the neural crest, Gjessing (1963c) has explored the possibility that cystathionine may be present as a metabolite in such tumor tissues. In 3 cases of adrenal neuroblastoma, Gjessing (1963c) obtained values of 6.7, 5.3, and 8.0 μg cystathionine per gm of tissue. These values were clearly higher than those of 0.7 μg/gm of tissue for normal human liver, kidney, or adrenals, but in the same range as the value of 8.0 μg/gm of human brain. In a later study (1964), Gjessing obtained values of 30, 20, 53, and 80 μg cystathionine per gm of tissue for 4 neuroblastomas. An adrenocortical carcinoma was not

found to contain any cystathionine. Gjessing (1968) has stated that neither ganglioneuromas nor pheochromocytomas are characterized by high levels of cystathionine, although the data on which this conclusion is based are sparse.

The higher concentrations of cystathionine in neuroblastoma may be attributed to the increased methylation of dopa and dopa derivatives produced in the tumor, or to lessened cystathionase activity, either because of insufficient amounts of pyridoxal phosphate in the tumor or because of combination of pyridoxal phosphate with the excessive amounts of catechols that are produced in the tumor (Gjessing, 1968).

C. *Urinary Catecholamines and Their Metabolites in Neuroblastoma and Ganglioneuroma*

The urinary excretion of the catecholamines and their metabolites in ganglioneuroma and neuroblastoma have been studied by several groups of investigators, including Greenberg and Gardner (1960), Gjessing (1963a,d, 1964), Sourkes *et al.* (1963) Gjessing and Borud (1965), and Greer *et al.* (1965). We have recalculated the comprehensive and detailed data of von Studnitz *et al.* (1963) and of Greer *et al.* (1965) to show the distribution of the magnitudes of urinary excretion of the catecholamines and their metabolites in patients with neuroblastoma (Table 7-4). There is fairly good agreement between the results of the two groups. For example, 100% of the sum of excretions of epinephrine and norepinephrine were less than 1.5 μg/mg creatinine. The excretion of 3-methoxy-4-hydroxymandelic acid was equally abnormal in both studies, above the upper limit of normal in 94% of the patients according to Greer *et al.* (1965) and in 100% of the patients according to von Studnitz *et al.* (1963).

Comparison of the pattern of urinary excretion in neuroblastoma with those of other neural crest tumors is difficult because of the paucity of data on the other tumors. Nonetheless, Table 7-5 attempts such a comparison. It may be noted that 94% of the patients with neuroblastoma had an elevated excretion of dopamine, and 67% had an elevated excretion of HVA. These results are to be contrasted with the 9 cases of benign pheochromocytomas in all of whom dopamine and HVA excretions were within normal limits. These frequencies can be shown to be significantly different by the χ^2 test. Table 7-5 also shows that high excretions of dopamine and HVA occur more frequently in the malignant tumor, neuroblastoma, than in its benign counterpart, ganglioneuroma.

The reader will recall the discussion of the side reactions involved

TABLE 7-4

Urinary Excretion of Catecholamines and Their Metabolites in Patients with Neuroblastoma

Compound	Control value on 15 subjects in μg/mg creatinine[b]	No. of patients	Percent of patients having daily excretion of catecholamines in μg/mg creatinine[a]						
			Above upper limit of normal	0.0–1.5	1.6–7.5	7.6–31.5	31.6–128	129–513	513–2050
Epinephrine	—	14[a]	—	100	—	—	—	—	—
Norepinephrine	—	14[a]	—	100	—	—	—	—	—
Epinephrine + norepinephrine	0.025–0.049[b]	21[b]	95	100	—	—	—	—	—
Dihydroxyphenylalanine (dopa)	0.016–0.091[b]	14[a]	—	79	14	7	—	—	—
		21[b]	90	77	23	0	—	—	—
Dopamine	0.127–0.259[b]	14[a]	—	29	58	7	7	—	—
		21[b]	95	42	34	15	10	—	—
HVA	3.9–39.9[b]	14[a]	—	—	—	12	19	57	12
		17[b]	72	—	—	30	30	36	6
3-Methoxy-4-hydroxymandelic acid	1.2–9.5[b]	16[a]	—	6	—	13	32	25	26
		21[b]	95	—	—	19	62	15	5

[a] The distribution of these values has been calculated from the original data of Greer *et al.* (1965).
[b] The distribution of these values has been calculated from the original data of von Studnitz *et al.* (1963).

TABLE 7-5

Frequency of Increased Excretion of Dopamine and HVA in Patients with Different Tumors of Chromaffin and Neural Origin[a]

Type of tumor	Total No. of cases	Increased dopamine and/or HVA excretion (%)	Normal dopamine excretion (%)	Increased dopamine excretion (%)	Normal HVA excretion (%)	Increased HVA excretion (%)
Pheochromocytoma, benign	9	0	100	0	100	0
Pheochromocytoma, malignant	1	100	0	100	0	100
Neuroblastoma	36	94	6	94	33	67
Ganglioneuroblastoma	4	75	25	75	50	50
Ganglioneuroma	6	17	83	17	83	17

[a] Percentages calculated from data of Käser (1966).

in the biosynthesis and metabolism of the catecholamines (Fig. 7-4). A number of the products listed there are also methylated at the 3-hydroxyl position to yield the 3-methoxy derivatives (Käser, 1966). The urinary excretion of these compounds has been studied in some detail (Gjessing, 1963a, 1964; Gjessing and Borud, 1965) so that, together with studies on metabolites of the direct pathway, additional diagnostic information might be elicited. The following indications were obtained (Gjessing, 1964): The dopamine metabolites which include 3-methoxy-4-hydroxyphenylalanine, 3-*O*-methyldopamine, and 3-methoxy-4-hydroxyphenylethanol were generally elevated in ganglioneuroma, whereas the metabolites of dopa, dopamine, epinephrine, and norepinephrine were all elevated in neuroblastoma (Gjessing, 1964). As has been previously indicated, pheochromocytoma is distinguished by increased excretion of epinephrine and norepinephrine metabolites including 3-methoxy-4-hydroxyphenylglycol.

We have already indicated that the excretion of 3-*O*-methylated dopa derivatives such as HVA and 3-methoxy-4-hydroxymandelic acid is much more predominant in patients with neuroblastoma than in those with ganglioneuroma, pheochromocytoma or pheochromoblastoma. Table 7-6 shows average values and ranges for the excretion of these two compounds in the various neural crest tumors. Catechol *O*-methyltransferase activity has been found in neuroblastoma tissue (La Brosse and

TABLE 7-6

Daily Excretion of HVA and 3-Methoxy-4-hydroxymandelic Acid in Patients with Neural Crest Tumors[a]

Type of tumor	No. of patients	HVA (μg/mg creatinine per 24 hours)		VMA (μg/mg creatinine per 24 hours)	
		Average	Range	Average	Range
Neuroblastoma	10	263	41–1000	374	15–1300
Pheochromocytoma	4	6.5	3–10	72	4–220
Ganglioneuroma	2	73	20–125	24	5–42
Normal children	—	—	3–16	—	2–12
Normal adults	—	—	2–4	—	1.5–3

[a] Based on data of Gjessing (1964).

Karon, 1962), although it appeared not to be present in ganglioneuroma tissue (Greenberg and Gardner, 1960). When tritiated norepinephrine is added to neuroblastoma in tissue culture, the major metabolic product is tritiated 3-methoxy-4-hydroxyphenylglycol (MHPG). This situation probably also exists *in vivo*, for it has been found in one instance that the tumor content of MHPG was about nine times that of 3-methoxy-4-hydroxymandelic-acid and the blood concentration was two times that of 3-methoxy-4-hydroxymandelic acid (La Brosse, 1970). These data indicate that norepinephrine is synthesized within the neuroblastoma tumor and metabolized to MHPG. The latter is released into the blood and subsequently metabolized to 3-methoxy-4-hydroxymandelic acid. La Brosse (1970) has studied the rate and extent of conversion of MHPG to 3-methoxy-4-hydroxymandelic acid in a patient with neuroblastoma.

Since dopamine-β-hydroxylase (DβH) catalyzes the conversion of dopamine to norepinephrine, the possibility arises that an alteration in this enzyme activity may affect the urinary excretion pattern of catecholamines in patients with neuroblastoma and may account for the variability in the urinary excretion among patients. Indeed, Goldstein *et al.* (1972) have found that the mean values of serum activity were significantly higher in patients with neuroblastoma than in control groups of children of comparable age. Ten of the 22 patients with neuroblastoma had extremely high values of serum DβH activity, and most of these excreted high amounts of 3-methoxy-4-hydroxymandelic acid. There was no correlation between the serum DβH activity and the urinary excretion levels of dopamine or HVA.

D. Cystathioninuria in Neuroblastoma and Ganglioneuroma

We have previously noted that cystathionine is present in neuroblastoma tissue in much higher concentrations than in control tissues or in ganglioneuroma. This state is reflected in excessive excretion of cystathionine in the urine. In 10 cases with neuroblastoma, the preoperative excretion ranged from 5 to 900 μg/mg creatinine (Gjessing, 1963c, 1964). When the metastases were extensive, the excretion was at the upper limit of this range. In one of these cases, successful removal of the tumor was followed by a steady decline of cystathionine excretion during a period of 4 months from a level of 200–240 μg/mg creatinine to complete absence. Another case of neuroblastoma also showed zero excretion of cystathionine 1 year after operation. One patient with ganglioneuroma had only a trace of cystathionine in the urine, whereas another patient with a mixed semimalignant ganglioneuroblastoma excreted relatively small amounts, ranging from 10 to 25 μg/mg creatinine. These results indicate that the degree of cystathioninuria is related to the malignancy of the tumor and the extent of its metastases. The presence of cystathioninuria in patients with neuroblastoma has been confirmed by von Studnitz (1965).

Cystathioninuria may be found in conditions other than neuroblastoma, but it is not generally characteristic of other tumors. In 1959, Harris *et al.* found that it existed as a rare genetic disorder in man, and other cases have since been reported. But it has also been found as a secondary characteristic, and in small amounts in vitamin B_6 (pyridoxine)-deficiency syndrome (Scriver and Hutchinson, 1963), in a case of argentaffinoma (Gjessing, 1963d), in hepatoblastoma (Gjessing and Mauritzen, 1965), following a large dose of thyroxine (Gjessing, 1968), in other genetic conditions such as galactosemia and maple syrup urine disease (Shaw *et al.*, 1967), and in a cretin infant on a vitamin-deficient diet (Fourman *et al.*, 1966). The involvement of the liver in some of these conditions raises the possibility that the cystathioninuria of neuroblastoma may be the result of disturbed liver function resulting from metastases to the liver (Gjessing, 1968). Obviously, it is necessary to study further the association of liver function impairment and cystathioninuria in patients with neuroblastoma.

E. Biochemical Diagnostic Procedures for Ganglioneuroma and Neuroblastoma

We have previously noted the frequency of neuroblastomas in childhood. In 1959, Greenberg and Gardner suggested that the urinary estima-

tion of 3-methoxy-4-hydroxymandelic acid might have utility in the diagnosis of ganglioneuroma and neuroblastoma. In 1966, Bell applied the screening test of Gitlow *et al.* (1960) to a group of 18 patients with neuroblastoma and to a group of 66 controls. In brief, a volume of urine containing about 0.5 mg creatine was acidified with hydrocholoric acid and extracted 3 times with ethyl acetate. The extracts were combined, evaporated to dryness, and the residue dissolved in water and potassium carbonate and followed by addition of diazotized *p*-nitroanaline. The color complex was extracted and read against water at 450 and 500 nm. The ratios of the readings, OD 450/OD 550, were used as the distinguishing criterion. The range of specimens for the control children was 1.2–2.15. The 18 cases of neuroblastoma gave ratios between 0.49 and 1.0.

More recently, a test strip has been advocated by Leonard *et al.* (1972). The strips are prepared by wetting filter paper in a solution consisting of equal volumes of 0.2% *p*-nitroanaline, 0.2% sodium nitrite, and 20% potassium carbonate. The strips are dried *in vacuo* for 4 hours. Dipped into urine, the strips are sensitive to a concentration of 20 μg/ml of either 3-methoxy-4-hydroxymandelic acid or normetanephrine. This concentration gives a violet color which increases to a purple color at higher concentrations. The strips are negative to other metabolites such as dopamine, norepinephrine, or epinephrine. Of 20,000 patients who were screened at the University of Minnesota hospitals and in neighboring local regions, 50 had positive tests, of which 30 became negative by testing the next morning. Of the 20 patients who remained positive, 15 were cancer patients on some type of chemotherapy, and 5 had lymphoma. Quantitative analysis of the urine revealed normal 3-methoxy-4-hydroxymandelic acid levels. The test, therefore, had a "false-positive" incidence of 1:400. Of a group of 24 patients with neuroblastoma, all tested positive by the strip test. Quantitative 3-methoxy-4-hydroxymandelic acid analysis revealed values higher than 20 μg/ml in each instance.

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8

Gastrointestinal Tract, Liver, and Pancreas

I. Introduction

The present chapter will include not only neoplasms of the digestive tract proper but also of the accessory organs, the pancreas and the liver. Although much biochemical investigation has been done on the latter two organs, the digestive system proper has received relatively little attention in this respect. Yet, neoplasms of some of these organs of the digestive system are among the most common of all tumors. For example, neoplasms of the colon and rectum, combined, are among the leading causes of death from cancer in the United States (Silverberg and Holleb, 1973; Segi and Kurihara, 1972; Ackerman and del Regato, 1970; World Health Organization, 1970). Without going into the many factors that influence the incidence and rate of mortality, such as sex, race, and geography, we may present in Table 8-1 a brief survey of the recent values for the overall incidence of and mortality from neoplasms of the various sites in the digestive system. Nonetheless, it is well to appreciate the wide variation resulting from the factors we have mentioned. For example, in the United States, the deaths per 100,000 population in 1967 resulting from malignant neoplasms of the stomach were 10.47 and 14.73 for white and nonwhite males, respectively, and 6.68 and 7.04 for white and nonwhite females, respectively. The similar rates in Japan were 59.56 for males and 35.93 for females.

TABLE 8-1

Incidence and Mortality of Neoplasms of the Digestive System in the United States

Organ of digestive system	1967 Mortality[a]	Estimated, 1973[b]	
		Mortality	Incidence
Buccal cavity and pharynx	5,906	7,600	15,400
Esophagus	4,438	6,400	6,800
Stomach	14,529	14,700	16,400
Small intestine	644	750	1,200
Large intestine	30,618	37,000	57,000
Rectum	9,645	10,400	22,000
Liver and biliary passages, primary	6,059	7,200[c]	7,300[c]
Liver, secondary and unspecified	3,926	—	—
Pancreas	15,234	19,200	19,400

[a] From Segi and Kurihara (1972).
[b] From Silverberg and Holleb (1973).
[c] Specified as primary (Silverberg and Holleb, 1973).

Since little biochemical information is available on the biochemical aspects of neoplasms of the buccal cavity, pharynx, and esophagus, we shall proceed directly to those of the stomach.

II. Neoplasms of the Stomach

A. Introduction

Carcinoma of the stomach constitutes the vast majority of gastric neoplasms. Sarcomas of the stomach make up only 1–5% of all gastric neoplasms, and about 60% of these are lymphosarcomas (Ackerman and del Regato, 1970). There appears to be little information concerning the nature of cellular disturbances in human gastric carcinoma, and most investigations have involved the chemical composition of the gastric juice.

B. Gastric Secretion

1. Introduction

The amount and the character of the gastric secretion is normally influenced by a variety of neurogenic, chemical, and hormonal factors which have been the subject of study for many years. For details, the

reader may refer to any one of a number of physiological texts or monographs as, for example that edited by Shnitka, Gilbert, and Harrison, (1967). Here, we shall refer only to those characteristics which are affected in carcinoma of the stomach.

The normal adult secretes an average of 2500 ml of gastric juice per day. The concentrations of electrolytes also vary widely, but the following may be given as mean values in milliequivalents per liter: sodium, 50; potassium, 10; and calcium, 2.5. Other ions, such as magnesium, phosphorus as phosphate and bicarbonate are also present (Bodansky and Bodansky, 1952). A number of enzymes are found in gastric juice; these include lipase, urease, rennin, lysozyme, acid phosphatase, and lactate dehydrogenase.

The most clinically important component is hydrogen ion, or as it is more practically known, hydrochloric acid. The various methods for the determination of this component have been based on the titration of the gastric juice with 0.1 *N* sodium hydroxide to various pH levels, more specifically, with dimethylaminoazobenzene to a pH of about 3.0 to determine "free acidity" and with other dyes to a pH of about 7.0–8.0 to determine the "total acidity." Obviously, the simplest as well as most rational procedure is to determine the pH of the gastric juice electrometrically, and this value can be converted by suitable tables to milliequivalents per liter (Spiro, 1970). In the older work, the gastric acidity was expressed as "chemical units" or the number of milliliters of 0.1 *N* sodium hydroxide required to bring 100 ml of gastric juice to the desired end point. This expression is fortuitously the same as the more modern terminology of the number of milliequivalents of acid per liter of gastric juice (Bodansky and Bodansky, 1952). As we shall presently see, the total gastric acidity has also been expressed as the number of milligrams of hydrochloric acid secreted during a stated period.

The gastric acidity has most often been measured, not only in a basal 12-hour or 1-hour period but also in response to a stimulus. Originally, a test meal was employed as the stimulus, but later a subcutaneous injection of histamine or histamine hydrochloride was used. Stimuli in current use include: (a) histamine acid phosphate given subcutaneously in a dosage of 0.04 mg/kg of body weight and preceded by an antihistaminic; (b) Histalog, a histamine analog; and (c) Pentagastrin (Spiro, 1970).

2. Gastric Acidity in Carcinoma of the Stomach

There are many observations in the literature demonstrating that the free and total acidity of gastric juice is decreased in patients with

carcinoma of the stomach (Polland, 1933; Comfort *et al.*, 1947; Levin *et al.*, 1948). For example, in 50 studies of 33 normal subjects of a 12-hour nocturnal and presumably "basal" gastric secretion, the "free acidity" ranged from 1 to 90 and averaged 29 clinical units, as contrasted with a range of 0–32 and an average of 5 units in 12 patients with carcinoma of the stomach. The total amount of free hydrochloric acid secreted during 12 hours in the normal subjects ranged from 14 to 3417 and averaged 661 mg. In contrast, the patients with carcinoma of the stomach ranged from 0 to 719 and averaged 82 mg.

The incidence of achlorhydria is much higher in patients with gastric carcinoma (Polland, 1933; LaDue, 1967). For example, LaDue (1967) reported that 51% of 135 patients with this neoplasm had achlorhydria, as compared with 23% in a group of control subjects of approximately the same age. In the series studied by Levin *et al.* (1948), 69% of patients with gastric carcinoma had concentrations less than 10 clinical units, whereas 47% of the patients with gastric ulcer, 24% of normal persons, and none of the patients with duodenal ulcer were below this level.

There appears to be no prospective evidence that the occurrence of anacidity in apparently normal individuals predisposes to the development of carcinoma of the stomach. Of relevance in this connection are the observations made of gastric analysis tests in 79 persons who had no evidence of carcinoma of the stomach on initial examination but who subsequently developed this disease (Comfort *et al.*, 1947). An average of 6 years intervened between the two events. Before cancer developed, 38% of these 79 subjects had achlorhydria, as determined after a test meal. In the remaining 62%, the mean concentration of free gastric acidity was 27.1 units. The incidence of achlorhydria was greater by 22.1%, and the mean incidence of free hydrochloric acid was less by 14.2 units than the expected values for a group of normal persons, matched for age and sex. After the development of cancer, the incidence of achlorhydria rose to 64.6%.

3. Enzymes in Gastric Secretion

As we have already noted, the gastric secretion contains a number of enzymes. Indeed, the older literature explored in some detail the possibility that the peptic activity of gastric juice may be altered in patients with ulcer (Polland and Bloomfield, 1929; Osterberg *et al.*, 1933; Vanzant *et al.*, 1933).

However, the activities of other enzymes have also been studied in the gastric secretion of patients with carcinoma of the stomach. Changus and Dunlap (1949) reported an increased activity of acid phosphatase.

The presence of excessive lactic acid in such instances has been explained by the glycolytic actions of bacteria on carbohydrate food materials which would be encouraged in the absence of free hydrochloric acid, that is, by pH levels approaching 7.0. But this finding is not specific since it was obtained in noncancerous disease of the stomach, and may result from long periods of stagnation in the stomach (Dodds and Robertson, 1930). However, the question may arise whether gastric lactic acid production in cases of carcinoma may not be elevated as a consequence of the well-known increased glycolytic capacity of neoplastic tissues. Shacter *et al.* (1949) observed that, following the intravenous injection of 100 ml of 50% glucose into patients with gastric carcinoma, the concentration of lactic acid in the gastric juice rose during 1 hour from an average value of 5 mg per 100 ml to one of 15 mg per 100 ml. In contrast, patients with gastric ulcer showed insignificant rises, namely, from about 2 to 3 mg per 100 ml.

The lactate dehydrogenase (LDH) activity of gastric juice in patients with various diseases of the digestive system has been of interest for a number of years (Schenker, 1959; Piper *et al.*, 1963). More recently, Faulk *et al.* (1972) reported that the levels of this enzyme activity were elevated in ulcer and gastritis and, to a greater extent, in carcinoma of the stomach (Table 8-2). Since no measure of variation was given,

TABLE 8-2

Lactate Dehydrogenase Activity in Gastric Juice in Human Stomach Cancer[a]

Group	No. of cases	LDH activity (units/ml)	LDH activity (units/hour)[b]
Normal	25	98	5,500
Ulcer, gastric	19	341	13,650
Gastritis			
Superficial	11	195	6,400
Hypertrophic	12	128	6,800
Atrophic	16	363	11,800
Mixed	27	448	16,600
Neoplasms			
Benign	4	470	16,000
Sarcoma	2	320	16,050
Carcinoma	7	870	41,800

[a] From Faulk *et al.* (1972) with modifications. Reproduced by permission of The Lancet Ltd.

[b] Activity in units/ml × volume of juice secreted in 1 hour.

it is difficult to determine whether these elevations are statistically significant. Simon and Figus (1973) have stressed the view that the total LDH activity of gastric juice depends on the general condition of the gastric mucosa and increases in parallel with the severity and extent of mucosal atrophy.

4. Gastric Glycoproteins

The reader may refer to Chapter 1, Section II,E,1 for a brief discussion of the structure of mucoproteins and glycoproteins. The presence of these compounds in the gastric mucosa and secretion has been of interest for many years, and the subject was reviewed by Glass in 1964. Employing fractionation of gastric juice by a resin column, Richmond *et al.* (1955) obtained mean values for several components in the gastric secretions of normal individuals, patients with gastric ulcers, duodenal ulcers, and carcinoma of the stomach (Table 8-3). The values for total hexoses, fucose, and sialic acid were significantly higher in the group of patients with carcinoma.

More recently, Schrager and Oates (1968, 1971a,b), using gel chromatography, have studied the isolation and composition of the major glycoprotein from human gastric aspirates and from gastric mucosa. These investigators found that the glycoproteins isolated from 4 proteolyzed normal gastric mucosae had practically the same carbohydrate and amino acid composition as the principal glycoprotein from gastric aspi-

TABLE 8-3

Content of Carbohydrates and Proteins in Human Gastric Juice[a] (mg per 100 ml)

Group	Protein	Hexoses	Hexosamine	Fucose	Sialic acid	Glucuronic acid
Normals	(11)[b] 330	(16) 32.1	(10) 32.7	(15) 13.8	(13) 7.3	(12) 2.0
Duodenal ulcers	(19) 212	(26) 34.7	(21) 30.2	(20) 9.7	(20) 8.1	(22) 1.8
Gastric ulcers	(10) 288	(12) 36.0	(9) 45.9	(7) 16.7	(8) 9.8	(9) 1.9
Gastric carcinoma	(10) 482	(11) 81.2[c]	(9) 52.4	(9) 30.7[c]	(9) 20.8[c]	(8) 2.1

[a] From Richmond *et al.* (1955) with modifications. Reproduced by permission of Williams & Wilkins.

[b] Figures in parentheses are the number of cases studied.

[c] Statistically significant as compared with mean values for gastric juice from normal persons.

rates. Galactose, fucose, glucosamine, and galactosamine were present in approximately molar ratios of 4:3:3:1. In addition to this basic common pattern, additional sugar residues were present that were associated with blood group specificity which was the same as that of the host's red cells (Schrager, 1972).

The acid hydrolysates of the glycoproteins isolated from the extracts of the 20 gastric neoplastic mucosae revealed significant differences from the pattern in extracts from normal stomach. As we saw above, the ratio of galactose/glucosamine/galactosamine in each of the normal individuals was 4:3:1. In the 20 patients with carcinoma of the stomach, only 9 cases showed this ratio. One case had a ratio of 1:1:1, a second case had a ratio of 2:1:1, and the remaining 9 had a ratio of 3:2:1. Moreover, whereas the blood group specificity of the gastric extract in the normal individual was the same as that of the host's red cells, in 11 of the 20 patients with carcinoma it differed from that of the host's red cells. Schrager (1972) has suggested that the mutation of the normal mucous cell to a neoplastic cell has changed the enzymic system synthesizing the glycoprotein.

5. Other Biochemical Findings

Later in this chapter, in connection with consideration of primary hepatoma, we shall discuss the α-fetoglobulin test. Here, we may note that elevated concentrations of serum α-fetoglobulin have been reported in patients with gastric carcinoma (Mehlman *et al.*, 1971; Kozower *et al.*, 1971; Alpert *et al.*, 1971). In these cases, liver metastases were demonstrable either at operation or autopsy. A malignant tumor of the stomach, which was not carcinoma but carcinoid, also produced elevated serum α-fetoprotein (Žižkovský *et al.*, 1972). Here, too, autopsy revealed hepatic metastases.

It has long been appreciated that gastric carcinoma is associated with a decrease in the concentration of total serum protein (Homburger and Young, 1948) and, more concretely, with marked decreases in the concentration of serum albumin and concomitant increases in the concentrations of serum α_1- and α_2-globulins (Petermann and Hogness, 1948). A recent study (Suga and Tamura, 1972) reaffirms these findings. As we pointed out in Chapter 1 (Section II,C,2), this altered pattern is characteristic of all types of advanced cancer and, indeed, for noncancerous, serious diseases such as tuberculosis, rheumatoid arthritis, and sarcoidosis (Seibert *et al.*, 1947; Mider *et al.*, 1950; Bodansky, 1956). Suga and Tamura (1972) have recognized the nonspecificity of their findings but have suggested that the level of the α-globulins in patients

with gastric cancer during chemotherapy might, nonetheless, serve as a parameter for the objective evaluation of effectiveness of therapy. As we have already seen in the case of various serum enzyme activities and shall have occasion to note again, there are a number of other nonspecific biochemical parameters which may serve this function.

III. Neoplasms of the Small Intestine

Reference to Table 8-1 will recall to the reader the extremely low incidence of neoplasms of the small intestine. Indeed, there are approximately 14 stomach cancers and 48 cancers of the large intestine for each small bowel tumor. Lowenfels (1973) has pondered the basis for this rarity, has reviewed the reasons that have been given, and has suggested several others. These include the fluid nature and rapid transit of small bowel contents which tend to reduce the intensity of exposure of the small bowel to an oral carcinogen, the relative sterility of the small bowel as compared with the colon, the presence of IgA-producing lymphoid tissue in the small intestine which would protect against cancer formation, and detoxification of ingested carcinogen through the action of the microsomal enzymes which are present in high concentration in the small intestine. Lipkin (1973) has also noted that another factor may be the greater rate of turnover and removal of cells from small intestine as compared to the rate in the distal colon where carcinoma is more frequent.

In this connection, it may be noted that a group of closely related microsomal enzymes exist which are capable of metabolizing a wide variety of compounds not normally present in the body. These enzymes have been demonstrated to be present, not only in the animal liver but also in the tissues that comprise the major portals of entry to the body such as the gastrointestinal tract, lung, skin, and placenta (Gelboin and Blackburn, 1964; Wattenberg and Leong, 1962). For example, benzpyrene hydroxylase activity is present in the duodenal mucosa of several mammals, including man (Wattenberg *et al.*, 1962). Wattenberg and Leong (1970) found that in rats the oral administration of β-naphthoflavone greatly increased the benzpyrene hydroxylase activity of the small intestine and inhibited almost completely the formation of pulmonary adenoma by benzpyrene. Dietary components may also increase the benzpyrene hydroxylase activity of the small intestine and, thus, possibly influence the response of the organism to exposures to polycyclic hydrocarbon carcinogens (Wattenberg, 1971).

The benign tumors of the small intestine occasionally evoke clinical

symptoms. They include polyps, leiomyomas, fibromas, hemangiomas, lipomas, and lymphangiomas. The 4 main types of malignant neoplasms are carcinoma, lymphoma, argentaffinoma (carcinoid), and sarcoma. In a series of 139 benign and malignant small bowel tumors seen at the Barnes Hospital in St. Louis from the years 1925–1968, the incidences were carcinoma, 8.6%; lymphoma, 28.8% carcinoid, 10.1%; and sarcoma, 8.6% (Ackerman and del Regato, 1970).

We have already discussed the biochemical aspects of carcinoid (Chapter 6) and of those instances of lymphoma associated with α-chain disease (Chapter 5). Little biochemical information is available concerning the remaining types of lymphomas, the carcinomas, and the sarcomas of the small intestine. However, all types of tumors of the small intestine are capable of leading to defective absorption and thus inducing the malabsorption syndrome. Kahn *et al.* (1972) found that 33% of patients with small bowel lymphomas had this syndrome. Malabsorption was also present in 3 patients with small intestinal lymphomas associated with α-chain disease (Bonomo *et al.*, 1972).

Primary or secondary metastatic neoplasms of the small intestine may be associated with the malabsorption syndrome and its biochemical abnormalities (Sleisinger, 1971). The clinical manifestations of the malabsorption syndrome of small intestine include weight loss, anorexia, abdominal distention, borborygmi, muscle wasting, and passage of abnormal stools that are light yellow to gray, greasy and soft. A large variety of biochemical procedures may be used as aids in diagnosis. In general, the concentrations of serum albumin, carotene, calcium, cholesterol, potassium, and magnesium are decreased. Tolerance tests involving the oral ingestion of D-xylose lead to decreased urinary excretion of this pentose, and those procedures in which glucose or vitamin A are ingested result in flat blood curves. Stool fat determinations involving the chemical determination after an oral intake of 100 gm of fat daily, or the fraction of excretion of ingested [^{131}I]triolein or [^{131}I]oleic acid show increases in the malabsorption syndrome. These changes are illustrated in a case recently reported by Benisch *et al.* (1972) of malabsorption resulting from involvement of the small intestine by metastatic melanoma.

The presence of neoplasm in the small intestine may give rise to biochemical effects not necessarily related to the site of tumor. For example, Stolbach *et al.* (1972) have reported the case of a 69-year-old patient presenting with symptoms and x-ray findings compatible with mechanical small bowel obstruction. Immediately following resection of a localized jejunal lymphoma, the serum alkaline phosphatase was found to be substantially elevated to 34.8 KA units. No liver metastases had been observed at operation and skeletal x-rays revealed no evidence of bone

metastases. In contrast, a substantial portion of the serum alkaline phosphatase was found to consist of the placental-like or Regan isoenzyme. The concentration of the latter dropped exponentially with a calculated half-life of approximately 10 days. It was undetectable 4 months postoperatively and remained so at the time of the report, 2 years after operation. This case represents the ectopic production of alkaline phosphatase by an intestinal tumor.

IV. Neoplasms of the Colon and Rectum

A. Introduction

The colon is the site of several types of polyps and benign tumors such as leiomyomas, fibromas, lipomas, and hemangiomas. Although the colon may also harbor such malignant neoplasms as sarcomas, melanocarcinomas, and argentaffinomas, these are rare and approximately 98% of all malignant neoplasms in the colon are carcinomas (Robbins, 1968). Deaths from cancer of the colon in the United States during 1968 were 44,345, second only to those, 59,367, resulting from neoplasms of the lung. It has been estimated that, in the United States in 1973, the incidence of cancers of the colon and rectum combined, will be approximately 79,000, equal to the estimated incidence of neoplasms of the lung (Silverberg and Holleb, 1973). Although there is some fluctuation, the death rates per 100,000 population are about the same or even somewhat higher in Great Britain and in the countries of Western Europe (Segi and Kurihara, 1972). Yet, in spite of these high incidences and death rates, the area of colorectal cancer has until recently received relatively little biochemical study.

B. Proliferation and Differentiation of Colonic Cells

Even when the individual reaches its full grown adult state, cell division continues in many organs, replacing cells which are extruded from the tissue. It has been shown that epithelia lining the digestive tract undergo continuous renewal of their constituent cells (Messier and Leblond, 1960; Bertalanffy, 1960). Studies of mitotic indices and radioautographic studies with [^{3}H]thymidine have suggested turnover times of human intestinal epithelium of the order of several days (Cole and McKalen, 1961; Bertalanffy and Nagy, 1961). Employing autoradiography after injection of [^{3}H]thymidine, Lipkin *et al.* (1963a) reported that the mean proliferative time of epithelial cells of colon and rectum

was about 1–2 days divided into a phase of DNA synthesis (S phase) lasting 11–15 hours; a postsynthetic phase, premitotic phase (G_2 phase) that, combined with mitosis, lasts 1–2 hours; and a postmitotic, presynthetic phase (G_1 phase) that lasts 10–30 hours. This cell proliferation takes place in the lower and middle third of the crypts of Lieberkuhn of the colon and rectum. Above the proliferation compartment, mature cells migrate to the crypt surfaces and are extruded into the lumen of the colon. It is during this migration that these cells undergo differentiative changes and attain mature development. It may be noted that similar studies have been carried out on the proliferation and migration of cells in the stomach and ileum (Lipkin *et al.*, 1963b).

C. Nucleic Acid Metabolism of Normal and Neoplastic Colonic Cells

1. Introduction

The proliferation and differentiation of colonic cells naturally raise questions concerning the nature of the biochemical changes associated with these processes. The distribution of several enzymes at various histological levels of the intestinal epithelium have been studied in the rat (Fortin-Magana *et al.*, 1970; Imondi *et al.*, 1969), and such studies have served as a model for similar studies in the normal human colon and in neoplastic lesions of the colon (Troncale *et al.*, 1971). Since most of the enzyme activities studied have been those involved in the intermediary metabolism of purine and pyrimidine metabolism, it may be appropriate to review these briefly.

2. Enzymes in Purine and Pyrimidine Metabolism

The sequence of reactions involved in the *de novo* biosynthesis of purines starts with the interaction of ribose 5-phosphate and ATP to form phosphoribosyl pyrophosphate and ends with the ribonucleotide, inosinic acid. Enzymes in this pathway do not as yet appear to have been employed in the study of proliferation or differentiation of intestinal or colonic cells. However, there are other pathways of purine nucleotide formation as well as pathways for interconversion of purine nucleotides, and some of the enzymes involved in these sequences have been utilized (Imondi *et al.*, 1969; Troncale *et al.*, 1971).

These have included purine nucleoside phosphorylase, adenine phosphoribosyltransferase, and hypoxanthineguanine phosphoribosyltransferase. Enzymes involved in the catabolism of purines such as adenylate

deaminase and adenine deaminase have also been employed in studies of differentiation of rat intestinal epithelium (Imondi *et al.*, 1969).

The biosynthesis of pyrimidines involves the following sequence of interactions: (a) the formation of *N*-carbamylaspartic acid from carbamyl phosphate and L-aspartic acid, catalyzed by aspartate carbamyltransferase; (b) the ring closure of L-ureidosuccinic, catalyzed by dihydroorotase, to yield L-dihydroorotic acid; (c) the oxidation of L-dihydroorotic acid by dihydroorotic acid dehydrogenase to form orotic acid; (d) the coupling of orotic acid and phosphoribosyl pyrophosphate, catalyzed by orotodine-5′-phosphate pyrophosphorylase to yield pyrophosphate and orotidine 5′-phosphate; and (e) the latter is then decarboxylated enzymically to form uridine 5′-phosphate (UMP).

From this point on many interconversions may occur. Uridine 5′-phosphate is successively phosphorylated, through kinase action, to form the diphosphate, UDP, and the triphosphate, UTP. The general pathway of interconversion of pyrimidine nucleotides may be summarized as follows (Balis, 1968; White *et al.*, 1968):

$$\text{UTP} \rightarrow \text{CTP} \rightarrow \text{CDP} \rightarrow \text{dCMP} \rightarrow \text{dUMP} \rightarrow \text{dTMP}$$

and

$$\text{UDP} \rightarrow \text{dUDP} \rightarrow \text{dUMP} \rightarrow \text{dTMP}$$

M, D, and T immediately before P represent the monophosphate, diphosphate, or triphosphate forms, respectively, of the ribosides; T and C represent thymidine and cytidine, respectively; and d designates the deoxyriboside forms. Pyrimidine deoxyribonucleotides and ribonucleotides can also be formed from the corresponding nucleosides (International Union of Biochemistry, 1965).

Of particular relevance to the study of human colonic cells have been determinations of activity of the enzymes, thymidine kinase and thymidine phosphorylase (Troncale *et al.*, 1971). Thymidine is the deoxyribonucleoside of thymine (5-methyl-2,4-dioxypyrimidine), and thymidine kinase (TdR kinase) catalyzes its phosphorylation to a deoxyribonucleotide:

$$\text{Thymidine} + \text{ATP} \rightarrow \text{thymidine 5′-phosphate} + \text{ADP}$$

Thymidine phosphorylase catalyzes the following reaction:

$$\text{Thymidine} + \text{phosphate} \rightarrow \text{thymine} + \text{2-deoxy-D-ribose 1-phosphate}$$

3. Enzymes of Purine and Pyrimidine Metabolism in Normal and Neoplastic Colon

Employing a tissue-planing apparatus developed by Imondi *et al.* (1969), Troncale *et al.* (1971) obtained layers of normal colonic

mucosa that were divided into three approximately equal layers and were histologically identified as upper, middle, and lower thirds of the colonic crypts. Figure 8-1 shows that the average activity of thymidine kinase in the lower third of the crypt is approximately fourfold that of the activity in the upper third. The changes shown in Fig. 8-1 indicate that thymidine kinase and phosphorylase were highest in young proliferating cells and decreased during differentiation and migration of the cells to the mucosal surface. In contrast, the activities of phosphoribosyltransferase were lowest in the young proliferating cells and increased during cell differentiation. In connection with the pattern of proliferation previously described, it may be said in summary that, during migration of normal colonic epithelial cells through the middle third of the colonic crypt, DNA synthesis and mitosis cease as the cells undergo differentiation (Lipkin, 1971, 1973).

The proliferation and differentation of neoplastic cells in the human colon have been explored in several studies by Lipkin and his associates (Troncale *et al.*, 1971; Lipkin, 1971). They have observed that these cells have variable replication rates with many cells proliferating at near normal, or slower than normal rates. The G_2 premitotic phase of the proliferative cell cycle was found to be prolonged in villous adenoma and carcinoma cells. Table 8-4 shows that the enzyme, thymidine kinase,

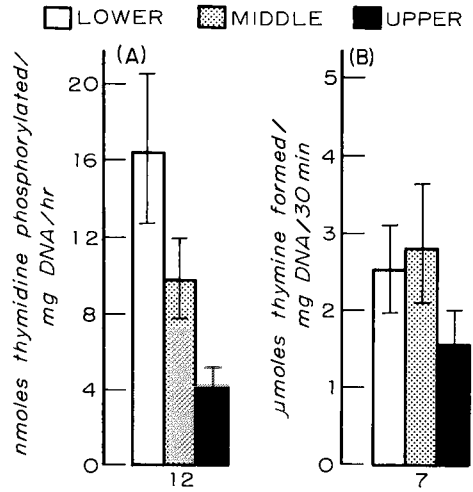

Fig. 8-1 Change in activity (mean ±SE) of enzymes in human colonic crypts measured when the crypts were separated into lower, middle, and upper third by a razor planning instrument. The number of colon specimens studied is in parentheses: (A) thymidine kinase and (B) thymidine phosphorylase. After Troncale *et al.* (1971). Reproduced by permission of Cancer Research, Inc.

TABLE 8-4

Thymidine Kinase Activity in Surface Cells of Normal Colon and Colonic Neoplasms[a]

Tissue	nmoles Thymidine phosphorylated per hr per mg DNA		
	Number[b]	Mean	± SE
Normal colon	22	8	3
Small polyps	11	11	2
Large polyps	9	10	2
Villous papillomas	2	37	8[c]
Carcinomas	7	21	7[c]

[a] After Lipkin (1971). Reproduced by permission of J. B. Lippincott Co.

[b] Numbers of specimens assumed to be the same as those reported by Troncale *et al.* (1971).

[c] Significantly higher ($p < 0.05$) than in other specimens.

which is involved in DNA synthesis, has significantly higher activity in the surface cells of villous papillomas and carcinoma than in those of normal colon and small and large polyps. Lipkin (1971, 1973) has pointed out that these enzyme results might indicate that preneoplastic and neoplastic cells of the colon continue DNA synthesis and proliferative activity throughout their life span and that normal differentiation is decreased. Salser and Balis (1974) have found that thymidine kinase in normal and neoplastic intestinal epithelium is present in two forms: one soluble and the other particle-associated. The activities of the soluble form in tumors in the stomach, ileum, and caecum were not increased by phospholipase C, whereas the corresponding normal tissues were stimulated an average of about 2.5-fold. The increases in the activity of thymidine kinse from carcinomas of the colon and rectum were moderate, an average of 1.6- and 2.0-fold, respectively. Studies on heat lability and degree of activation by mercaptans indicated the possibility that the enzyme in colon carcinoma was not identical with that in normal colon. Regulation of cell division in gastrointestinal cancer did not appear identical with that of rapidly dividing crypt or fetal intestinal cells. The necessity for further study of the sequence and broad range of changes occurring in the cells of normal colon and benign and neoplastic lesions of the colon appears obvious. Peterson and Lipkin (1974) have recently reported studies of the activities of thymidine kinase and thymidine synthetase in crypts of the normal and neoplastic human colon.

Their findings indicate a regulatory malfunction in epithelial cells in patches of colonic mucose where neoplastic lesions were forming.

D. Carcinoembryonic Antigen (CEA)

Attempts have been made for many years to produce tumor-specific antibodies in sera obtained from animals immunized with preparations of human cancer. In 1965 and in the following few years, Gold and his associates (Gold and Freedman, 1965a,b; Thomson *et al.*, 1969) developed the concept that human neoplasms had tumor-specific antigens, that in the case of colorectal carcinoma this was a definite protein–polysaccharide of consistent amino acid and carbohydrate composition, that this substance could engender antibodies in the rabbit or goat, and that the antiserum thus produced could, through a suitable radioimmunoassay, detect the presence in the entire circulation of only a few micrograms of a substance coming from a colorectal carcinoma, hopefully when such a tumor was not clinically evident. The efforts to document this concept have been truly prodigious.

On the basis of a sensitive radioimmunoassay which they developed, Thomson *et al.* (1969) designated as a positive test one showing a concentration of serum CEA greater than 2.5 ng/ml. They reported that 97% of 36 patients with colorectal cancer and only 9% of 32 patients with cancer of other digestive organs were positive. None was positive in groups with noncancerous digestive disease or with cancer of nonenteric organs. In this study, CEA was characterized as a protein–polysaccharide complex that was soluble in 1.0 *M* perchloric acid and 50% saturated ammonium sulfate.

Subsequent investigations failed to yield the sharp discriminations between different groups that had been obtained by Krupey *et al.* (1968). Employing some technical modifications, Lo Gerfo *et al.* (1971) found that elevated levels of CEA, namely, levels greater than 2.5 ng/ml, were obtained in serum specimens from 87 of 101 patients (86%) with known colonic adenocarcinoma, 79% of 57 patients with cancer of other digestive organs, 56% of 98 patients with extragastrointestinal cancer, and 12% of 98 patients with noncancerous digestive disease. The results of Zamcheck's group (Dhar *et al.*, 1972; Zamcheck *et al.*, 1972) affirmed this lack of specificity. Of 127 patients with colonic cancer, tumor was present at the time of assay in 79 patients, and CEA was positive, namely, had concentrations greater than 2.5 ng/ml, in 57, or 72%, of these 79. The incidences of positive tests in other groups of patients were: cancer of other digestive organs, 70% of 27 patients; noncancerous digestive disease, 22% of 45 patients; and cancer of nonenteric organs,

46% of 13 patients. Finally, the same trend was illustrated by a large collaborative study by five university centers in Canada and in the United States under joint auspices of the National Cancer Institute of Canada and the American Cancer Society with the Montreal General Hospital laboratory (Dr. Gold's) acting as reference laboratory (Collaborative Study, 1972).

To summarize, these studies show that the incidence of positive CEA tests in patients with colorectal carcinoma decreased with each succeeding group of investigators. Conversely, the incidence of positive tests in the groups of patients with nonenteric cancer, with nonneoplastic digestive disease, etc., rose from zero incidence in the original report to levels of approximately 40–60% in the final collaborative study. More recent studies have again shown the same trend. For example, employing essentially the procedure applied by Lo Gerfo *et al.* (1971) and a value of 5 ng/ml plasma as the upper limit of normal, Fleisher *et al.* (1973) found the following incidences of positive tests in various cancers: lung, 74%; gastrointestinal tract, 54%; uterus, 63%; prostate, 60%; and breast, 54%.

It may be concluded, therefore, that the CEA tests lack sensitivity as a diagnostic procedure for cancer of the colon, even in established cases, and lack specificity as well. It has been stated that the CEA test may, nonetheless, be utilized as a diagnostic test for the presence of cancer, no matter what the type, and might indicate the clinical status of the patient such as after the resection of the tumor (Zamcheck *et al.*, 1972). But, in both of these connections, it would not appear to possess any advantages over other procedures we have discussed earlier in this volume, such as serum enzyme procedures (Chapter 2, Section II,D). Indeed, the CEA test may not possess the ease of technical performance that characterizes the enzyme tests.

E. Other Biochemical Changes

A few miscellaneous biochemical changes that have been reported in connection with cancer of the colon may be briefly described. It has been noted that serum alkaline phosphatase activity may be moderatlely elevated in patients with colorectal cancer when no metastases to the liver can be found (Baden *et al.*, 1971). Fabricuis-Bjerre *et al.* (1972) confirmed this finding and observed that this enzyme activity in tissue homogenates was 2–3 times higher in tumors than in the surrounding mucosa in 18 patients with colonic or rectal cancer. However, there was no correlation between the enzyme activities in tumor or serum, no postoperative fall in serum levels, and no difference between

enzyme activities in blood draining tumors, blood draining normal segments of the colon, and blood from peripheral vessels. There was no clear evidence, therefore, that the elevated serum alkaline phosphatase could be ascribed to the production of this enzyme by the tumor.

The relationship of cancer of the colon to the composition of the diet has claimed some attention, particularly with relation to geographic differences in incidences of this tumor (Wynder *et al.*, 1969). Hill and Aries (1971) have reported that the concentrations of urobilin, neutral steroids, and acid steroids were much higher in the feces of English and Scottish individuals than in the feces of Ugandans and Indians. These differences largely reflect the nature of the diets. The English and Scottish live on diets containing a high proportion of fat and animal matter and have a high incidence of cancer of the colon, whereas the Ugandans and Indians ingest diets that are largely vegetarian and have a low incidence of cancer of the colon. At the present time, these data are inadequate to establish a causal relationship.

It has been reported by several investigators that the higher transfer ribonucleic acid methylase activity (tRNA methylase) in extracts prepared from malignant cells is a result of the absence of inhibitors of the enzyme in those cells (Bergquist and Matthews, 1962; Chaney *et al.*, 1970). This enzyme catalyzes the methylation of the bases in intact tRNA and other types of RNA. Chaney *et al.* (1970) showed that a dialyzable inhibitor was present in normal adult tissue and absent in those malignant tissues examined, and this group of investigators subsequently purified the inhibitor and showed it to be nicotinamide (Halpern *et al.*, 1971). It has now been found that 0.005 *M* nicotinamide can inhibit crude preparations of tRNA methylases from human colon adenocarcinoma to the extent of about 41–57%. However, this effect can also be obtained for other human tumors (Buch *et al.*, 1972), but there is no effect on the enzyme in surrounding normal tissue.

V. Neoplasms of the Liver

A. Introduction

Cancers of the liver may be classed into 2 major groups, namely, primary carcinoma (malignant hepatocarcinoma) and secondary or metastatic carcinoma. In turn, primary carcinomas of the liver may be divided into 2 types: (a) liver cell carcinoma or malignant hepatoma, and (b) adenocarcinoma of the bile duct type or cholangiocarcinoma. The former are much more common. These 2 types may, however, be commingled in the same tumor. Microscopically, the liver cell carcinomas

show varying degrees of differentiation ranging from almost normal liver tissue, but without a proper lobular pattern, to anaplastic growth with great cellular pleomorphism and much degeneration and hemorrhage. The cholangiocarcinomas resemble carcinomas of the extrahepatic bile ducts (Ackerman and del Regato, 1970; Becker *et al.*, 1963). The liver is the organ most frequently involved in metastases. Of 1000 consecutive autopsied cases with carcinomas, the liver was affected in 49.4% of the cases, and carcinomas of organs responsible for these metastases were in descending order of frequency: breast, ovary, kidney, and lung and the group of stomach, colon, rectum, and pancreas (Abrams *et al.*, 1950).

The number of deaths from primary cancer and biliary passages in the United States in 1967 was 6059 whites and 669 nonwhites. The proportion of females dying from this disease was greater among whites than among nonwhites (Segi and Kurihara, 1972). It has been estimated that the incidence in 1973 will be approximately 7300 and the number of deaths will be 7200 (Silverberg and Holleb, 1973). The geographic distribution of primary cancer of the liver varies widely, and this is best expressed per 100,000 population. Thus, the death rates for various countries in 1966–67 for males were: United States, white, 5.42; nonwhite, 6.68; Japan, 13.06; Austria, 12.87; England and Wales, 4.59; South Africa, 5.26; and Australia, 3.40. The corresponding rates for females were: United States, white, 5.96; nonwhite, 4.25; Japan, 9.59; Austria, 17.93; England and Wales, 4.90; South Africa, 5.78; and Australia, 4.35 (Segi and Kurihara, 1972). The number of deaths in the United States from secondary and "unspecified" neoplasms of the liver was listed as 4581 for the year 1967 (Segi and Kurihara, 1972) but, as has been pointed out, the number of persons dying with autopsy evidence of metastatic lesions of the liver is much larger (Abrams *et al.*, 1950).

Several biochemical aspects of liver neoplasms have already been discussed as, for example, the elevation of various enzyme activities (Chapter 2, Section II,D and Chapter 3, Sections III,B,2 and IV,C,2). In the following pages, we shall consider chiefly hypoglycemia and serum α-fetoprotein in primary liver cancer but, in connection with this type as well as with secondary (metastatic) carcinoma of the liver, we shall also discuss alterations in other biochemical parameters of liver function.

B. Primary Carcinoma of the Liver

1. Hypoglycemia

The occurrence of hypoglycemia in primary carcinoma of the liver was first reported in 1929 (Nadler and Wolfer, 1929). Working in Hong-Kong, McFadzean and Yeung (1956) studied a series of 27 Chinese

patients with this condition; 23 had hepatomas of a massive type, and the remaining 4 had cholangiocarcinomas. Blood sugar studies were done in 25 patients, 9 of whom developed persistent hypoglycemia (Group I); the lowest blood sugar level, determined by the relatively nonspecific Folin-Wu method, ranged between 20 and 51 mg per 100 ml for these 9 patients. The incidences of the various symptoms of hypoglycemia in this group were hunger, 8; cold sweats, 7; weakness, 8; dizziness, 7; restlessness, 2; apprehension, 4; mental disturbance, 2; coma, 3; and convulsions, 3. The duration of observations in the 16 patients without hypoglycemia (Group II) lasted from 2 to 17 weeks. The fasting blood sugar was estimated at intervals not greater than 7 days, and the mean values ranged from 72 to 109 mg per 100 ml.

The rates of disappearance of intravenously injected glucose from the peripheral blood were determined in the patients of each group and were compared with the findings in 14 healthy controls. The average rate in each group of patients was not distinctly different from the average encountered in the healthy controls; the blood glucose reached a maximum about two- to threefold the fasting level within 5 minutes after injection, and receded to the pre-injection level within 2 hours. The 4 patients with cholangiocarcinoma were in Group II, namely, those without hypoglycemia. After the intravenous glucose, the decline from the maximum was slow and reached a plateau level, with the level at 2 hours significantly higher than the fasting level. This phenomenon occurs in other diseases of the liver. McFadzean and Yeung (1956) submitted no values for the liver weights or for the extent of the tumor in the liver, so that no correlation between these values and the occurrence of hypoglycemia was possible.

The mechanism of hypoglycemia in heptocellular carcinoma was further investigated by McFadzean and Yeung (1969). They observed that a series of 142 consecutive patients could be divided into 2 distinct types. In 124 of the patients (or 87%), the tumor grew rapidly and was poorly differentiated; rapid wasting and profound muscle weakness were characteristic of the patient. In this group, designated as type A, hypoglycemia was absent for almost the entire course, developing in only 20 cases within 2 weeks of death. This hypoglycemia was elicited slowly on fasting and could be readily controlled by the administration of glucose. Type B consisted of 18 of the total of 142 cases of hepatocellular carcinoma. The tumor grew slowly and was well differentiated. There was neither wasting nor muscle weakness. Hypoglycemia developed early in the course of the disease, within 2 to 10 months of death, was associated with a precipitous fall in blood glucose levels, and was difficult to control.

The results of a series of biochemical procedures may be briefly summarized. In type A patients, when compared with normal controls, the lower assimilation upon intravenous injection of glucose, the smaller decrease in serum inorganic phosphate, the lower content of glycogen in the liver and muscle, the lower insulin response on oral administration of glucose, and the lower response to glucagon all indicated a diversion of glucose from peripheral assimilation by the liver dominantly, but not exclusively, to the tumor; this diversion was greater the more massive the tumor. In type B patients, another factor additional to metabolic diversion of glucose was superimposed. In these patients, the glycogen content of the tumor and of the residual liver tissue was substantially higher than in the type A patients. The glycogen in the tumor and liver of the type B patient did not disappear on incubation at 37°C for 4 hours, whereas substantial *in vitro* glycogenolysis occurred in the liver of type A patients. Moreover, the response to glucagon injection in type B patients was even lower than in type A. These findings indicated that an acquired glycogenosis in type B patients was the additional factor responsible for the differences in the development of the two forms of hypoglycemia.

2. Other Biochemical Characteristics

Several studies of patients with primary hepatocarcinoma have provided biochemical evidence of liver impairment. The following have been reported: moderate to marked elevations in activity of serum alkaline phosphatase, serum glutamic oxaloacetic transaminase, and leucine aminopeptidase; moderate elevations (20–25% at 45 minutes) in bromsulfophthalein retention; and elevated serum cholesterol with increase in the free fraction (Schonfeld *et al.*, 1961; Becker *et al.*, 1963). The occurrence of polycythemia in a substantial fraction of Chinese patients with primary hepatocarcimoma and, more rarely, in other cases may result from the production of an erythropoietic substance by the liver tumor (Schonfeld *et al.*, 1961), and the report of a case with hypercalcemia, in addition to hypoglycemia and polycythemia, may reflect an example of pseudohyperparathyroidism, a subject which we shall discuss in a later chapter (Becker *et al.*, 1963).

It has been shown that cholesterol synthesis in animals is subject to a negative feedback control system. Thus, in the sequence

Acetyl CoA → β-hydroxy-β-methylglutaryl CoA → mevalonic acid → cholesterol

the single step which involves the reduction of β-hydroxy-β-methylglutaryl CoA to mevalonic acid is depressed when exogenous cholesterol

is introduced (Siperstein and Fagan, 1962). Cholesterol synthesis *in vitro* in biopsy material from liver of individuals on a high cholesterol diet, achieved by the addition of 3–4 gm cholesterol per day, is almost completely suppressed (Bhattathiry and Siperstein, 1963). This feedback mechanism was, however, found to be absent in rat hepatomas and two human hepatomas (Siperstein and Fagan, 1964; Siperstein *et al.*, 1966). For example, in normal individuals on a low cholesterol dietary intake of approximately 70 mg/day, [2-^{14}C]acetate per mg tissue was converted to an average of 309 pmoles cholesterol per mg liver in 2 hours. On a high cholesterol intake of 3–4 gm/day, this conversion was practically completely suppressed to 4 pmoles/mg per 2 hours. In a patient with hepatoma, the conversion of acetate on a low cholesterol diet was somewhat low, namely, 94 pmoles cholesterol formed per mg of hepatoma in 2 hours, and the administration of a high cholesterol diet failed to depress the conversion below 50 pmoles/mg of tissue per 2 hours. In contrast to patients in the United States with hepatoma, this effect could not be demonstrated in Ugandan patients since, on a low cholesterol diet, the synthesis was below approximately 17 pmoles/mg tissue in 2 hours, and a high cholesterol diet could not have visibly depressed these low levels any further (Bissell and Alpert, 1972).

3. Serum α-Fetoprotein

In 1956, Bergstrand and Czar reported that paper electrophoresis of sera from fetuses ranging from 10 to 26 cm in length revealed a new protein situated between albumin and α_1-globulin. The concentration rose from negligible levels at about 6–7 weeks of gestation to reach a maximum of approximately 280 mg per 100 ml at approximately 13 weeks, then declined to reach levels of less than 2% of the maximum by 34 weeks gestation (Gitlin and Boesman, 1966). The small concentration present in the newborn at term has an average half-life of 5 days during the first week and of 3 days during the succeeding weeks. Thus, the concentration decreases to a level of about 1.0 mg per 100 ml in about 12 days. This rapid decrease suggests a sharp curtailment of α-fetoprotein synthesis either at birth or during the few weeks before birth.

Although this protein is ordinarily absent from the serum in the normal adult, Tatarinov (1966) first detected its presence in the serum of 4 patients with primary carcinoma of the liver. Thereafter, several papers appeared in rapid succession to confirm this observation and to determine whether it was specific with respect to metastatic cancer of the liver, malignant neoplasms at other sites, and, indeed, other noncancerous disease (Abelev *et al.*, 1967; Abelev, 1968; Purves *et al.*, 1968; Masopust

et al., 1968). Incidentally, some of these reports have used the term "α-globulin" instead of "α-fetoprotein." Instead of describing the results of these and many other reports, it appears more advisable to summarize the results obtained in the collaborative study of O'Conor *et al.* (1970) and the more recent review article of Abelev (1971).

In 1966, the International Agency for Research on Cancer at Lyons, France initiated a collaborative study to evaluate and compare the specificity and sensitivity of the α-fetoprotein (AFP) test in human primary liver carcinoma. Serum samples were collected from centers in Kenya, Uganda, Republic of Congo, Nigeria, Senegal, Singapore, and Jamaica. The sera were tested at institutes at Villejuif, France and at Moscow and Astrakhan, USSR (O'Conor *et al.*, 1970). Antisera against AFP were prepared by immunization of rabbit, and a modified Ouchterlony technique of double diffusion in agarose was used for identification of AFP. Table 8-5 shows that, of 247 patients diagnosed clinically and/or histologically as having primary liver cancer, 151, or 61.1%, had positive tests in all three test laboratories; 80, or 32.4%, were negative in these laboratories and 16, or 6.5%, were subject to disagreement. There were 566 control cases, and these included hepatic cirrhosis, hepatitis, bile duct carcinoma, other liver lesions, primary cancer at extrahepatic sites, and infectious, nutritional, and metabolic diseases. As may be seen, only 12, or 2.1%, were positive in all three laboratories, and 1.9% were subject to disagreement. Of the 12 patients who had positive tests, 2 had teratocarcinomas of the testicle, the only type of tumor, in addition to primary hepatocarcinomas, which may have a substantial incidence (21%) of positive AFP tests (Abelev *et al.*, 1967).

TABLE 8-5

Results of Serological Test for α-Fetoprotein[a]

Test results	Cases of primary hepatocarcinoma		Controls	
	Number	Fraction (%)	Number	Fraction (%)
Positive by 3 test labs	151	61.1	12	2.1
Negative by 3 test labs	80	32.4	543	96.0
Disagreements	16	6.5	11	1.9
Grand totals	247	100.0	566	100.0

[a] Based on data of O'Conor *et al.* (1970). Reproduced by permission of J. B. Lippincott Company.

As has been noted, the cases with clinical and/or histological evidence of primary hepatocarcinoma had a percentage of 61.1% positive tests. O'Conor *et al.* (1970) have noted that, if only those cases with histological confirmation of primary hepatocarcinoma were considered, the percentage of positive cases increased to about 75%, which is essentially in agreement with the results of Masopust *et al.* (1968) and of Purves *et al.* (1968). More striking than this sensitivity is the specificity of the test. We have noted (Table 8-5) that the percentage of positive tests in conditions other than primary liver cancer was less than 2%. Abelev *et al.* (1967) reported that, outside of testicular carcinoma, there were no positive tests in a group of 233 cases which included the following conditions: benign liver tumor; metastatic liver tumor; malignant lymphoma; mammary carcinoma; cancers of the lung, stomach, or colon; hypernephroma; chorioepithelioma; a variety of children's tumor; and liver diseases other than tumors. Abelev's (1971) recent review of the world literature reaffirmed these general trends, although the highest frequency of elevated serum AFP in primary hepatocarcinomas is found in areas where this condition is endemic such as South Africa (78%) (Purves *et al.*, 1970), Indonesia (87%) (Kresno *et al.*, 1970), and Senegal (79%) (Uriel *et al.*, 1967). The proportion of AFP-positive hepatomas is smaller in most parts of Europe (Foli *et al.*, 1969; Abelev *et al.*, 1967) and in the United States (Smith and Todd, 1968).

It has recently been noted that a radioimmunoassay procedure is more sensitive than the double diffusion method, that hepatoma sera found negative by the latter method were positive by radioimmunoassay, and that metastatic cancer of the liver also yielded positive results by immunoassay. In 12 of 14 patients with primary cancer, the AFP levels by radioimmunoassay ranged from 0.1 to 840 μg/ml and most were more than one-thousand times above the upper normal level of 25 ng/ml. In 14 of 17 patients with secondary carcinoma of the liver, the serum levels were below 25 ng/ml, and the other 3 ranged from 60 to 400 ng/ml (Ruoslahti *et al.*, 1972). Obviously, if a higher level as, for example, 500 ng/ml, were taken as the differentiating point, 9 of 14 patients, or 64%, of primary cancer, would be positive.

Whether the double diffusion or the radioimmunoassay method is used, it would appear that the AFP test is fairly sensitive and highly specific for primary liver cancer and possesses substantial diagnostic usefulness. Moreover, in patients who undergo surgery for removal of their primary hepatocarcinoma, the serum AFP decreases sharply and may fall below the limit of quantitation, but rises again when tumor growth recurs. This determination is, therefore, a potential indicator of the growth and regression of hepatocarcinoma upon treatment of

the patient, whether by surgery or by other means (McIntire *et al.*, 1972).

C. Hepatic Function in Metastatic Cancer of the Liver

We have already discussed in some detail alterations of serum alkaline phosphatase activity (Chapter 3, Section III,B,2) and serum 5′-nucleotidase activity (Chapter 3, Section IV,C,2) in metastatic disease of the liver. The reader will also recall the data submitted on the frequency of serum elevations of ubiquitous enzymes in metastatic carcinoma of the liver and that glucosephosphate isomerase showed an incidence of 84%, higher than that of any other enzyme studied (Chapter 2, Section II,D).

Perhaps some of the most striking biochemical changes occur in extrahepatic obstruction of the biliary tract, and the older literature contains many references to such examples. Thus, in a series of 19 cases of obstruction resulting from carcinoma of the head of the pancreas, the distribution of serum bilirubin values were: no cases at 0–1 mg bilirubin per 100 ml; 11% at 1.1–5.0 mg per 100 ml; 21% at 5.1–11.0 mg per 100 ml; and 68% at 11.1–25.0 mg per 100 ml (Bodansky and Bodansky, 1952). The occurrence of hypercholesterolemia is also marked. For example, in a series of 82 cases of obstruction resulting from carcinoma of the head of the pancreas or of metastases to the lymph nodes, 88% of the cases had levels above 250 mg cholesterol per 100 ml as compared with 9% in a group of 117 normal individuals (Bodansky and Bodansky, 1952). It is to be expected that other biochemical manifestations of extrahepatic obstruction will characterize this type of metastasis or extension to the liver. These include the presence of bilirubinuria; the diminished excretion of fecal urobilinogen; the higher proportion of conjugated serum bilirubin; negative serum flocculation tests; and, as we have noted in Chapter 3 (Sections III,B,2 and IV,C,2), greatly increased activities of serum alkaline phosphatase and 5′-nucleotidase.

Hepatic function was studied in some detail in a series of 99 cancer patients with proven intrahepatic metastases and compared with a control group of 61 cancer patients who had no such metastases (Mendelsohn and Bodansky, 1952). The serum protein, blood urea nitrogen, thymol turbidity, total cholesterol, and hemoglobin were not significantly affected by the presence of liver metastases. In contrast, the values for cephalin-flocculation, serum bilirubin concentration, bromsulfophthalein retention, and serum alkaline phosphatase activity were significantly elevated.

VI. Neoplasms of the Pancreas

A. Introduction

Cancer of the pancreas is one of the more common malignant neoplasms. In 1967, it accounted for 9,685 deaths in males and 7,176 in females in the United States (Segi and Kurihara, 1972). It has been estimated that the incidence will be approximately 19,400 in this country in 1973, and that the deaths will amount to 19,200 (Silverberg and Holleb, 1973). Some anatomic detail of the pancreas is necessary to understand the effects of neoplastic growth. The pancreas is a reddish yellow organ, 4–6 inches long, weighs from 60 to 100 gm, extends transversely across the abdomen from the concavity of the duodenum to the spleen, and is not subject to direct physical examination. The right portion of the pancreas, called the head, is somewhat globular in shape, is covered anteriorly by the pylorus and transverse colon, and fits snugly into the bend of the duodenum. The common bile duct passes either through a groove or through the substance of the head of the pancreas. This portion of the pancreas is separated from the main part, or the body of the pancreas, by a short neck, and the body then tapers off into a short tail. The substance of the pancreas is composed largely of alveolar cells which secrete a juice containing various electrolytes and enzymes into ducts which merge into the principal duct of Wirsung; this passes through the entire pancreas and terminates at the papilla in the duodenum. This system constitutes the exocrine portion of the pancreas. Scattered throughout the pancreas are the islands (or islets) of Langerhans. These are composed of several types of cells, of which the best known are the beta cells, associated with the secretion of insulin.

B. Cancer of the Pancreatic Ducts

1. Introduction

As we have noted, the majority of pancreatic neoplasms arise in the ducts. Approximately 60–70% of these are in the head of the pancreas, 20–30% in the body, and 5–10% in the tail (Arkin and Weisberg, 1949; Cliffton, 1956; Gullick, 1959; Green *et al.*, 1958). Extension of the tumor is direct, by invasion of the remaining gland or duodenum and by adherence to adjacent structures. Extension through the lymphatic and blood vessels involves the following organs in order of decreasing frequency: liver, lungs, intestine, adrenals, bone, and other organs. The tumors

in the head of the pancreas frequently produce biliary obstruction, and we have just discussed the biochemical manifestations of this phenomenon.

However, other biochemical aspects are concerned with alterations in the nature of the pancreatic secretion. In the acinar cell, where the formation of the secretion begins, ribonucleoprotein particles are associated with chymotrypsinogen, trypsinogen, ribonuclease, and amylase. Siekevitz and Palade (1960) have studied in some detail the cytochemistry of the guinea pig pancreas. Several of the important steps appeared to be the formation of zymogen granules containing the various enzymes, the migration to the apical region of the cell, and the discharge of the granules into the lumen of the pancreatic ductules, which then empty into the main duct of Wirsung.

The volume and the composition of the pancreatic secretion depend upon the number of physiological stimuli, but some average values may be given. The volume ranges from about 1200 to 2000 ml/day and the approximate mean value for the concentrations of the major electrolyte components in milliequivalents per liter are sodium, 148; bicarbonate, 80; chloride, 80; potassium, 7; and calcium, 6 (White *et al.*, 1968). Amylase, carboxypeptidase, trypsinogen, and chymotrypsinogen and the conversion products, trypsin and chymotrypsin, are present in substantial activities. Other components present in minor amounts or activities include phospholipases A and B, alkaline phosphatase, phosphorus, and magnesium.

2. Biochemical Findings in Cancer of the Pancreatic Duct

Many of the older investigations concerned themselves with the nature of the duodenal secretion. Comfort *et al.* (1939) observed that in 73% of their series the duodenal trypsin activity was less than 2 arbitrary units, as compared with 12% of a series of normal individuals and 25% in patients with liver disease or where the obstruction resulted from stone. Comfort *et al.* (1939) also found that, in about 67% of patients with obstruction resulting from carcinoma of head of the pancreas or of the ampulla of Vater, the duodenal secretion had a low amylase activity, as compared with 12% in normal individuals, 23% in liver disease, and 52% in patients with obstruction resulting from stone. However, these were relatively small series, and evaluation of statistical significance was not carried out.

Within recent years, evocative tests, which depend on the stimulation of pancreatic secretion, have been studied in some detail in order to

differentiate between different types of pancreatic disease (Wirtz, 1961; Howat, 1970; Dreiling, 1971). After proper preparation of the patient and passing of a double-lumen gastroduodenal tube, secretin or secretin and pancreozymin in order are given intravenously and the duodenal aspirate is collected for a stated period, usually 60 minutes. Secretin is a polypeptide composed of 27 amino acids, and pancreozymin is another peptide consisting of 37 amino acids.

The secretin-pancreozymin test appears to be a sensitive test of impaired pancreatic function in both chronic pancreatitis and cancer of the pancreas (Howat, 1970). For example, in acute pancreatitis the incidences of decreases of various parameters were rather low: volume of secretion, 5%; maximal bicarbonate concentration, 15%; maximal bicarbonate output, 18%; and amylase output in the postpancreozymin phase, 8%. In patients with pancreatic carcinoma, the incidences of decreases were: maximal bicarbonate concentration, 68%; maximal bicarbonate output, 76%; and amylase output, 82%. Although Howat (1970) considered that there were also differences between pancreatic carcinoma and chronic pancreatitis, these differences were small, and no statistical evaluation concerning their significance was submitted.

Evidence of a disturbed carbohydrate metabolism in cancer of the pancreas has been at hand for many years (Mirallié, 1893). According to the composite data of reports from 14 authors during the period 1893–1941, the incidence of glycosuria was 9.4%, and of 25 cases, 60% had abnormally high fasting sugars or hyperglycemia (Dashiell and Palmer, 1948). A later report indicates an incidence of 38% of hyperglycemia in a series of 50 patients (Masley *et al.*, 1960). The incidence of decreased glucose tolerance has been reported in series of studies as ranging from 25 to 80% (Dashiell and Palmer, 1948; Green *et al.*, 1958; Gullick, 1959; Masley *et al.*, 1960). Glycosuria has been variously reported as 9.4% in one composite report (Dashiell and Palmer, 1948) and up to 29% in others (Green *et al.*, 1958; Gullick, 1959). Indeed, it has been held that the occurrence of one or more of the findings of hyperglycemia, decreased glucose tolerance, or glycosuria may be of aid in making the diagnosis of pancreatic carcinoma (Kowlessar, 1967).

Several different mechanisms for the occurrence of these evidences of disturbed carbohydrate metabolism have been offered (Masley *et al.*, 1960). In Chapter 1 (Section III,B) it was pointed out that this disturbance is present generally in many types of cancer, and it is possible that its occurrence in pancreatic carcinoma is not particularly specific. Other possibilities that have been proposed are (a) trypsin is released into the circulation by the disruption of acinar tissue by the growing tumor and inactivates insulin being furnished by intact islet beta cells,

(b) the growing tumor interferes with the escape of insulin from intact beta cells, and (c) the beta cells are encroached upon and damaged or destroyed by the growing tumor (Masley *et al.*, 1960). At the present time, there are no data that would give predominant support to any of these possible mechanisms.

C. Pancreatic Islet Cell Neoplasms

1. Introduction

The islets of Langerhans, the pale areas larger than the individual acini, are distributed throughout the pancreas. In normal man, the islets contain 3 types of cells: (a) the beta cells, about 75% of the total, which are responsible for the production of insulin; (b) the alpha cells, about 20% of the total, which are involved in the synthesis of glucagon; and (c) D cells, approximately 5% of the total, which secrete gastrin (Williams, 1968).

Microscopically, the adenomas consist of cells resembling those of normal islets, arranged in clumps and ribbons and sometimes showing acinar or ductular grouping. They are often not encapsulated and may show marginal infiltration of surrounding pancreatic tissue. This infiltration may be so extensive as to classify the tumor as an islet carcinoma.

The existence of pancreatic islet cell tumors was first described by Nicholls in 1902. In 1935, Whipple and Frantz reviewed 75 cases of hyperinsulinism. In 62 of these cases, tumors were found in the pancreas and of these, 3 were carcinomatous and had metastases. By 1960, a total of some 600 of these tumors had been described in the world literature (Hanson, 1960). By far the large majority, approximately 90%, were nonmalignant and of the simple islet cell adenoma type. The remaining 10% were chiefly malignant. As will be discussed presently, islet cell tumors may also include tumors of the alpha cells. In view of the latter possibility, the islet cell tumors of the beta cells, frequently referred to as adenomas and carcinomas of the islets, should more properly be termed "insulinomas" (Hanson, 1960).

2. Beta-Cell Islet Tumors (Insulinomas)

It has been estimated that the pancreas of a normal man contains about 4 units of insulin per gm of pancreas or a total of 200 units. The normal secretion is about 50 units/day (Williams, 1968). The international unit consists of the amount of insulin which will lower the blood sugar of a normal fasting 2-kg rabbit to the convulsion level of

45 mg per 100 ml in 5 hours. Human crystalline insulin has an activity of about 22 units/mg (Beaser, 1958; Levine, 1967). Although it is generally assumed that more insulin is produced by beta-cell tumors of the islets, there appear to be no data on the content or secretion of insulin by these tumors.

It is beyond the scope of this volume to discuss in detail the biochemical characteristics of the manifold actions of insulin. The latter have been admirably and critically summarized by Levine in his Joslin lecture (1967) and may be listed as follows: (a) stimulates membrane transport of glucose in skeletal muscle, cardiac muscle, fat cells, and fibroblasts; (b) increases the conversion of the transported glucose to glycogen, probably as a result of the activation of glycogen synthetase; (c) inhibits rate of lipolysis in adipose tissue; (d) increases potassium uptake by muscle; (e) increases the cellular uptake of amino acids and their incorporation into muscle protein; and (f) influences the rate of sugar output and uptake by the liver cells, even though the liver cell membrane appears to lack a glucose transport system.

The literature has not recognized until recently that insulinomas are only one kind of adenomas or carcinomas of the islets. It had been recognized that the presence of adenomas or carcinomas was not invariably associated with hypoglycemia (Whipple and Frantz, 1935; Howard *et al.*, 1950; Scholz *et al.*, 1960). For example, of 398 cases recorded in the literature and reviewed by Howard *et al.* (1950), 91 of 361 patients with benign adenomas or suspiciously malignant neoplasms had nonfunctioning tumors. Of 37 cases with carcinoma of the islets, 22 were functioning, 12 nonfunctioning, and evidence was not available in 3 cases. Consequently, as will be noted presently in detail, this, as well as other series, may have contained adenomas which did not produce insulin.

As has already been noted and will be brought out again in the course of this volume, hypoglycemia may arise from any one of a number of causes other than insulinoma. These include (a) deficient blood supply, (b) certain hepatic disorders, (c) deficiency of insulin-counteracting hormones, (d) disorders with abnormal secretion of insulin, (e) extrapancreatic neoplasms, (f) various nervous system disorders, and (g) various drugs such as sulfonylureas. The clinical signs and symptoms associated with hypoglycemia are essentially the same, regardless of the cause and, in the early stages, consist of restlessness, irritability, apprehension, sweating, and mental confusion. In the later stages, agitation, profuse sweating, and abnormal psychological behavior may ensue and culminate in coma and convulsions.

The extent of hypoglycemia in tumors of the islets of the pancreas has been observed by many investigators (Howard *et al.*, 1950). For

example, in the early series of Whipple and Frantz (1935), the fasting blood sugar was determined, presumably by a variety of methods, in 28 cases. The values ranged from 4 to 58 mg per 100 ml, and in 26, or 93% of the cases, the concentration was less than 50 mg per 100 ml. The cases with carcinoma did not exhibit more marked hypoglycemia than did the patients with simple adenoma. Again in the series of Scholz *et al.* (1960) on 85 adenomas and 10 carcinomas, observed at the Mayo Clinic during the years 1927 through 1958, the distribution of the concentrations of blood sugar after overnight fasting in 91 cases in whom the determination was done was as follows: no patients less than 25 mg per 100 ml, 3 at 26–35 mg per 100 ml, 24 at 36–45 mg per 100 ml, 18 at 46–55 mg per 100 ml, 20 at 56–65 mg per 100 ml, and 26 at more than 65 mg per 100 ml. The procedure used was the Folin–Wu method with a normal range of 80–120 mg per 100 ml. Varying periods of fasting up to 48 hours were necessary to produce a hypoglycemic attack. Table 8-6 shows the distribution of the lowest levels of blood glucose levels attained during hypoglycemic attacks.

The preceding considerations form the basis for a clinical–biochemical diagnostic criterion which has come to be known as Whipple's Triad (Whipple, 1944; Clarke *et al.*, 1972). This criterion consists of (a) hypo-

TABLE 8-6

Lowest Level of Blood Sugar during Hypoglycemic Attacks in Patients with Adenoma or Carcinoma of the Pancreatic Islets[a]

Concentration of blood sugar in mg/100 ml	Number of patients with	
	Adenoma	Metastasizing carcinoma
20 or less	0	0
21–25	15	0
26–30	18	4
31–35	27	5
36–40	17	1
41–45	7	0
More than 45	0	0
No record	1	0
Total	85	10

[a] Data of Scholz *et al.* (1960). Reproduced by permission of the Mayo Association.

glycemic attacks precipitated by fasting or exertion, (b) fasting blood sugar concentrations below 50 mg per 100 ml, and (c) symptoms relieved by oral or intravenous administration of glucose.

If the pancreatic islet beta-cell tumors, which we have been discussing, secrete abnormal amounts of insulin as they have been presumed to do, then it might be expected that patients with such tumors would have a high rate of removal of glucose by the peripheral tissues (glucose assimilation). Conard *et al.* (1953) developed an equation to express this phenomenon:

$$kg = \frac{\log_e 2}{t/2} \times 100$$

Fifty milliliters of a 50% solution of glucose is injected intravenously during a period of 90–120 seconds and capillary blood samples are obtained before the injection, at the midpoint of the injection, and at 5–10 minute intervals for the next 60–90 minutes. The time taken for the blood glucose to fall to one-half its value is designated as $t/2$ and is expressed in minutes; "kg" is the "glucose assimilation coefficient" and its dimensions would be in min^{-1}. The mean value for the coefficient was 1.72 ± 41 (SD) min^{-1} in a series of 10 controls (Marks and Marrack, 1962). In 8 of 10 patients with insulinomas, the coefficient was normal or low. It was suggested that the defect in homeostasis in such patients is the result of impaired release of glucose by the liver under the influence of insulin arriving in the portal vein.

Other criteria have been used to elicit the mechanism of hypoglycemia in pancreatic islet cell tumors. The administration of L-leucine, which has no effect on blood glucose in normal persons, has been shown to accentuate hypoglycemia in a number of conditions such as "idiopathic hypoglycemia" of infants (Cochrane *et al.*, 1956), in healthy and diabetic subjects after repeated prior administration of chlorpropamide (Fajans *et al.*, 1960), and in patients with pancreatic islet cell tumors (Flanagan *et al.*, 1961; Marks and Klein, 1961; Field, 1964). The decreases in this last condition may amount to from 21 to 76% below already low levels ranging from 20 to 40 mg per 100 ml and may be accompanied by a rise in plasma insulin levels. The antidiabetic compound, sulfonylurea, accentuates the hypoglycemia and increases the glucose assimilation constant of patients with pancreatic islet cell tumors (Marks and Marrack, 1962; Field, 1964; Williams *et al.*, 1969). It would, therefore, appear that although insulin is being excessively produced in beta-cell tumors further stimulation of the cells is possible.

Removal of the beta-cell tumor results in the excessive rise of the blood glucose, often to levels of 300 mg/ml or more. This rise may

be evident within 2 hours after operation (Field, 1964), and the level begins to fall toward normal levels in the succeeding several days (Williams *et al.*, 1969). However, this phenomenon does not occur to the same extent when there are multiple adenomas in the pancreas and it is not possible to remove them all, or when metastases to the liver have occurred. The blood glucose may rise only to moderate levels after operation, and hypoglycemia may set in again within a few hours postoperatively.

The possibility that assay of plasma insulin might aid in the detection of insulinomas or in postoperative follow-up of the patients has been explored by several groups (Samols and Marks, 1963; Khurana *et al.*, 1971). Using a specific immunoassay, Samols and Marks (1963) found that the range for fasting plasma insulin in 100 control subjects was 2–63 μU (microunits) of insulin per ml with a mean value of 19 μU $\pm$ 7.5 (SD). In 9 of 10 patients with insulinoma, there was at least one high fasting plasma insulin level, but in 4 of these, there were occasions when hypoglycemia was not accompanied by a high plasma insulin. Indeed, in 1 patient, 3 plasma samples, each drawn during hypoglycemias of 13–36 mg glucose per 100 ml, showed normal levels of plasma insulin, ranging between 31 to 46 μU/ml. The rise in plasma insulin after intravenous injection of tolbutamide (1-butyl-3*p*-tolylsufonylurea) or after ingestion of L-leucine was greater in the patients with insulinoma than in control subjects and was abolished by the removal of the tumor.

In 1971, Khurana *et al.* reviewed the literature on the blood sugar and serum insulin in 59 published cases of proven insulinoma prior to and after oral glucose, intravenous tolbutamide, oral leucine, and/or injected glucagon. Figure 8-2 shows the insulin and blood glucose levels before and at various times after the intravenous injection during 2 minutes of 1.0 gm sodium tolbutamide dissolved in 20 ml sterile water. It was found that marked fasting or spontaneous hypoglycemia was present in only about one-half of the patients with insulinoma and, as has been pointed out, is not specific. Fasting insulin levels are elevated in only one-third to one-half of the patients with insulinoma. It may be seen further that, in the tolbutamide tests performed in patients with this tumor, false negative values occur in one- to two-thirds of the time points and false negative insulin values predominate.

3. Alpha-Cell Tumor (Glucagonoma)

In their review of islet cell tumors of the pancreas, Howard *et al.* (1950) noted that of 154 patients who were diagnosed at autopsy as having benign adenoma, 5 had been listed as having diabetes, with

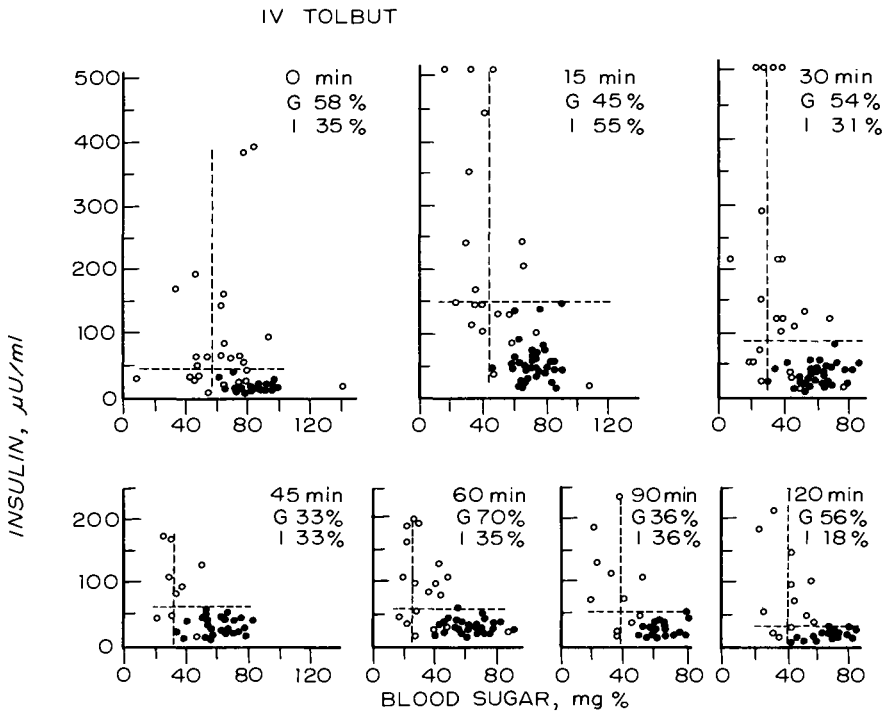

Fig. 8-2 Results of intravenous tolbutamide tolerance tests in (○) insulinoma patients and in (●) healthy young adults. Blood glucose and serum insulin levels of insulinoma patients overlapped in varying degree those of the control subjects. "G" refers to percentage of insulinoma blood glucose values and "I" indicates percentage of insulinoma serum insulin values within the control range, i.e., the percentage of false negatives. From Khurana *et al.* (1971). Reproduced by permission of Charles B. Slack, Inc.

a fasting blood sugar being determined in 1 case and having been found to be 236 mg per 100 ml. Again, in a group of 15 nonfunctioning carcinomas of the islets, 5 patients had shown normal fasting blood sugars ranging from 76 to 117 mg per 100 ml. These data give rise to the possibility that several of the 398 islet cell tumors which Howard *et al.* (1950) reviewed were not beta-cell insulin-producing tumors.

In 1966, McGavran *et al.* reported the case of a 42-year-old woman who was admitted because of bullous and eczematoid dermatitis of the hands. In the course of her work-up, she was found to have a 2-plus test for sugar in the urine and a diabetic glucose tolerance curve. Eighteen months later, she was admitted because of an episode of severe right-sided pleuritic pain and was found to have an enlarged, hard liver

that elevated the right diaphragm and extended 7 cm below the costal margin. Radiographic examination confirmed these findings. Biopsies of a mass in the tail of the pancreas and of metastases in the right lobe of the liver were taken. These were first diagnosed as undifferentiated carcinoma of the pancreas, but, on later review, were considered to be of islet origin. Eight months later, the patient was re-explored, and it was possible to resect the primary tumor in the pancreas, but not the hepatic metastases. Additional diagnostic determinations showed normal values for the volume, pH and acid content of overnight gastric secretion, for serum potassium and for 24-hour excretion of 5-hydroxyindoleacetic acid, 3-methoxy-5-hydroxyvanylmandelic acid [*sic*], and epinephrine. The 24-hour urinary excretion of norepinephrine was slightly elevated. These findings tended to exclude the possibilities of Zollinger–Ellison syndrome, carcinoid, ACTH-secreting tumors, and pheochromocytoma.

Histochemical and electron microscopic examination revealed that the tumor cells were different from the beta cells of the islets and resembled alpha cells. Radioimmunoassay showed a concentration of 14 μg of glucagon per gm of tumor tissue, as compared with a value of about 20 μg for the entire normal human pancreas or about 0.2 μg/gm of normal pancreas.

Some blood biochemical parameters obtained in this patient are shown in Table 8-7. It may be seen that the fasting plasma glucagon was

TABLE 8-7

Insulin, Glucagon, and Glucose Parameters in Patient with Glucagon-Secreting Alpha-Cell Carcinoma of the Pancreas[a]

	Controls	Patient
Fasting plasma glucagon (ng/ml)	36–55	<2
Fasting plasma insulin (μU/ml)	18[b]	80
Increase in plasma glucose over base line in response to intravenous glucagon (mg/100 ml)	44–60	0–8
Increase in plasma insulin in response to intravenous glucagon (μU/ml)	80–215	0–10
Increase in plasma insulin in response to 25 gm intravenous glucose (μU/ml)	—	110
Increase in plasma insulin at 1 hour in response to oral glucose (30 gm/m²)(μU/ml)	From 20 to 115[c]	From 80 to 180[c]

[a] From McGavran *et al.* (1966).

[b] These data were obtained from Vance *et al.* (1969).

[c] Estimated from Figs. 6 and 7 of McGavran *et al.* (1966).

greatly depressed in this patient, whereas the plasma insulin was elevated. The patient showed little response in the levels of blood glucose or plasma insulin after injection of glucagon, whereas normal controls showed substantial rises. In response to ingestion of glucose, the patient had an initial decrease in plasma insulin, but the differential rise, i.e., the increase between the fasting and maximal levels, was of the same order in controls and in the patient.

The alpha-cell type of islet tumor appears to be very rare, but several other cases of this neoplasm have been described since the initial report (Vance *et al.*, 1969; Croughs *et al.*, 1972; Sturner, 1972). One of its more interesting aspects is that it may appear as part of an endocrine adenomatosis. For example, Vance *et al.* (1969) studied the members of a family with this condition in which the pancreatic islets, the parathyroid, and the adrenal cortex were involved. Of 6 subjects representing three generations, all had excessive secretion of insulin, 3 showed excessive plasma glucagon response to oral administration of glucose, and 4 had elevated plasma gastrin levels. These manifestations of multiple islet activity suggested that a genetic defect resulted in a proliferation of the primordial cells of the islets of Langerhans (nesidoblastosis). A patient studied by Croughs *et al.* (1972) was diagnosed as having Cushing's syndrome as a result of an adrenal adenoma and hyperparathyroidism. In the course of an operation to remove this adenoma, the pancreas was examined and found to have a tumor located superficially in the head of the pancreas. The tumor was removed and found to weigh 1.1 gm. Histochemical studies suggested classification as the alpha-cell type. Biochemical studies of the tumor extract showed absence of gastrin in a 0.3-gm specimen, the presence of glucagon at a concentration of 1.15 mg/gm as determined by radioimmunoassay, and the capacity to increase plasma glucose and insulin levels on injection into a dog.

4. The Zollinger–Ellison Syndrome

In 1955, Zollinger and Ellison reported two instances of primary nonspecific ulcers associated with marked gastric hypersecretion and hyperacidity and the presence of non-beta-cell tumors of the pancreatic islets. Four other cases that had been reported in the literature were recognized to fall into this category. These observations stimulated many others to appreciate the existence of this syndrome and, by 1968, approximately 500 cases had been reported (Zollinger and Moore, 1968).

Gregory and his associates (1960, 1967), who were instrumental in isolating the gastrin from the mucosa of the pyloric gland area of the hog stomach, isolated a very similar substance from the pancreatic tumors

in the Zollinger–Ellison syndrome. This was a pair of heptadecapeptide amides. Subsequently, Gregory and Tracy (1972) isolated, in addition, a pair of larger, less acidic, gastrin peptides from Zollinger–Ellison tumor tissue. These consisted of the heptadecapeptide amide with an *N*-terminal glutaminyl residue instead of a pyroglutamyl residue. The pair of "big" gastrins were also potent stimulants of gastric acid secretion. The existence and nature of these "big" gastrins and of a "big, big" gastrin have received further investigative attention (Renderknecht, 1972; Rehfeld and Stadil, 1972; Yalow and Berson, 1972).

The establishment of the presence of gastrins in the Zollinger–Ellison tumor and of their activity as gastric secretagogues stimulated investigation of a third type of cell in the pancreatic islets. The existence of such a cell in the normal human islet had been recognized since 1931 and was designated as the α_1 cell and later as the D cell. The presence of gastrin in the islet D cell of the normal human pancreas was identified by means of immunofluorescence techniques (Lomsky *et al.*, 1969; Greider and McGuigan, 1971). Employing immunofluorescent, cytochemical, and electron microscopic techniques, Polak *et al.* (1972) have studied the cells in samples from the central and fundic mucosa of the stomach and from the body or tail of the pancreas in patients with the Zollinger–Ellison syndrome. It was found that the patients could be divided into 2 groups, as shown in Table 8-8. We shall presently illustrate these 2 groups with specific biochemical findings and shall also consider the relation of this classification to a group of cases with non-beta-cell tumors of the pancreas which are characterized by watery diarrhea, hypokalemia, and achylia and have been designated as the "WDHA syndrome."

One of the outstanding biochemical findings in the Zollinger–Ellison

TABLE 8-8

Comparison of Gastric pH, Hourly Basal Flow, and Acid Secretion in Normal Subjects and in Patients with the Zollinger–Ellison Syndrome[a]

Group	No. of patients	Basal average pH	Basal average flow (ml/hour)	Basal acid (mEq/hour)	Histamine augmented average acid (mEq/hour)
Normals	350	5.3	66	2.3	28.7
Zollinger–Ellison	10	1.0	405	44.3	57.5

[a] From Dreiling and Greenstein (1972).

syndrome is the excessive secretion of gastric juice and the high acidity of this secretion. For example, in the first patients reported by Zollinger and Ellison (1955) and admitted to the hospital in 1952, a 12-hour nocturnal gastric aspiration yielded a total volume of 2000 ml and a total free acid of 164 mEq. About 2 months later, after removal of an obstructing intestinal lesion, the 12-hour gastric secretion had a volume of 3170 ml; free HCl, 86 clinical units, and total free HCl, 272 mEq. These values are greater than the normal mean values which, it will be recalled (Section II,B,2), are free acidity, 29, with a range of 1–90 clinical units and total free HCl, 18 mEq with a range of 0.5–95 mEq. Recent and comprehensive studies by Dreiling and Greenstein (1972) show even more strikingly the extent of the gastric hypersecretion (Table 8-8). These investigators also observed that both the basal and secretin-stimulated volume and bicarbonate concentration of pancreatic secretion were substantially higher in patients with the Zollinger–Ellison syndrome than in normal individuals.

In their original report, Zollinger and Ellison (1955) suggested that the pancreatic islet tumor might elaborate into the peripheral circulation a humoral substance capable of stimulating gastric acid production. With the identification of the chemical nature of gastrin, it became feasible to search for its presence in serum. Employing a radioimmunoassay technique, McGuigan and Trudeau (1968) found that the serum concentration in a control group of 24 hospitalized patients without recognized gastrointestinal disease ranged from 245 to 668 and averaged 425 $\pm$ 136 (SD) pg/ml. The serum levels in 3 patients with clinical evidence of and histologically verified Zollinger–Ellison syndrome were 3550, 7800, and 21,000 pg/ml, values considerably above the upper limit of normal. Thompson *et al.* (1972) have more recently reported a series of 15 proven cases of the syndrome. Their upper limit of normal was 250 pg/ml and 3 of their patients showed values ranging between 157 and 500 pg/ml; 2 patients had concentrations ranging between 747 and 867 pg/ml and 10 patients had concentrations, on at least one occasion, greater than 1000 pg/ml. Though operative removal of the tumor may result in reduction of the serum gastrin level, the presence of metastases may counteract such a reduction, and continued spread may result in a continuous increase of the level. For example, in a patient with initial preoperative values of about 1000 pg/ml, removal of a mass in the distal portion of the pancreas decreased this level slightly. A larger mass, 4 $\times$ 6 cm, had been visualized in the liver and this was apparently the continued source of gastrin. The serum concentration rose in the course of 600 days to a level of 30,500 pg/ml.

A number of interrelationships between the level of serum gastrin

and other biochemical parameters have been elicited recently. For example, in a patient with the Zollinger–Ellison syndrome and carcinoma of the parathyroid, parathyroidectomy resulted in a sensational fall of the concentration of serum gastrin from approximately 12,000 to 600 pg/ml, while the serum calcium dropped from about 12 to slightly more than 6 mg per 100 ml. Conversely, substantial increases in serum gastrin concentration and, as was to be expected, in serum calcium concentration were induced by calcium infusion (Trudeau and McGuigan, 1969). This phenomenon has been applied as a possible prognostic procedure in deciding whether a calcium-stimulated gastrin elevation may be of aid in providing a means for determining residual pancreatic D-cell islet function after gastrectomy. It had been observed that the long-term survival rates for patients with the Zollinger–Ellison syndrome treated by total gastrectomy have been far better than would be anticipated, and it has been suggested that removal of the end organ (stomach) eliminates a stimulus for tumor growth. Of 6 patients tested in this connection by calcium infusion, 3 showed either no rise or only a modest increase in serum gastrin, whereas the others demonstrated dramatic increases amounting to several thousand picograms per milliliter of serum (Sanzenbacher *et al.*, 1973). It was concluded that the calcium-stimulated gastrin procedure might be of value in detection of retained functioning tumor after gastrectomy.

Another interesting interrelationship concerns the effect of secretin, the peptide that stimulates pancreatic secretion. Whereas this compound inhibits stimulated gastric acid secretion in normal man, a patient with the Zollinger–Ellison syndrome has been described in whom secretin increased gastric acid secretion and caused elevations of serum gastrin and serum calcium (Isenberg *et al.*, 1972).

5. The Watery Diarrhea-Hypokalemia-Achlorhydria (WDHA) Syndrome

Another type of pancreatic islet cell tumor is that first clearly described by Priest and Alexander (1957). The outstanding clinical symptom was a long-continued watery diarrhea with muscle weakness, anorexia, nausea, occasional vomiting, and upper abdominal pain. This condition has also been termed "pancreatic cholera." Microscopically, the cells of the tumor are somewhat larger than in the tumor of the Zollinger–Ellison syndrome, have a clearer cytoplasm and large nuclei which contain less chromatin material. The cells do not appear to be of either the alpha- or beta-cell type (Chears *et al.*, 1960; Longmire *et al.*, 1968; Zollinger *et al.*, 1968). The tumor is rare and by 1968 a total of only

15 cases had been reported in the English literature (Longmire *et al.*, 1968). Since then, additional cases have continued to be recorded (Zollinger *et al.*, 1968; Lopes *et al.*, 1970; Sircus *et al.*, 1970; Gjone *et al.*, 1970; Karacadag *et al.*, 1972; Elias *et al.*, 1972; Jacobs and Halperin, 1972). Forty cases had been reported by 1972 (Jacobs and Halperin, 1972).

The two major biochemical findings are hypokalemia and achylia or diminished gastric secretion. In contrast to the excessive gastric secretion and acidity that characterize the Zollinger–Ellison syndrome, the WDHA syndrome is featured by absent or very low gastric acidity. For example, in one of the cases reported by Zollinger *et al.* (1968), the basal values showed absence of hydrochloric acid, and the total production in 1 hour after histamine stimulation was 5.0 mEq on one occasion and 1.40 mEq on another. These values are to be contrasted with basal values of 2.3 and 44.3 mEq in normal individuals and patients with the Zollinger–Ellison syndrome, respectively, and with 28.7 mEq/hour in normals and 57.5 mEq/hour in Zollinger–Ellison patients (Table 8-8). In a review of 8 cases in 1967, Marks *et al.* found 4 cases with very low gastric acidity and 4 with complete achlorhydria.

The second striking feature of the WDHA syndrome is the hypokalemia. In the 15 cases reviewed by Longmire *et al.* (1968), all had low serum potassium concentrations, and death was attributable to this hypokalemia in 9 cases. For example, in the case of the patient reported by Chears *et al.* (1960), the serum potassium fluctuated between 1.8 and 5.1 mEq potassium during eight admission periods, some of which followed attempts for correction of dehydration and potassium depletion. Eight of fifteen determinations recorded during these periods were 3.0 mEq/liter or lower. In the case described more recently by Karacadag *et al.* (1972), the following serum biochemical parameters were abnormal on admission: K^+, 1.7 mEq/liter; Na^+, 128 mEq/liter; Cl^-, 86 mEq/liter; HCO_3^-, 19.6 mEq/liter; calcium, 7.8 mg per 100 ml; and phosphorus, 1.7 mg/100 ml. In a recent review of 26 patients with the WDHA syndrome (Kraft *et al.*, 1970), the patients' stool volumes were measured daily in 20 cases and averaged 5.8 liters/day. In 14 patients, the stool potassium excretions were 350 mEq/day, as contrasted with an average normal excretion of 15 mEq. The other low levels of electrolytes in the serum occurred later than hypokalemia but were probably also manifestations of their excessive losses of electrolytes in the stools.

The possibility that these biochemical changes result from the action of a hormone elaborated by the tumor has been explored. Zollinger *et al.* (1968) extracted pancreatic tumor and metastases from 2 cases of WDAH and found the extracted material to produce a secretinlike

pancreatic response when injected into dogs. However, diarrhea resulted only occasionally. Barbezat and Grossman (1971) suggested that both gastrin and glucagon might be involved. On the other hand, immunofluorescence studies of primary tumor and metastases from a case studied by Elias *et al.* (1972) gave strongly positive results only for antibodies to porcine "gastric inhibitory polypeptide" and was negative to antibodies of gastrin, secretin, glucagon, and several other peptides. Gastric inhibitory polypeptide was isolated from extracts of porcine intestine, shown to inhibit gastric acid secretion, and had a 43-amino acid sequence (Brown *et al.*, 1969; Brown and Dryburgh, 1971).

VII. Extrapancreatic Tumors Producing Hypoglycemia

A. Introduction

It would be repetitious to discuss extrapancreatic tumors in connection with each organ in which they are likely to occur. It is convenient to consider them at this point because they possess the common feature of hypoglycemia. By 1972, approximately 200 such cases had been reported (Chandalia and Boshell, 1972). They may be divided into two large classes, those of epithelial origin and those of mesodermal origin. The hepatocellular carcinomas which we have discussed and the adrenocortical carcinomas which we shall consider later in this volume fall into the former group. We shall here concern ourselves with those of mesodermal origin. By 1964, 32 cases of these had been listed out of a total of 57 cases of extrapancreatic tumor (Silverstein *et al.*, 1964).

The presenting symptoms are usually those of hypoglycemia, but the size of the tumor mass may be so impressive as to outweigh the former. Of the 32 patients reviewed by Silverstein *et al.* (1964), 23 had an abdominal tumor and 9 had an intrathoracic tumor. The tumors were very large, ranging in size from 14 by 14 by 14 cm to 36 by 38 by 25 cm and in weight from 770 to 9000 gm. Histologically, 14 were designated fibrosarcomas, 3 mesotheliomas, 3 fibromas, 3 spindle cell sarcomas, 2 questionable low grade islet cell tumors, 1 rhabdomyofibroma, 1 liposarcoma, 1 leiomyosarcoma, and 1 hemangiopericytoma. Three sarcomatous lesions were difficult to classify. The levels of blood glucose were low, 25–50 mg 100 ml, during periods when the usual symptoms of hypoglycemia such as nervousness, irrational behavior, muscular twitchings, convulsions, and coma were manifest. Since the 1964 review by Silverstein *et al.*, many other cases have been reported which, in general,

exhibit the same characteristics (Boshell *et al.*, 1964; Carey *et al.*, 1966; Mars *et al.*, 1969; Miller *et al.*, 1970; Frerichs *et al.*, 1970).

B. Mechanisms of Hypoglycemia

Approximately ten different mechanisms have been submitted within the past few years to account for the hypoglycemia in extrapancreatic tumor, both of mesodermal and epithelial origin (Unger, 1966; Silverstein *et al.*, 1966; Nissan *et al.*, 1968; Mars *et al.*, 1969; Miller *et al.*, 1970; Frerichs *et al.*, 1970). These include: (a) excessive utilization of glucose, that is, consumption and storage of glucose by the tumor; (b) production by the tumor of a substance that stimulates insulin production by the pancreas; (c) production by the tumor of a noninsulinase-inhibiting hypoglycemia-producing substance different from insulin; (d) production of substances that inhibit insulinase or compete with insulin for insulinase; (e) passage of leucine from the tumor into the blood of a leucine-sensitive person, stimulating endogenous insulin production by the beta cells of the pancreatic islets; (f) production by the tumor of insulin or an insulinlike material; (g) production of an insulin-potentiating or insulinoid substance; (h) neurogenic manifestations resulting from pressure secondary to the tumor; (i) release from tumor of a suppression of a counter-regulating hormone; and (j) production or release of tryptophan as a hypoglycemic agent.

In 1966, Unger critically reviewed some of these theories in light of the studies then available. With regard to the mechanisms involving release of a suppressor of counter-regulatory hormones, such as an inadequate secretion of growth hormone, ACTH, or glucagon, he noted that no evidence had been submitted to support this formulation and, indeed, some evidence existed that was contrary. As for the release of a β-cytotropin, such as certain amino or keto acids, that would stimulate the beta cells to produce more insulin, the evidence, except for a single instance (Oleesky *et al.*, 1962), was overwhelming that plasma insulin was low in such cases and could not be elevated by either glucose or tolbutamide. A third possibility, that insulin is released from the tumor, was contravened by the failure to find any immunoassayable insulin in any of several cases in which this was attempted. On the other hand, a fourth formulation, that the tumor may release insulinoid (insulinlike material) or an insulin potentiator, was supported, in Unger's opinion, by the finding that insulinlike activity, calculated to exceed a total of 10 units/tumor, was demonstrated in 6 of 15 tumors studied by the rat epididymal fat pad method and in 2 of 11 tumors assayed by the rat hemidiaphragm technique. The inability to find this activity

in the remaining tumors was attributed to the presence of a dialyzable inhibitor of the insulinlike activity.

A review of recent reports indicates that two of the formulations presented above are preferred, namely, the excessive utilization of glucose by the massive neoplasm (Nissan *et al.*, 1968; Frerichs *et al.*, 1970) or the production by the neoplasms of insulinlike substances or insulin potentiators (Unger, 1966; Silverstein *et al.*, 1966). These conclusions are most frequently derived on the basis of a study of individual cases, and it is possible that different mechanisms of hypoglycemia may be operative in different patients (Chandalia and Boshell, 1972).

One of the other formulations, namely, that L-tryptophan or its metabolites may be released from the tumor and thus cause hypoglycemia, deserves some mention at this point. The observation that the administration of L-tryptophan and some of its metabolites to normal rats causes hypoglycemia (Mirsky *et al.*, 1957) led to the study of this amino acid and its metabolites in the serum and urine of 3 patients with extrapancreatic tumors (Silverstein *et al.*, 1966). In general, it was found that these patients did indeed have increased concentrations of tryptophan and tryptophan metabolites in serum and urine during periods of hypoglycemia.

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9

The Leukemias and Lymphomas

I. Introduction*

The leukemias are a group of diseases characterized by widespread proliferation of the precursors of the leukocytes in bone marrow and other blood forming tissues. Leukemias may be classified as acute or chronic and, under each heading, as lymphocytic, myelocytic, or monocytic, according to the type of cell involved. Conditions primarily affecting the lymph nodes are generally designated as lymphomas or lymphoproliferative disorders and these, in turn, may be classified into lymphosarcoma, reticulum cell sarcoma, Burkitt tumor (African lymphoma), and Hodgkin's disease. Of interest also are the myeloproliferative disorders, a designation which has been applied to conditions involving overgrowth of one or more lines of bone marrow elements.

The deaths from leukemia in the United States in 1967 were 7,599 for males and 5,683 for females (Segi and Kurihara, 1972), and it was estimated that, during 1973, the incidences would be about 11,000 for males and 8,000 for females, and the death rates, 8,600 and 6,700, respectively (Silverberg and Holleb, 1973). Leukemia and lymphomas accounted for about 10% of all cancer deaths in 1968, and were the chief cause of cancer deaths under the age of 15. There is some variation

* The following abbreviations are used most commonly in the present chapter: AL = acute leukemia; ALL = acute lymphoblastic leukemia; CLL = chronic lymphocytic leukemia; CML = chronic myelocytic leukemia; TMP = thymidylic acid (thymidylate). The terms "granulocytic" and "myelocytic" have been used alternatively by different authors.

from country to country; for example, the death rate in Japan is about 70% of that in the United States.

Biochemical study of the leukemias is facilitated since the circulating leukocyte is readily available, very often undergoes biochemical alteration, and may thus help to reveal the nature of the neoplastic process in this group of diseases. For example, the mature polymorphonuclear granulocytes contain a great variety of enzymes. The azurophilic granules, visualized on staining, appear to be lysosomes and to contain many of the enzymes, such as acid phosphatase and lysozyme, characteristic of these intracellular structures (Weissman, 1965; Bainton and Farquhar, 1968a,b). Other biochemical characteristics of granulocytes, lymphocytes, and plasma cells will be discussed subsequently.

II. Total and Isoenzyme Activity of Leukocytic Acid Phosphatase

Table 9-1 shows that, in spite of considerable interindividual variability, leukocytic acid phosphatase was elevated in chronic granulocytic leukemia and tended to be decreased in chronic lymphocytic leukemia and in acute leukemia (Valentine and Beck, 1951; Beck and Valentine, 1951).

Employing 0.005 *M* sodium α-naphthyl acid phosphate as substrate, Li *et al.* (1970a), found the average leukocytic acid phosphatase activity

TABLE 9-1

Leukocytic Acid Phosphatase of Normal Subjects and Patients with Leukemia[a]

Group	No. of patients	No. of determinations	Range	Mean
Normals	23	23	14–37	22
Leukocytosis	30	30	7–66	26
Chronic granulocytic leukemia	14	22	16–61	35[b]
Chronic lymphocytic leukemia	12	16	1–106	18
Acute leukemia	8	14	0–46	9[c]

[a] Data of Valentine and Beck (1951) and of Beck and Valentine (1951). Activities are expressed as mg phosphorus liberated in 1 hour by 10^{10} cells from a reaction mixture at pH 5.0, containing a final concentration of 0.02 *M* sodium β-glycerophosphate as substrate and 1 mg/ml of saponin to lyse the leukocytes.

[b] Significantly higher ($p < 0.01$) than normals.

[c] Significantly lower ($p < 0.01$) than normals. Statistical treatment by author (OB).

in 5 normal persons to be 1219 nmoles of naphthol liberated per hour per 10^7 cells at pH 5.0 and 25°C. Electrophoresis of normal leukocytic preparations at pH 4.0 on acrylamide gel revealed the existence of 4 isoenzymes, proceeding from the cathodic, designated as No. 1, to the most anionic, No. 4. The mean normal values for the distribution of activity among these isoenzymes were No. 1, 37.8%; No. 2, 29.2%; No. 3, 11.5%; and No. 4, 21.5%. These mean values and their ranges are shown in Fig. 9-1. It was subsequently determined that preparations of lymphocytes or of platelets gave only one electrophoretic band of isoenzyme activity, namely, No. 3 (Li *et al.*, 1970b). It followed, therefore, that isoenzymes No. 1, No. 2, and No. 4 resulted from neutrophils and monocytes. Normally, the neutrophils constitute about 70–80% of all leukocytes, and the acid phosphatase activities of neutrophils or monocytes were sufficiently high so that as little as 5% of either cell type in the blood of patients could yield clear evidence of isoenzymes No. 1, No. 2, and No. 4.

Li *et al.* (1970a) found that, in agreement with Valentine and Beck (1951), the total leukocytic acid phosphatase was decreased in acute leukemia (granulocytic or lymphocytic). However, contrary to the findings of Valentine and Beck (1951), no elevation in the mean value for chronic granulocytic leukemia occurred. The mean activity in 7 cases of chronic lymphocytic leukemia was 284 nmoles of naphthol liberated per hour per 10^7 cells, much lower than the normal mean value of 1219 nmoles of naphthol per hour per 10^7 cells. A different substrate, β-glycerophosphate, had been used in the studies of Valentine and Beck (1951).

Figure 9-1 shows the distribution of the 4 isoenzymes in normal individuals and in patients with various types of leukemia. Although there were not enough cases for statistical evaluation, certain definite tendencies may be noted. In acute granulocytic and chronic lymphocytic leukemias, the fraction of isoenzyme No. 3 was increased, whereas that of isoenzyme No. 2 was decreased substantially. The fraction of isoenzyme No. 4 was also decreased in chronic lymphocytic leukemia. In addition to the 4 bands of acid phosphatase isoenzyme activity in normal leukocytes, another isoenzyme may appear. In a case of acute granulocytic leukemia with 100% blast forms, only 1 electrophoretic band of activity was manifest; this migrated between bands 3 and 4 and was therefore designated as 3b (Li *et al.*, 1970b).

A related condition in which acid phosphatase isoenzymes play a role is leukemic reticuloendotheliosis, also known as "reticulum cell leukemia," "lymphoid reticular cell neoplasia," and "hairy cell disease" (Lee *et al.*, 1969; Mitus, 1971). This disease is characterized by massive spleno-

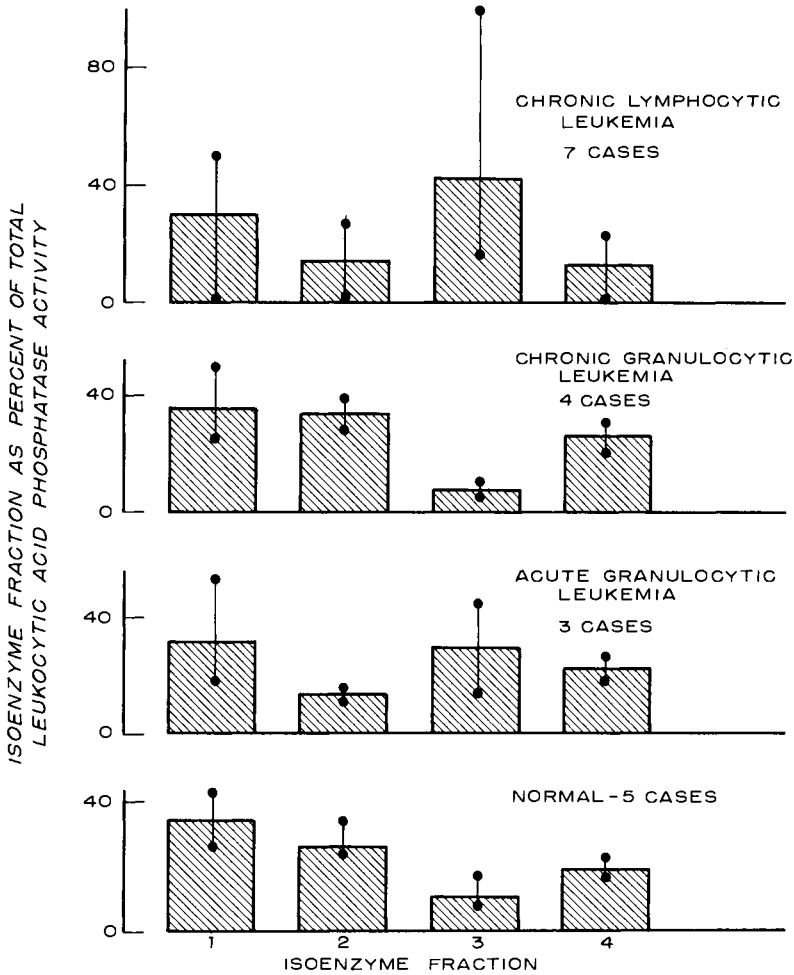


Fig. 9-1 Leukocytic acid phosphatase isoenzyme activities in leukemia, as percent of total leukocytic acid phosphatase activity. Drawn from data of Li *et al.* (1970a). The vertical lines at the midblock represent the range of individual values. The total acid phosphatase activities and the ranges, expressed as nanomoles of naphthol liberated from α-naphthyl phosphate per hour per 10^7 cells were normal 1219 (926–1650); acute granulocytic leukemia, 649 (120–1300); chronic granulocytic leukemia, 1242 (826–1700); and chronic lymphocytic leukemia, 284 (33–836).

megaly resulting from invasion of lymphoid reticular cells. The peripheral blood and bone marrow also contain large numbers of these large cells, 12–20 μm in diameter, with a round or oval, occasionally kidney-shaped, eccentrically placed nucleus and a plentiful, very faintly basophilic and

cloudy cytoplasm (Lee *et al.*, 1969). Ten of a series of 25 patients, reported by Lee *et al.* (1969) had moderate anemia, and all but 3 had varying degrees of thrombocytopenia. At the first visit, 14 of the 25 patients had reduced total white blood cell counts, less than 5000 per mm^3. A normal or high white cell count was characterized by the presence of significant numbers of the specific "reticulum cell."

In 1 patient with leukemia reticuloendothelosis, a preparation of the peripheral blood, in which 98% of the leukocytes were reticulum cells, showed only 1 isoenzyme of leukocytic acid phosphatase. This was designated as No. 5 since it migrated anionically to isoenzyme 4. In patients with a differential white cell count with lesser numbers of reticulum cells and greater numbers of lymphocytes and neutrophils, isoenzymes No. 1, No. 2, No. 3, and No. 4 were also present. For example, in a case with 54% reticulum cells, 20% neutrophils, 1% monocytes, and 25% lymphocytes, the relative isoenzyme activities were No. 0, 0%; No. 1, 31%; No. 2, 19%; No. 3, 10%; No. 3b, 8%; No. 4, 11%; and No. 5, 22% (Li *et al.*, 1970b).

L(+)-Tartrate (0.05 *M*) inhibited isoenzymes Nos. 0–4, including No. 3b, but had no appreciable effect on the reticulum cell isoenzyme, No. 5 (Yam *et al.*, 1971; Mitus, 1971). In cytochemical studies of blood smears from patients with leukemic reticuloendotheliosis, the nature of the acid phosphatase activity in monocytes, eosinophils, neutrophils, and other cells that could be definitely identified as lymphocytes did not differ appreciably from that of the corresponding cells of normal subjects, and the enzyme was completely inhibited by L(+)-tartrate. The neoplastic reticulum cells showed varying degrees of acid phosphatase activity, with most cells staining strongly positive and not inhibited by L(+)-tartrate.

These findings have been recently extended as a potential diagnostic measure in differentiating leukemic reticuloendotheliosis from lymphosarcoma and reticulum cell sarcoma (Katayama *et al.*, 1972). Surgical specimens including biopsies of spleen or lymph nodes were used. Some exceptions were found in specimens involved by Hodgkin's disease and in an adrenal of 1 patient with aldosteronism.

III. Leukocytic Alkaline Phosphatase

In normal individuals, the levels of leukocytic alkaline phosphatase activity, expressed as milligrams of phosphorus liberated in 1 hour by 10^{10} cells from a reaction mixture containing 0.02 *M* β-glycerophosphate barbiturate buffer at pH 9.9, ranged from about 13.4 to 58.0 mg and averaged 25.8 mg in a group of 23 normal persons. A series of 30 patients with leukocytosis and usually clinical evidence of infection had elevated alkaline phosphatase activities, ranging from 35 to 277

mg and averaging 119 mg of phosphorus liberated per hour by 10^{10} cells (Beck and Valentine, 1951).

The alkaline phosphatase activity was substantially decreased in chronic granulocytic leukemia. In a series of 22 determinations on 14 patients, the activities ranged from 0.0 to 14.4 mg and averaged 4.0 mg of phosphorus liberated per hour by 10^{10} cells. In 16 determinations on 12 patients with chronic lymphocytic leukemia, the activities ranged from 2.5 to 68.2 mg with a mean value of 20.8 mg. Patients with acute leukemia had very low levels of activities. In 14 determinations on a series of 8 patients, the values ranged from 0.0 to 6.2 mg with a mean value of 1.6 mg.

The activity of leukocytic alkaline phosphatase has been shown to alter with the clinical status of the patient with chronic granulocytic leukemia. Several groups of investigators (Valentine *et al.*, 1957; Block *et al.*, 1963) noted that the low leukocytic alkaline phosphatase activity, characteristic of chronic granulocytic leukemia, may return to normal during the remission phase, particularly in association with treatment. More recently, Rosner *et al.* (1972) reported a series of 38 patients with chronic granulocytic leukemia who were followed during various stages of their disease. Prior to treatment, all patients tested had very decreased or zero leukocytic alkaline phosphatase activity. Remission was achieved in 16 patients, and of the 29 determinations performed on these patients, 5 normal and 1 increased enzyme values were obtained. Three patients in the blastic stage of the disease and 3 patients who became aplastic in the course of their chemotherapeutic treatment had substantial increases in their leukocytic acid phosphatase activity. These results show that normal enzyme activity is achieved in only about one-fourth of remissions. The basis for the increased production of alkaline phosphatase activity in the leukocytes of these patients is unknown.

An alkaline phosphatase (phosphatase N), distinguishable from the usual alkaline phosphatase by its inability to hydrolyze S-substituted monoesters of thiophosphoric acid, has been recently reported as constituting fractions up to 100% of the total serum alkaline phosphatase in patients with acute or chronic lymphatic leukemia. It is absent from normal serum (Neumann *et al.*, 1974).

IV. Lysozyme

A. Introduction

Lysozyme (EC 3.2.1.17) is also known as muramidase, and its function is to hydrolyze the β-1,4 links between *N*-acetylmuramic acid and *N*-acetylglucosamine residues in a mucopolysaccharide or mucopolypeptide or in chitin. In the present discussion, we shall use the recommended trivial name "lysozyme" (International Union of Biochemistry, 1965).

N-Acetylmuramic acid consists of the 6-carbon amino sugar acetylglucosamine in ether linkage with the 3-carbon acid, lactic acid.

In 1922, Fleming discovered lysozyme as a bacteriolytic element present in various tissues and secretions. Leukocytes were a particularly rich source. Since then, the enzyme has been reported to be present in a wide variety of plants, bacteria, viruses, invertebrates, and vertebrates, including man (Thompson, 1940; Perri *et al.*, 1963).

A frequently used assay has been that based on the change in absorbance produced per minute in standardized acetone dried cell suspensions of *Micrococcus lysodeikticus* by lysozyme-containing tissue extract. The activity of the unknown was determined from the change in absorbance per minute against a standard curve obtained with egg white lysozyme; the activities were expressed in milligrams equivalent to egg white lysozyme per gm of wet tissue (Perri *et al.*, 1963).

B. Serum and Urinary Lysozyme in Cancer

1. Introduction

About 1966, a substantial interest arose in the lysozyme activity of serum and urine in patients with various types of leukemia. During the next several years, this aspect was pursued very actively with a view to exploring the diagnostic potentiality as well as to understanding the mechanisms involved in this phenomenon. We shall presently consider these studies in some detail. In 1954, Fogelson and Lobstein had observed that the blood of patients with localized or generalized carcinomatosis had a higher level of lysozyme activity than normal adults. Barbieri and Genesi (1954) also reported on the activity of lysozyme of plasma and leukemic leukocytes. It was subsequently noted that certain tissues, particularly the kidney of tumor-bearing mice and rats, had increased levels of lysozyme activity (Cappucino *et al.*, 1962). Removal of the tumor from rats bearing Jensen sarcoma was followed by a rapid return to normal levels of renal lysozyme activity (Perri *et al.*, 1963).

2. Serum Lysozyme in Leukemia

In 1966, Osserman and Lawlor reported the electrophoretic isolation of an exceptionally basic, cationic protein from the urine of 10 consecutive cases of monocytic and monomyelocytic leukemia. The protein was found to possess the enzyme properties of lysozyme, and was apparently identical with the lysozyme of normal tears, saliva, serum, and leukocytes but different from the lysozyme of hen's egg white.

Employing an agar plate method for quantitating lysozyme activity in small samples, Osserman and Lawlor (1966) found that the concentration of lysozyme in the serum of normal individuals was approximately 7 μg/ml, and the activity of the urine was barely detectable. In patients with monocytic leukemia, the serum concentrations ranged from 40 to 150 μg/ml, and the concentrations in the urine from 25 to 420 μg/ml. Elevated serum and urinary lysozyme levels were also found in other conditions, but these were not as high as those encountered in monocytic leukemias. For example, patients with chronic renal disease, particularly those with the nephrotic syndrome, showed a urinary excretion of 3–5 μg/ml. Moderately elevated levels of 10–30 μg/ml in serum and urine were obtained in chronic infections and sarcoidosis. Normal levels were observed in myelocytic leukemias without associated monocytosis, and reduced levels were found in cases of lymphatic leukemia. It may be noted that the serum rather than the leukocyte reflected lysozyme activity in the various leukemias.

More comprehensive studies by Perillie and his associates (1968) and by Wiernik and Serpick (1969) are shown in Table 9-2. Although the

TABLE 9-2

Serum and Urinary Lysozyme Activity in Leukemias

Group Control or type of leukemia	Investigator[a]	No. of patients	Mean value ± SE in μg/ml[b]	
			Serum	Urine
Control	P	30	8.2 ± 0.3	—
	W	15	16.4 ± 1.0	2.7 ± 1.5
Acute lymphocytic	P	27	4.2 ± 0.4	—
	W	21	7.9 ± 1.2	2.3 ± 1.1
Acute myelocytic	P	19	32.1 ± 7.5	—
	W	24	20.5 ± 1.6	4.8 ± 3.2
Acute myelomonocytic	P	10	61.3 ± 13	—
	W	9	64.0 ± 5.3	27.5 ± 10.2
Acute monocytic	P	13	134 ± 23	—
	W	10	134 ± 4.6	128 ± 23
Chronic lymphocytic	P	24	10.3 ± 2.2	—
	W	7	9.1 ± 1.1	2.0 ± 1.2
Chronic myelocytic	P	23	38.6 ± 6	—
	W	6	51.6 ± 12.9	5.9 ± 3.9

[a] P and W designate values of Perillie *et al.* (1968) and of Wiernik and Serpick (1969), respectively.

[b] Expressed in terms of microgram-equivalents of egg white lysozyme.

control values obtained by the 2 groups of investigators differed greatly, similar results for the leukemias were obtained by both. Thus, in both investigations, the mean values for serum lysozyme in the groups of acute and chronic myelocytic, acute myelomonocytic, and acute monocytic leukemias were significantly higher than the mean value for the controls. The mean value for acute lymphocytic leukemia was significantly lower than that of the control group. The urinary excretions of lysozyme in the acute monocytic and myelomonocytic leukemias were substantially and significantly higher than the excretions in the controls or other types of leukemia.

The mechanism of alteration of lysozyme activity in serum is naturally of interest. Of the various types of cells present in perpiheral blood and bone marrow, only monocytes and mature neutrophils and their precursors extending back to the progranulocyte contain significant amounts of lysozyme (Briggs *et al.*, 1966). Moreover, the presence of these cells in the marrow appeared to play a role in the levels of lysozyme in the plasma or serum. In a series of 15 hematologically normal persons, the following mean and standard deviation values were obtained: blood plasma lysozyme activity, 104 ± 53 μg/ml; bone marrow plasma lysozyme, 237 ± 107 μg/ml; and ratio of marrow plasma lysozyme activity to blood plasma lysozome activity, 2.92 ± 132 (Hansen *et al.*, 1969). This ratio, greater than unity, suggested intramedullary destruction of myeloid cells under normal conditions. Patients with granulocytopenia with an increased number of granulocytes in the bone marrow had elevated serum lysozyme activity, whereas those with hypocellular bone marrow had reduced levels of this serum enzyme (Fink and Finch, 1966).

But there also appears to be a relationship between the lysozyme activity of serum and that of the circulating leukocyte. Noble and Fudenberg (1967) determined the serum and leukocyte lysozyme activities in 20 normal control subjects and in 9 patients with acute myeloblastic leukemia. In general, a parallelism existed between the maturity of the granulocytes and their lysozyme content. As may be readily appreciated, a parallelism also exists between the conditions which show elevated leukocyte lysozyme activity and those which show elevated serum lysozyme activity (Table 9-2; Fig. 9-2). A similar relationship was elicited by determining the ratio of serum lysozyme activity to the white cell count (Wiernik and Serpick, 1969). The ratio, serum lysozyme (μg/ml):white cell count (10^3 cells per mm^3) and its SE value was 2.1 ± 0.8 for 15 controls. The ratios for leukemias were acute lymphocytic, 21 cases, 2.0 ± 0.5; chronic lymphocytic, 7 cases, 0.8 ± 0.7; chronic myelocytic, 6 cases, 1.1 ± 0.6; acute myelocytic, 24 cases, 6.3 ± 1.4; acute monomyelocytic, 9 cases, 24.8 ± 6.1; and acute mono-

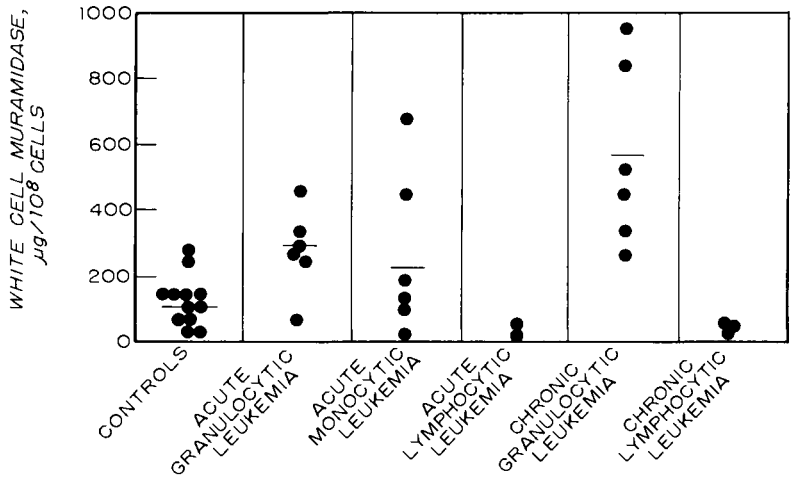


Fig. 9-2 Leukocyte lysozyme (muramidase) activity in controls and patients with various types of leukemia. From Perillie *et al.* (1968). Reproduced by permission of the American Medical Association.

cytic, 10 cases, 17.7 ± 5.5. Not only were the mean values higher in the last 3 groups than in the controls or in the lymphocytic leukemias but so were the proportions of cases that had ratios higher than 4.0. For example, 8 of 10 cases with acute monocytic leukemia and all of 9 cases with acute myelomonocytic leukemia had ratios higher than this value, whereas none of 21 patients with acute lymphocytic or of 9 patients with chronic lymphocytic leukemia achieved ratios greater than 4.0. When serum lysozyme activity was plotted against the logarithm of the granulocyte count in 98 patients with various hematological disorders, a positive correlation was obtained for a direct relationship between these two parameters (Jollés, *et al.*, 1965).

3. Urinary Lysozyme in Leukemia

The average pretreatment excretion of this enzyme in various types of leukemia is shown in Table 9-2. In patients with serum lysozyme activities greater than 50 μg/ml, the ratios of urinary to serum lysozyme activities were 1.23 ± 0.20 (SE) in those with acute monocytic leukemia and 0.583 ± 0.21 (SE) in patients with acute myelomonocytic leukemia. These were much higher than the ratio, 0.054 ± 0.110, in chronic myelocytic leukemia (Wiernik and Serpick, 1969). This finding led to a consideration of the possibility that a renal tubular defect might be present

in acute monocytic leukemia. Osserman and Lawlor (1966) had noted that several of their patients with monocytic or myelomonocytic leukemia had exhibited significant hypokalemia. In a subsequent study of 8 patients with monocytic and myelomonocytic leukemia, hypokalemia was also observed, about 2.0–3.0 mEq potassium per liter, on several occasions during the course of each patient. Serum and urinary lysozyme activities were, of course, high. Balance studies performed in 3 of the patients with hypokalemia demonstrated marked lysozymuria and unusually high rates of renal potassium loss. Limitation in titratable acid excretion was present in one patient and glycosuria and hyperuricosuria in another (Muggia *et al.*, 1969).

The urinary excretion of lysozyme in the various leukemias and in other conditions we shall presently consider raises the question of whether the kidney itself is the source of this enzyme or whether there is a renal tubular defect which allows the excretion of low molecular weight proteins, including lysozyme, that are formed, perhaps in excessive amounts, in other tissues of the body.

4. Serum and Urinary Lysozyme in Other Types of Cancer and in Other Diseases

The increase in serum lysozyme activity is not necessarily specific for the types of leukemias we have noted. Fogelson and Lobstein (1954) found increased whole blood lysozyme activities in patients with various types of neoplasms. Of 77 normal individuals, only 13% had levels above 8 μg/ml of whole blood in contrast to 54% of 35 patients with cancer.

Nor is the high excretion of urinary lysozyme specific for the acute myelocytic and myelomonocytic leukemias or, indeed, for other neoplasms. In 1961, Butler and Flynn studied the occurrence in urine of a post γ-globulin protein in a variety of conditions. This protein was later identified by Osserman and Lawlor (1966) as consisting largely of lysozyme. The incidence of occurrence of this post-gamma urinary protein in various other conditions may be illustrated as follows: Fanconi syndrome (various types), 19 of 21 cases; renal tubular acidosis, 3 of 3 cases; multiple myeloma, 9 of 33 cases; and organic-aciduria syndrome, 2 of 2 cases. In chronic nephritis and Hodgkin's disease, the occurrence of this protein was less frequent (Butler and Flynn, 1961). More generally, increased serum and urinary levels of lysozyme have been observed in patients with these conditions and in cadmium poisoning (Muggia *et al.*, 1969; Piscator; 1966). The mode of renal excretion of lysozyme is not completely known. It may be assumed that, because of its small molecular weight, lysozyme would normally be filtered at the glomerulus and reab-

sorbed by the tubules. However, in the presence of tubular damage, lysozyme might appear in the urine along with other low molecular weight proteins (Piscator, 1966). Hypokalemia, glycosuria, hyperuricosuria, and aminoaciduria are present in some patients and may also be evidence of tubular dysfunction.

The increase in serum and urinary lysozyme in patients with generalized carcinomatosis, multiple myeloma, or Hodgkin's disease may reflect overproduction of the enzyme as the result of proliferation of tissue elements such as macrophages. The sequence of events in cadmium poisoning in man and in animals is marked by proliferation of alveolar macrophages, a cell-type rich in lysozyme, accumulation of lysozyme in the proximal tubular cells, proteinuria, lysozymuria, and several renal tubular defects (Muggia *et al.*, 1969; Kazantzis *et al.*, 1963). In experimental animals, lysozyme concentration in kidney tissue has been shown to rise to a much greater extent than in all other tissues following the administration of egg white lysozyme, immunization, transplantation of malignant tumors, or exposure to cadmium (Muggia *et al.*, 1969). Although, as has been indicated, this may reflect tubular damage and the accumulation of lysozyme in the tubular cells, it is also possible that the accumulation of lysozyme in the kidney may reflect overproduction. In a study of human autopsy material from 22 patients who died of a variety of metastatic cancers, Perri and Faulk (1963) found that kidneys contained elevated levels of lysozyme. One patient with gastric carcinoma and another in the blastic stage of chronic granulocytic leukemia had 10 times the normal amount of lysozyme. Elevations 2–4 times the normal level were observed in patients with acute leukemia, Hodgkin's disease, carcinoma of the tongue, carcinoma of the palate, and an embryonal testicular carcinoma.

V. Metabolism of Purines and Pyrimidines in Cancer

A. Introduction

The goal of determining whether cancer in man affects the biosynthesis of purines or pyrimidines to nucleic acids or the degradation of the latter to uric acid and various pyrimidines and purines has been approached at several points. These include the incorporation of precursors into purines and nucleic acids of isolated cancer cells, preferably human; the study of the excretion of purines and uric acid in patients with various types of cancer; and the effects of various chemotherapeutic agents on the pattern of pyrimidine, purine, and uric acid excretion.

B. Biosynthesis of Purines in Normal and Leukemic Leukocytes

One of the most available systems for the study of metabolism in man is the leukocytes. The incorporation of suitably labeled precursors may be used to measure the synthesis of leukocyte RNA and DNA. In 1959, Wells and Winzler reported the extent of incorporation of formate into the acid soluble fraction and the RNA and DNA components of leukocytes (Table 9-3). It may be seen that these were greater in the acid soluble and RNA components of the cells from patients with chronic lymphocytic leukemia (CLL) than in the cells from normal individuals or those with chronic myelocytic leukemia (CML). A substantial difference also existed between CLL and CML cells with respect to incorporation into DNA. Amethopterin present in the incubation mixture with CML cells inhibited the incorporation of formate into DNA thymine by about 98% and into RNA guanine and adenine by about 50%. No inhibition or slight stimulation occurred on incubation of amethopterin with cells of normal individuals or of patients with CLL (Wells and Winzler, 1959).

The pathway of *de novo* purine synthesis in general involves glycine, 5-phosphoribosyl pyrophosphate, formate, and the amide-nitrogen of glutamine. Human leukocytes cannot apparently accomplish the early steps of purine synthesis since [^{14}C]glycine is not incorporated into soluble purine nucleotides or nucleic acid purines (Laszlo *et al.*, 1970). Scott (1962) reported that leukocytic nucleic acid purine incorporation of [^{14}C]formate reflects the utilization of formate carbon for the closure of the purine ring from the precursor, 5-amino-4-imidazole carboxamide ribotide, and the subsequent incorporation of the labeled purine into RNA adenine and guanine. Such a synthesis could not be demonstrated for DNA purines. In the absence of added carboxamide ribotide, *de novo* synthesis of RNA purines was present only in acute leukemic leukocytes. Addition of the ribotide increased the synthesis in this system severalfold, as compared with moderate increases in CLL and CML leukocytes.

Nucleic acid purines may be derived either from preformed purines or from purines synthesized by the cells. Scott (1962) undertook to evaluate the relative proportions of these two pathways in the biosynthesis of the nucleic acids of various types of leukemic leukocytes. [^{14}C]Adenine was incorporated into both ribonucleic acid adenine and guanine by each of the leukocyte populations examined. These specific activities were found to be at least 10 times those of the DNA purines of both chronic and acute leukemic leukocytes. Apparent DNA purine

TABLE 9-3

The *in Vivo* Incorporation of [^{14}C]Formate in the Purines and Pyrimidines of Normal and Leukemic Human Leukocytes[a,b]

Cell type	Acid soluble		RNA		DNA		
	Adenine	Guanine	Adenine	Guanine	Adenine	Guanine	Thymine
Normal	161	51	41	12	<0.5	<0.5	2.0
Chronic lymphocytic leukemia	372	324	66	35	<0.5	<0.5	1.0
Chronic myelocytic leukemia	188	94	36	10	1.24	1.26	172

[a] Data of Wells and Winzler (1959). Reproduced by permission of Cancer Research, Inc.

[b] Incorporation was expressed as RSA $= \dfrac{\text{counts/min/}\mu\text{mole compound}}{\text{counts/min/}\mu\text{mole added [}^{14}\text{C]formate}} \times 100.$

incorporation of this magnitude could be accounted for by contamination of the DNA fraction with highly labeled RNA purine.

Labeled formate incorporation into the nucleic acid purines of acute leukemic leukocytes took place preponderantly in the RNA fraction. DNA purine incorporation of [^{14}C]formate was not altered by the presence of either the preformed purines or the purine precursors. These results would indicate that the leukocyte nucleic acid incorporation of [^{14}C]formate reflects the utilization of formate carbon for closure of the purine ring. The extent of incorporation of formate into DNA thymine depended greatly on the type of leukocyte population. It was insignificant in CLL lymphocytes, was related in CML leukocytes to the percentage of cells capable of mitotic division (myelocytes, promyelocytes, and myeloblasts), but unrelated to the percentage of morphologically immature cells in the acute leukemias.

C. Biosynthesis of Pyrimidines in Leukemic Cells

1. Biosynthesis of Orotic Acid

The metabolic sequences involved in the biosynthesis of orotic acid may be summarized in Fig. 9-3. The enzyme activities involved in this

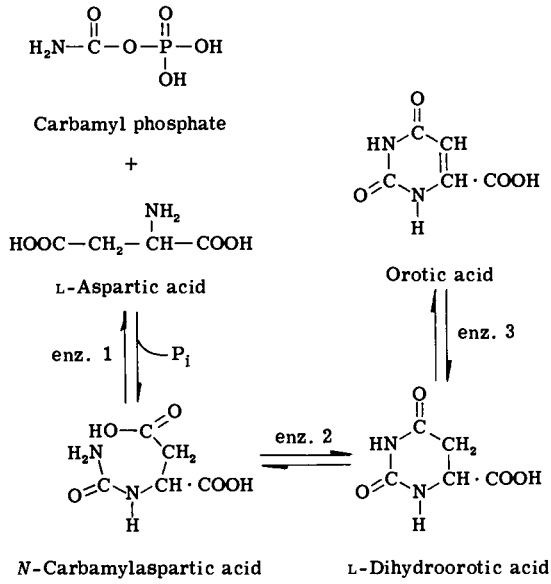
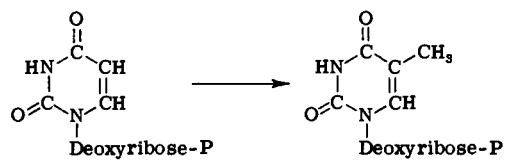


Fig. 9-3 Biosynthesis of orotic acid. Designation of enzymes: enz. 1, aspartate carbamyltransferase; enz. 2, dihydroorotase; and enz. 3, dihydroorotic dehydrogenase.

sequence have been studied in sonicates of leukocytes from various leukemias (Smith *et al.*, 1960). The aspartate carbamyltransferase and dihydroorotase activities were increased substantially in the sonicates of the leukocytes from all 5 patients with myelocytic leukemia, and dihydroorotic dehydrogenase was also increased in the sonicates of these patients. The increased activities tended to parallel cytologic evidence of immaturity. For example, the highest value of aspartate carbamyltransferase in the group of myelocytic leukemias was about 2200 units, expressed as nanomoles of carbamylaspartic acid (CAA) synthesized per 10^8 leukocytes; the average value for normal polymorphonuclear leukocytes was about 500 units. Leukocytes from patients with infection or with myeloproliferative disorders showed similar but lesser increases in ezyme activity. The enzyme, 5-carboxymethylhydantoinase, previously found in some bacteria, was absent from normal and abnormal red and white blood cells.

2. Thymidylate Synthetase

Thymidylate (TMP), an essential precursor of DNA, is formed by the interaction of deoxyuridylate and N^5,N^{10}-methylene tetrahydrofolate, catalyzed by the enzyme, thymidylate synthetase.



Deoxyuridylate (dUMP) — Thymidine 5′-phosphate (TMP) or thymidylic acid

Dihydrofolate is also formed and is recycled to tetrahydrofolate by the action of dihydrofolic reductase, and the addition of a methyl group, through the action of serine hydroxymethylase.

Silber *et al.* (1963b) undertook to purify and characterize the properties of thymidylate synthetase from human leukocytes and to determine the level of activity in normal and leukemic leukocytes. The enzymic reaction was assayed by means of the tetrahydrofolate- and deoxyuridylate-dependent incorporation of $H^{14}CHO$ into thymidylate. Thymidylate synthetase was not present in the leukocytes of 12 normal subjects. In 12 patients with chronic myelocytic (granulocytic) leukemia of whom

50% had had no therapy, the activity ranged from 0.06 to 0.34 nmoles and had a mean value of 0.19 ± 0.02 nmoles TMP formed per hour per mg protein. Very low levels, ranging from 0 to 0.15 and averaging 0.06 nmoles/hour/mg protein characterized 6 patients with acute lymphoblastic or myeloblastic leukemia. No activity was present in any of 8 patients with CLL.

3. Deoxythymidine Synthetase

The deoxyribonucleoside of thymine has been designated as "deoxythymidine" (Gallo and Perry, 1969), but White *et al.* (1968) have pointed out that the more proper name is "thymidine," since the corresponding pyrimidine, thymine, is primarily present in DNA. However, to be consistent with the literature to be cited, we shall employ the term "deoxythymidine." It has been known that pyrimidine products of DNA degradation such as deoxynucleotides, deoxynucleosides, and perhaps even the free pyrimidine bases may be reutilized for the synthesis of new nucleic acid molecules. The enzyme, *trans-N*-deoxyribosylase, which catalyzes deoxynucleoside synthesis, was well characterized in bacterial systems (Beck and Levin, 1963), but its presence in human tissues was more debatable (Gallo and Perry, 1969). However, evidence has been submitted that human spleen and normal human leukocytes contain an enzyme, deoxynucleoside phosphorylase, which catalyzes synthesis of deoxythymidine or deoxyuridine by two distinct mechanisms. The action could be, first, that of a *coupled deoxynucleoside phosphorylase:*

$$\text{(a)}\quad \text{XdR} + \text{P}_\text{i} \rightleftarrows \text{dR-1-P} + \text{X}$$
$$\text{(b)}\quad \text{T} + \text{dR-1-P} \rightleftarrows \text{TdR} + \text{P}_\text{i}$$

where X is any purine or pyrimidine deoxynucleoside and TdR is deoxythymidine. But it may also act as a *pyrimidine deoxyribosyltransferase:*

$$\text{T} + \text{PydR} \rightleftarrows \text{TdR} + \text{Py}$$

where T is thymine and Py is any other pyrimidine.

Gallo and Perry (1969) assayed the activities of these enzymes in normal and leukemic leukocytes. The mean values for both deoxythmidine phosphorylase and pyrimidine deoxyribosyltransferase activities in normal leukocytes, chiefly granulocytes, were about twofold the activities in CML and approximately fivefold those in normal lymphocytes or lymphocytes from patients with CLL. In contrast, leukocytes from CML showed elevated purine nucleoside phosphorylase activity, namely, 150–200% that of normal leukocytes. In CLL or in normal lymphocytes, the

activity of this enzyme was about 20% of normal. The deoxyribosyltransferase activities in CML, CLL, and normal lymphocytes were approximately 35, 20, and 20%, respectively, of the normal activities in granulocytes.

In both the normal and leukemic cells, the transferase activity could not be differentiated from the phosphorylase activity, indicating that the two activities were associated with the same protein. The leukemic enzyme could not be distinguished from the normal enzyme by pH optima, thermal stability, kinetic properties, K_m values, susceptibility to substrate inhibition by thymine, or activation by phosphate or arsenate. These findings would indicate that the decreases in activity in chronic myelogenous leukemia result from an actual decrease in the amount of enzyme in the cells or from a mixed cell population, one with a normal quantity of enzyme and the other with little or no active enzyme.

4. Thymidine Kinase

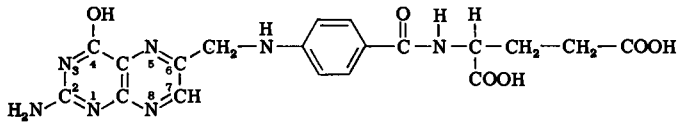
Thymidine kinase mediates the phosphorylation of the deoxyriboside, thymidine (TdR), to thymidylic acid (TMP). Bianchi (1962) observed that a 30-minute incubation of supernatant fractions of human homogenized normal and leukemic leukocytes, spleen and lymph nodes with [^{14}C]thymidine (T) and an ATP regenerating system resulted in a conversion of about 6–30% of the thymidine to thymidine triphosphate (TTP). When TMP was incubated with the supernatant, all of the substrate was converted to the triphosphate within 30 minutes. Obviously then, the production of TMP was a rate-limiting reaction, and all of the tissues tested contained limited amounts of thymidine kinase. No distinction could be drawn from these studies between normal leukocytes and leukemic cells.

The biosynthesis of pyrimidines in human leukocytes involves several points of regulatory control. Bresnick and Karjala (1964) found that the thymidine kinase activity in sonicated soluble preparations obtained from normal and leukemic leukocytes [both chronic myelogenous leukemia (CML) and chronic lymphocytic leukemia (CLL)] was inhibited by TMP, TDP, or TTP and by deoxycytidine triphosphate (dCTP). For example, 1.28 ± 10^{-4} *M* TDP or TTP inhibited the thymidine kinase of leukocytes from CLL or from normal persons by about 60%. It should be realized that this type of inhibition may affect measurements of thymidine kinase activity. Bresnick and Karjala (1964) also were unable to elicit differences in thymidine kinase activity or susceptibility to end product inhibition between normal and leukemic leukocytes.

5. Relationship between Pyrimidine-Purine Biosynthesis and Folic Acid Metabolism in Leukemia

We have already noted that the tetrahydrofolate-dependent enzymes mediate reactions in which 1-carbon units are incorporated in the course of purine and pyrimidine biosynthesis and, hence, play a role in nucleic acid biosynthesis. Indeed, it has long been considered that tetrahydrofolate-dependent enzyme systems provided potential targets for certain chemotherapeutic agents (Bertino *et al.*, 1963).

In 1957, Ellison and Hutchison observed that the activity of "citrovorum factor" of leukocytes was greater than normal in many cases of acute leukemia. Patients being treated with methotrexate (amethopterin; 4-amino-N^{10}-methylpteroylglutamic acid) showed values within the normal range. "Citrovorum factor" is a designation formerly used for folinic acid, one of the compounds related to folic acid which is involved in various transmethylation reactions. These can be briefly described here. Folic acid itself has the formula:



Pteroylglutamic acid (folic acid, PGH)

It may be seen that three residues are attached successively to each other: 2-amino-4-hydroxy-6-methylpterin, *p*-aminobenzoic acid, and glutamic acid. In dihydrofolic acid, the bond between position 7 and 8 is hydrogenated, and in tetrahydrofolic acid, the bond between 5 and 6 is also hydrogenated. Folinic acid (citrovorum factor) has a formyl group attached to position 5 in tetrahydrofolic acid and hence is known variously as 5-formyl tetrahydrofolate or 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid. As was previously noted (Section V,C,2), N^5-N^{10}-methylene tetrahydrofolate is involved as a coenzyme in the methylation of deoxyuridine 5′-phosphate to yield thymidine 5′-phosphate (thymidylic acid) under the influence of thymidilate synthetase. Substitution of an amino group for the OH group at position 4 of the pteridine ring of folic acid leads to the production of antifolic compounds which inhibit bacterial and tumor growth. Aminopterin has only one substitution, the amino group for the hydroxyl group at position 4. Methotrexate (aminopterin) has, in addition, a methyl group substitution at position 10.

Bertino *et al.* (1963) have studied the enzymes involving tetrahydrofolate in normal and leukemic leukocytes (Table 9-4). Formate-activating enzyme (formyltetrahydrofolate synthetase) is the enzyme that mediates the following reaction:

$$\text{Formate} + \text{ATP} + \text{tetrahydrofolate} \rightleftarrows N^{10}\text{-formyltetrahydrofolate} + \text{ADP} + P_i$$

N^5,N^{10}-methylenetetrahydrofolic dehydrogenase is TPN-dependent and catalyzes the reversible oxidation of N^5,N^{10}-methylenetetrahydrofolic acid to N^5,N^{10}-methylidyneltetrahydrofolic acid. Dihydrofolic reductase catalyzes the reversible reduction of 7,8-dihydrofolic acid to 5,6,7,8-tetrahydrofolic acid. As may be seen from Table 9–4, the leukocytes from patients with acute leukemia (AL) show significantly increased activities of all 3 enzymes, as compared with normal leukocytes. The leukocytes from patients with CML have significantly increased activities only of the formate-activating enzyme and the dihydrofolic dehydrogenase. In acute lymphoblastic leukemia (ALL) leukocytes, the activities of the formate-activating enzyme and the tetrahydrofolic dehydrogenase are significantly increased.

6. Thymidine Phosphorylase

Thymidine phosphorylase catalyzes the reversible interaction of thymidine and phosphate to form deoxyribose 1-phosphate and the free pyrimidine, thymine (Friedkin and Roberts, 1954). Using as a unit of activity that quantity of enzyme which, under defined conditions, catalyzed the formation of 1 μmole of thymine per hour, Marsh and Perry (1964) obtained the following mean values, expressed as units per 10^8 leukocytes: CML in relapse, 2.52 ± 0.15 SE; CLL, 1.02 ± 0.18 SE; and AL, 0.85 ± 0.23 SE. These values were all significantly lower than the value, 4.32 ± 0.25 SE, of normal leukocytes. The CML value was higher than those from CLL and AL. When the data were expressed as enzyme activity per milligram of homogenate protein, the following mean values were obtained: normal, 1.17 ± 0.10; CML (relapse) 0.83 ± 0.06; ALL, 0.97 ± 0.05; and AL, 0.33 ± 0.12. The values for CML and AL were still significantly lower ($p < 0.01$) than the normal value.

D. Pyridine Nucleotides in Cancer

The pyridine nucleotides act as coenzymes of electron transport in a wide variety of biological oxidation–reduction reactions. The structure

TABLE 9-4

Levels of Formate-Activating Enzyme, N^5,N^{10}-Methylenetetrahydrofolic Dehydrogenase, and Dihydrofolic Dehydrogenase in Normal and Leukemic Leukocytes[a]

Cell type	Formate-activating enzyme		N^5,N^{10}-Methylenetetrahydrofolic dehydrogenase		Dihydrofolic dehydrogenase	
	No. of subjects	μmole/hour/mg protein	No. of subjects	μmole/ml/hour mg protein	No. of subjects	μmole/ml/hour mg protein
Normal	14	0.38 ± 0.03	12	0.15 ± 0.01	15	About 0.001
CLL[d]	8	0.41 ± 0.03	6	0.15 ± 0.03	8	0.002 ± 0.001
CML	12	0.62 ± 0.08[b]	11	0.13 ± 0.02	10	0.042 ± 0.008[c]
AL	17	0.96 ± 0.08[c]	16	0.28 ± 0.04[c]	22	0.34 ± 0.004[c]
ALL (child)	10	0.76 ± 0.06[c]	6	0.27 ± 0.04[c]	—	—

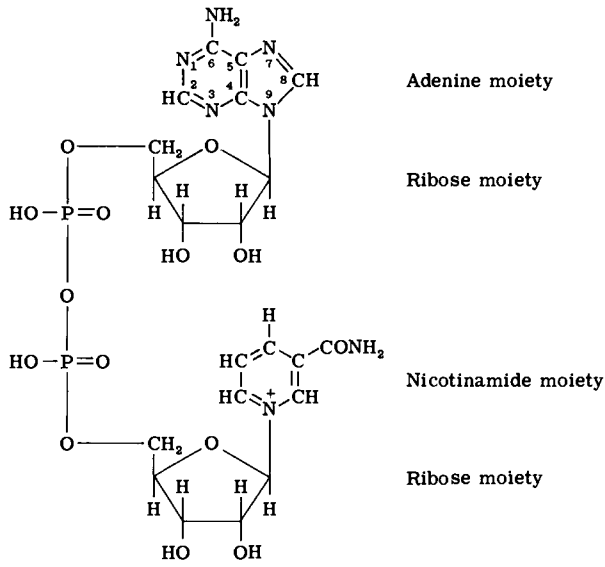
[a] Based on data of Bertino *et al.* (1963). Values are mean ± SE. Significance of difference between means for normal leukocytes and mean for various types of leukemic cells have been calculated and are shown as:

[b] <0.02.

[c] <0.01.

[d] CLL, chronic lymphocytic leukemia; CML, chronic myelocytic leukemia; AL, acute leukemia; and ALL, acute lymphoblastic leukemia.

of diphosphopyridine nucleotide, DPN^+, is more accurately described by the name, nicotinamide adenine dinucleotide (NAD), and is as follows:



Triphosphopyridine nucleotide, TPN^+, has a phosphate esterified with the OH on C′-2 of the ribose attached to the adenine moiety. This compound has also been designated as nicotinamide adenine dinucleotide phosphate (NADP).

In 1955, Weinhouse reviewed the literature on DPN^+ and DPNH levels in normal and tumor tissues of laboratory animals and presented his own data. Although there were considerable variations, neoplastic tissues tended to show lower levels than normal tissues in the mouse and rat. A more systematic study of pyridine nucleotide levels in normal and leukemic human leukocytes was presented by Silber *et al.* (1962). The mean values for the concentrations of DPN^+ in the leukocytes of CLL, CML, and AL were significantly higher than the mean value for normal cells (Table 9-5). The mean value for the concentration of TPNH in CLL cells was significantly less than the value in normal leukocytes.

In 1952, Colowick *et al.* demonstrated the transhydrogenation of pyrimidine nucleotides by an enzyme found in *Pseudomonas fluorescens.* Since then, this reaction has also been found in plant and animal tissues. TPNH-DPN (TD) transhydrogenase catalyzes the following reaction:

$$TPNH + DPN \rightleftarrows TPN + DPNH$$

TABLE 9-5

Levels of Pyridine Nucleotides in Leukemic Leukocytes[a]

Cell type	No. of samples	Nanomoles per 10^9 leukocytes			
		DPN^+	TPN^+	DPNH	TPNH
Normal	14	32 ± 2.0	8 ± 1.5	25 ± 2.3	24 ± 3.9
CLL[d]	10	43 ± 3.3[b]	6 ± 0.6	17 ± 3.6	11 ± 2.5[b]
CML	10	70 ± 9.5[c]	9 ± 1.2	23 ± 3.6	21 ± 4.9
AL	11	116 ± 22[c]	11 ± 2.6	25 ± 4.7	34 ± 6.7

[a] Data are those of Silber *et al.* (1962). Reproduced by permission of the American Society for Clinical Investigation, Inc. Values listed are mean ± standard error of the mean. p values for the significance of the differences between means for the normals and for the various groups of leukemic leukocytes have been calculated and are listed as:

[b] $p = <0.05$.

[c] $p = <0.01$.

[d] CLL, chronic lymphocytic leukemia; CML, chronic myelocytic leukemia; AL, acute leukemia.

DPNH-DPN (DD) transhydrogenase catalyzes a similar reaction:

$$DPNH + DPN \rightleftarrows DPN + DPNH$$

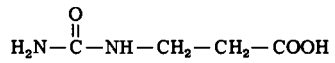
The levels of these transhydrogenases in normal and leukemic human leukocytes have been investigated chiefly by 2 groups (Silber *et al.*, 1963a; Evans and Kaplan, 1966). Although there were some differences employed in the preparations used for the assay of enzyme activities, the results were in general agreement. Calculation from these authors' results shows that there were no statistically significant differences between the mean values for the DD-transdehydrogenase activity of any of the leukemic cells and those of normal cells. However, both groups of investigators showed that the levels of TD-transdehydrogenase activity were significantly higher in the leukocytes of CLL and AL than in normal leukocytes. In addition, Evans and Kaplan (1966) found a significantly higher than normal level in the leukocytes of CML.

Some interesting characteristics of the TD and DD dehydrogenases have been described (Silber *et al.*, 1963a). Both these enzymes were inhibited about 50% by *p*-chloromercuribenzoate at 5×10^{-4} *M*, and the inhibitions were reversible by thiols. Thyroxine and triiodothyronine at 10^{-4} *M* inhibited the TD-dehydrogenase by about 30%, but did not affect the DD-enzyme. The possibility naturally exists that the two dehydrogenase reactions may play an important role in regulating levels

in vivo of the reduced forms of the two pyridine nucleotide coenzymes (Silber *et al.*, 1963a; Evans and Kaplan, 1966).

E. Catabolism of Pyrimidines in Leukemia

Present information on the catabolism of pyrimidines indicates that cytosine (2-oxy-4-aminopyrimidine) and methyl cytosine (5-methyl-2-oxy-4-aminopyrimidine) are deaminated to uracil and thymine, respectively. These compounds are then reduced to yield dihydrouracil and dihydrothymine, respectively. The enzyme, hydropyrimidine hydrase, mediates the hydrolysis of these compounds to form β-ureidopropionic and β-ureidoisobutyric acids, respectively. The formula of the former is shown below, and the latter contains a methyl group attached to the α-carbon:



β-Ureidopropionic acid

Upon further hydrolysis, β-ureidopropionic acid is broken down to yield carbon dioxide, ammonia, and β-alanine. β-Ureidoisobutyric acid similarly yields carbon dioxide, ammonia, and β-isobutyric acid.

The urinary excretion of various pyrimidines and their metabolic end products in normal individuals have been studied in some detail by Heirwegh *et al.* (1967) who have identified uridine, 6-hydroxymethyluracil, and pseudouridine compounds. Adams *et al.* (1960) observed in a few cases that the excretion of uracil (2,4-dihydroxypyrimidine) was substantially increased in AL, but only slightly in CML and CLL. The urinary excretion of 5-ribosyluracil (pseudouridine) was increased considerably in all these 3 types. Pinkard *et al.* (1972) have recently found that in Hodgkin's disease a positive relationship exists between high urinary levels of pseudouridine and features with a poor prognostic implication such as constitutional symptoms and the predominance of atypical histiocytes and Reed–Sternberg cells in biopsy specimens.

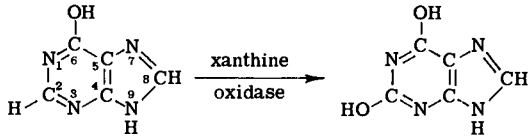
F. Catabolism of Purines in Cancer

1. Introduction

The degradation of free purines in the human organism is limited. Guanase is present in kidney, spleen, and other tissues and catalyzes the deamination of free guanine (2-amino-6-oxypurine) to xanthine (2,6-deoxypurine). The corresponding enzyme that would act upon adenine (6-aminopurine) is limited in amount. However, deamination does occur

very readily through the nucleoside or the nucleotide form. An adenylic acid deaminase present in muscle and other tissues acts upon adenylic acid to form inosinic acid. Guanosine and adenosine deaminases, which have also been found in animal tissues, mediate the conversion of the amino groups in the purine moieties of these nucleosides to hydroxyl groups. The cell is not freely permeable to nucleotides and cleavage to nucleoside or even further must precede outward passage.

Hydrolysis by adenase or guanase leads, respectively, to hypoxanthine and xanthine. The next step in the catabolism of these oxypurines is the oxidation, catalyzed by xanthine oxidase. This enzyme, which contains a flavinadenine nucleotide prosthetic group, catalyzes the conversion of hypoxanthine to xanthine and of the latter to uric acid (2,6,8-trioxypurine).



Hypoxanthine Xanthine

The production and fate of uric acid is of interest in a number of important human diseases, including gout, psoriasis, congenital juvenile hyperuricogenesis (Lesh–Nyhan syndrome), myeloproliferative diseases, and various types of cancer (Balis, 1967; Gutman and Yü, 1965). As determined by several investigators using the isotope-dilution method, the total amount of uric acid in the body of normal man, that is, the pool size, ranges from 0.87 to 1.59 gm and averages about 1.20 gm. Of this amount, approximately 45–85%, and averaging about 60%, is replaced daily by freshly elaborated uric acid. It follows, therefore, that in normal man uric acid is formed in quantities ranging from about 0.50 to 1.10 gm/day, averaging approximately 0.75 gm/day (Gutman and Yü, 1965). Not all of the uric acid that is normally ingested or formed is eliminated as such in the urine. Benedict *et al.* (1949) found that as much as 20% of ingested uric acid was broken down and excreted as compounds other than urate. Geren *et al.* (1950) observed that orally administered uric acid was extensively degraded to urea, whereas intravenously administered uric acid was excreted essentially unchanged. Sörensen (1952) reported that in one patient 69% of intravenously administered labeled uric acid was eliminated in 10 days as urate, about 11% as expired CO_2, and approximately 7% as fecal products.

Another factor which is important in the urinary excretion of uric acid is renal function. As the reader will recall, the rate of glomerular

filtration is about 125 ml/minute per 1.73 m^2 of surface area in human males. Substances like insulin, mannitol, and creatinine are filtered through the glomerulus, are neither reabsorbed nor secreted by the tubular epithelium, and have, therefore, a clearance value equivalent to the glomerular filtration. Uric acid appears freely filterable at the glomerulus, but about 98% is reabsorbed by the tubular epithelium. Most of the uric acid that appears in the urine is secreted, and the 2% that escapes reabsorption makes up about 20% of that excreted in the urine (Rastegar and Thier, 1972).

2. Urinary Excretion of Purines in Leukemia

There are relatively few data on the excretion of purines in leukemia. Employing two-dimensional paper chromatography followed by ultraviolet spectroscopy, Weissman *et al.* (1957) obtained the following average values, as milligrams excreted per day in a series of 9 normal male adults: hypoxanthine, 9.7; xanthine, 6.1; adenine, 1.6; guanine, 0.4; and 7-methylguanine, 6.5. Using ion exchange and filter paper chromatography, Adams *et al.* (1960) presented some fragmentary data on 1 normal subject and 4 patients with leukemia. The excretions of guanine in the leukemias ranged from 19 to 28 mg per 24 hours, as compared with a normal excretion of 22 mg per 24 hours. The excretions of adenine ranged from 10 to 60 mg per 24 hours, as compared with 4 mg per 24 hours in the normal individual. The excretions of hypoxanthine were also elevated above the normal value. These data were too sparse to draw any distinctions among the different leukemias.

Employing the method of Williams (1950), which determined the sum of xanthine and guanine, Sandberg *et al.* (1957) obtained a range of 20–50 mg and a mean of 40 mg for the 24-hour urinary excretion of these 2 purines in a group of 8 normal persons. A maximal urinary excretion of 20 mg per 24 hours was obtained in 1 patient with acute lymphoblastic leukemia and values of approximately 160 and 220 mg per 24 hours in 2 other cases with this condition. The administration of chemotherapy such as cortisone or 6-mercaptopurine caused marked increases in the excretion of these 2 purines. We shall presently consider the excretion of uric acid in leukemias, and, in that connection, submit other data on the excretion of oxypurines.

3. Urinary Excretion of Uric Acid in Leukemia

On a diet that is low in purines and moderately restricted in proteins, normal man excretes in his urine an average of 418 ± 70 mg of uric

acid per day (Gutman and Yü, 1957). The rate of glomerular filtration of uric acid averages 6.7 ± 1.4 mg/minute, and the excretion of uric acid in the urine averages 0.49 ± 0.16 mg/minute.

That the urinary excretion of uric acid may be increased in patients with leukemia has been appreciated since the report by Salkowski in 1870. However, there has been much inconsistency concerning the extent of increase. In 1957, Sandberg *et al.* considered the influence of such factors as the morphological type of leukemia, the degree of elevation of the leukocyte count, and the degree of enlargement of the spleen or other tissues (Table 9-6). In the group of patients with ALL, there was good correlation between the amount of uric acid excreted and the total leukocyte or lymphoblast count. No significant correlation was present between the uric acid excretion and the size of the spleen or the volume of packed red blood cells. The serum uric levels were determined in 3 of these patients, and were all elevated, ranging from 11.3 to 44.0 mg per 100 ml. During therapy with 6-mercaptopurine, amethopterin, or cortisone, the leukocyte count decreased and the urinary excretion of uric acid increased initially, then decreased. For example, in one patient with ALL, a leukocyte count of 120×10^3 per mm^3 and a basal uric acid excretion of approximately 950 mg per 24 hours, the administration of 6-mercaptopurine resulted in stepwise increases of uric acid excre-

TABLE 9-6

Uric Acid Excretion in Leukemias[a]

Group	No. of cases	White blood cell (count × 10^3)		Urinary uric acid (mg/24 hours)		Urinary uric acid (mg/kg/24 hours)	
		Mean	Range	Mean	Range	Mean	Range
Normals[b]	17	—	—	399	276–460	6.5[c]	5.2–7.3
ALL[d]	14	140	0.7–586	888	250–1840	30.2	11.9–74.8
AML	13	46	0.95–197	753	300–1250	13.0	6.2–20.5
CML	5	179	96–450	835	740–934	13.5	12.8–14.1

[a] Based on data of Sandberg *et al.* (1957).

[b] There were no differences in the urinary excretion between males and females, and the mean value and range are listed for the group as a whole.

[c] Data in the literature (Brøchner-Mortensen, 1940; Bishop and Talbott, 1953) indicate that when urinary excretion of uric acid in normal children is expressed as milligrams per kilogram body weight, the values are in the same range as those for normal adults.

[d] ALL, acute lymphoblastic leukemia; AML, acute myeloblastic leukemia; CML, chronic myelocytic leukemia.

tion up to levels of approximately 3500 mg per 24 hours, as the leukocyte count decreased from 180×10^3 per mm^3 toward normal levels.

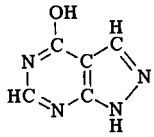
In 13 cases with acute myeloblastic leukemia, there was no correlation between the excretion and the leukocyte count. Here, too, the institution of antileukemic therapy frequently resulted in substantial increases of uric acid, but not as large as in the patients with ALL. In the absence of antileukemic therapy, the excretion of uric acid was moderately elevated in CML, but normal in patients with CLL regardless of the height of the leukocyte count.

The presence of hyperuricemia and of increased excretion of uric acid in leukemias and lymphomas has been amply confirmed (Weissberger and Persky, 1953; Holland *et al.*, 1959; Krakoff and Meyer, 1965). Moreover, Ultmann (1962) showed that hyperuricemia occurs in disseminated neoplastic disease other than lymphomas and leukemias. In a series of 79 patients, the level of serum uric acid ranged from 6.0 to 16.8 mg per 100 ml, as compared with normal levels up to 5.0 mg per 100 ml. None of the 79 patients had antecedent or concurrent renal impairment, and none gave a family history of gout. Of these 79 patients, 26 had carcinoma of the lung, 29 had carcinoma of the breast, and the remainder included cases of hepatoma, melanoma, leiomyosarcoma, and carcinoid. As evidence of the extensive progressive disease, liver metastases were present in 30 cases, and hypercalcemia in 31 patients. Only 5 patients had clinical evidence of gout. In some patients, progressive deterioration of renal function and uremia developed. Ultmann (1962) did not present any data on the urinary excretion of uric acid.

In 1953, Weissberger and Persky reported an incidence of uric acid calculi of 5.3% in 283 patients with acute and chronic leukemia, Hodgkin's disease, lymphosarcoma, or reticulum cell sarcoma. No calculi were found in a group of 100 patients with metastatic cancer, other than lymphoma. Many of the patients with lymphoma had been treated with roentgenray therapy, radioactive phosphorus, or nitrogen mustard. Signs of renal failure and uremia developed in some treated cases; these were characterized by oliguria and elevations not only in serum uric acid but also in serum urea nitrogen and creatinine. In 1958, Kritzler reviewed 10 cases of leukemia, including 3 reported by himself, who had developed anuria and chronic uremia following treatment with x-ray or various chemotherapeutic agents. The leukocyte count decreased greatly in each of these cases, and the blood nonprotein nitrogen (NPN) reached maximal peaks ranging from 82 to 244 mg per 100 ml. Where cystoscopy was done, examination revealed uric acid crystals in the bladder and evidence of blockage of the ureters. Eight of these patients died, and 2 survived following diuresis.

4. Allopurinol and Uric Acid Excretion in Leukemia

Elion *et al.* (1963) and Rundles *et al.* (1963) reported that the compound, allopurinol [4-hydroxypyrazolo(3,4-*d*) pyrimidine, HPP], which was an effective xanthine oxidase inhibitor, increased the therapeutic efficacy of 6-mercaptopurine (6MP) and related thiopurines in leukemia.



Allopurinol

The compound is an isomer of hypoxanthine in which the carbon and nitrogen atoms in the 7 and 8 positions of the purine ring are interchanged.

Rundles *et al.* (1963) demonstrated that allopurinol, presumably because of its capacity to inhibit xanthine oxidase, produced a decrease in serum and urine uric acid in normal and gouty humans and in patients with chronic granulocytic leukemia. Soon after these initial observations, several detailed studies appeared utilizing allopurinol in the treatment of hyperuricemia of gout (Yü and Gutman, 1964; Klinenberg *et al.*, 1965; Wyngaarden *et al.*, 1965; Rundles *et al.*, 1966) and of neoplastic disease (Krakoff and Meyer, 1965; Krakoff, 1966; De Conti and Calabresi, 1966). In a series of 15 patients with leukemia or lymphoma undergoing treatment with various agents or modalities, the administration of allopurinol in doses of 300–800 mg daily resulted in lowering of serum uric acid in each patient and a decrease in urinary uric acid excretion in each of 10 patients in whom it was measured (Krakoff and Meyer, 1965). For example, in a 59-year-old male with lymphosarcoma undergoing x-ray therapy and showing marked regression of an intra-abdominal tumor, the serum uric acid level was 21.9 mg per 100 ml, and the urinary excretion of uric acid per day was 3090 mg. During treatment with allopurinol, the serum uric acid decreased to 3.9 mg per 100 ml and the daily urinary excretion of uric acid to 527 mg. Similar changes are shown in greater detail during a period of 6 weeks in Fig. 9-4. By 1966, Krakoff had used allopurinol in 75 patients with leukemia or lymphoma to counteract hyperuricemia and excessive excretion of uric acid. No uric acid nephropathy occurred in this group, and allopurinol caused no significant toxicity.

Since allopurinol inhibits the conversion of the oxypurines, xanthine

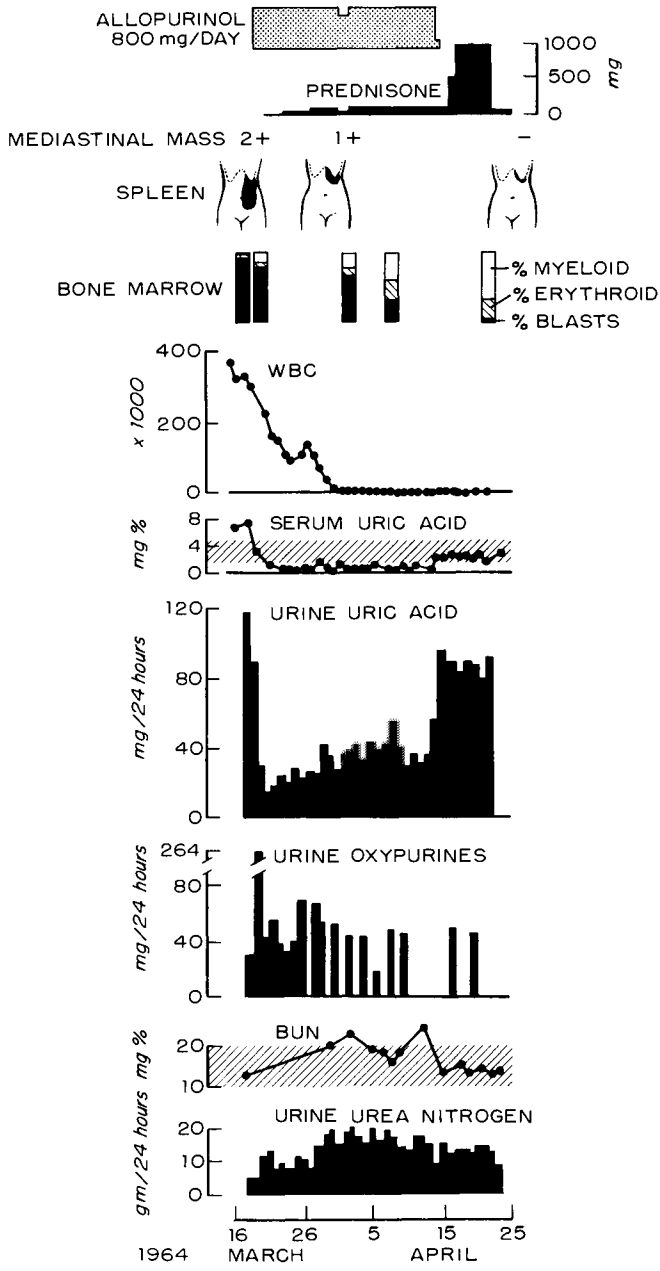


Fig. 9-4 Relation of serum uric acid, urine uric acid and oxypurines, and blood and urine urea nitrogen to response to therapy with vincristine and prednisone in patients with acute lympholastic leukemia. One dose vincristine, 0.04 mg/kg, was given 3/19/64. Urine total nitrogens paralleled urine urea nitrogen very closely. Effect of allopurinol is also shown. From Krakoff and Meyer (1965). Reproduced by permission of the American Medical Association.

and hypoxanthine, to uric acid, it is to be expected that these will reach high concentrations in the serum and may be excreted in the urine. Rundles *et al.* (1963) found that in patients receiving allopurinol, the serum levels of hypoxanthine and xanthine approached 300–400 μg per 100 ml, approximately 3 times the normal level. The renal clearances of oxypurines and uric acid were determined in 4 patients during allopurinol therapy, and it was found that the oxypurine clearance exceeded the uric acid clearance by 6–16 times. The increase in oxypurines, exclusive of uric acid, excreted per day during allopurinol therapy does not usually equal the decrease in uric acid output (Krakoff and Meyer, 1965; Yü and Gutman, 1964).

The studies that we have cited and many others have amply demonstrated the favorable effects of allopurinol in inhibiting the formation of uric acid and avoiding the hazards of uric acid nephropathy. The question arises whether the increased concentrations of oxypurines in the serum and urine may have unfavorable effects or whether allopurinol may affect enzymes of other metabolic sequences. Greene *et al.* (1969) have reported the case of a 16-year-old boy with the Lesch–Nyhan syndrome who, previous to allopurinol therapy, had a serum uric acid concentration of 10.0 mg per 100 ml and a 24-hour urinary excretion of 4.93 mmoles (828 mg) uric acid and 0.073 mmole of other oxypurines. He was placed on 50 mg allopurinol 4 times daily. The serum uric acid concentration decreased to 2.5 mg per 100 ml and the 24-hour urinary uric acid excretion to 0.857 mmole (144 mg). The oxypurines increased to 4.64 mmoles, of which 42% was xanthine and 58% was hypoxanthine. Two years later, the patient began to pass stones, of which several were yellow and were found to consist of about 85% xanthine and less than 4% uric acid.

Fox *et al.* (1970) have reported that the administration of allopurinol increases the urinary excretion of the pyrimidine nucleoside, orotidine. The biosynthesis of pyrimidines involves the coupling of orotic acid with 5′-phosphoribosyl-1-pyrophosphate under the influence of the enzyme, oritidine-5′-phosphate pyrophosphorylase (phosphoribosyltransferase) to yield oritodine 5′-phosphate. In turn, this compound is converted by orotodine-5′-phosphate decarboxylase to yield uridine 5′-phosphate (UMP). A small amount of orotodine 5′-phosphate is irreversibly dephosphorylated to yield the riboside, orotodine, which is normally excreted in the urine to the extent of about 2–4 mg/day. When allopurinol was administered to normal persons or to patients, the urinary orotodine excretion ranged from 30 to 60 mg/day, as compared with a normal excretion of 2–4 mg/day. Although allopurinol itself does not inhibit the decarboxylase reaction, prior incubation with erythrocyte hemolyzate

and phosphoribosyl pyrophosphate transforms it to an inhibitor of the enzyme. Fox *et al.* (1970) concluded that allopurinol *in vivo* is converted by a phosphoribosyltransferase to the ribonucleotide that inhibits orotodine-5′-phosphate decarboxylase and leads to the accumulation of orotodine 5′-phosphate. The dephosphorylation of this large concentration yields orotodine which is excreted in the urine.

G. Nucleic Acids in Leukemia

1. Introduction

There are few fields of biochemistry which are conceptually as relevant to the problem of human cancer as the biosynthesis, structure and function of the nucleic acids. The biochemistry of nucleic acids has been treated at great length in various monographs and texts (Watson, 1970; Lehninger, 1970; White *et al.*, 1973), and only those portions which pertain to certain aspects of human leukemia will be considered in somewhat greater detail at this point. These aspects will include: (a) RNA homology between human leukemic cells and a mouse leukemia virus (Hehlmann *et al.*, 1972), (b) patterns of isoaccepting phenylalanine transfer RNA species in leukemic lymphoblasts (Gallo and Pestka, 1970), and (d) methylation of nucleic acids in leukemic cells (Silber *et al.*, 1966).

2. RNA-Dependent DNA Polymerase and RNA Homologies in Human Leukemia

Originally, DNA was considered to transfer its information for the synthesis of ribonucleic acid. Subsequently, it was shown that the reverse process was also possible (Lee-Huang and Cavalieri, 1963; Baltimore, 1970; Temin and Mizutani, 1970). The latter two studies indicated strongly that the virions of RNA tumor viruses contained a new type of polymerase that catalyzed the incorporation of deoxyribonucleotide triphosphates into DNA from an RNA template. This polymerase has also been designated as "reverse-transcriptase."

The preceding findings were utilized by Spiegelman and his associates (Spiegelman *et al.*, 1970; Schlom *et al.*, 1971; Axel *et al.*, 1972a,b) in exploring the possibility that human cancers might contain RNA molecules or portions of RNA molecules that were homologous to various tumor virus RNA's in animals. This type of exploration bears several implications. The role of viruses in animal cancers can be determined by inoculation of virus into the animal and the subsequent observation of cancer development. This procedure is obviously not permissible in

man. The establishment of homologies between the RNA molecules of certain tumors of man and animals does not, of course, signify a viral etiology in man, but may provide evidence for the involvement of virus-related information in human tumors.

The extent of structural similarity between RNA molecules from two different sources can be determined by preparing a DNA homologous to RNA, utilizing the "reverse-transcriptase" method, then hybridizing the resulting DNA with the second type of RNA molecule. For example, Axel *et al.* (1972b) devised a procedure for detecting viral-specific RNA in a mouse mammary tumor. [^{3}H]DNA homologous to mammary tumor virus RNA was prepared by incubating purified virus in a suitable medium with dGTP, dCTP, dATP, and [^{3}H]dTTP. The resulting [^{3}H]DNA was purified and then denatured by incubation at 68°C in formamide for 10 minutes. Under these conditions, the hydrogen bonds break and the complementary strands separate. After quick chilling of the solution to 0°, the appropriate RNA, which had been prepared from nuclei or polysomes of normal or mammary tumor tissue, was added. This, constituting a hybridization mixture, was brought to 0.4 *M* NaCl-50% formamide in a suitable volume and incubated for 18 hours at 37°C. At the end of this annealing period, the reaction mixture was added to 5.5 ml of 5 m*M* EDTA mixed with an equal volume of saturated Cs_2SO_4 and centrifuged at 44,000 rpm for 60 hours at 20°C. 0.4 ml fractions were collected and assayed for trichloroacetic acid precipitable radioactivity. The formation of DNA-RNA hybrid complexes during the annealing period was manifested by movement of the radioactive DNA toward the RNA region of the density gradient. We shall discuss the similar work of Spiegelman and his associates (Spiegelman *et al.*, 1970; Schlom *et al.*, 1971; Axel *et al.*, 1972a,b) on breast carcinoma more fully in Chapter 17.

In a manner similar to the instance mentioned above, molecular hybridization with radioactively labeled DNA complementary to the RNA of the Rauscher leukemia virus was used to detect the presence of homologous RNA in the polysome fraction of human leukemic cells (Hehlmann *et al.*, 1972). These included acute and chronic lymphatic leukemias, acute myelogenous leukemias, and monocytic leukemias. The leukocytes of 24 out of 27 patients, or 89%, contained RNA possessing homology to that of the mouse leukemia agent. In contrast, no positive reaction was obtained in the polysomal RNA preparations from any of 33 normal tissues; these included normal leukocytes, phytohemagglutinin-stimulated lymphocytes, and various normal adult and fetal tissues. Moreover, no positive reactions were obtained when an attempt was made to hybridize the leukemic cell RNA's with [^{3}H]DNA complemen-

tary to the RNA of the mouse mammary tumor virus or of the avian myeloblastosis virus. These studies indicated, therefore, that human leukemic cells contain RNA sequences homologous to those found in a viral agent known to cause leukemias in an experimental animal. It is of interest that human sarcomas also contain this type of RNA (Kufe *et al.*, 1971). Complexes of 70 S RNA and reverse transcriptase have recently been reported to be present in the plasma of leukemia patients, more specifically, in 74% of 19 such patients as contrasted with none in 9 normal persons (Yaniv *et al.*, 1973).

3. Patterns of Isoaccepting Transfer RNA's in Human Leukemia and Lymphoma

It has been of interest to determine whether a transfer RNA that accepts the same amino acid (isoaccepting tRNA) is the same in different tissues of one species, in the same tissue of different species, or in normal tissue and its malignant counterpart. On comparison by $^{14}C,^{3}H$ double-labeling technique of the chromatographic profiles on methylated albumin kieselguhr, Taylor *et al.* (1967) found no major differences between tissues or species for alanine, leucine, lysine, phenylalanine, or threonine tRNA. However, differences were seen in the elution profiles of minor tRNA species for glycine and serine. Significant differences were obtained between Ehrlich ascites tumor cells and normal mouse tissues with respect to the elution profiles of phenylalanine-, serine-, and tyrosine-tRNA's. Similar studies have been carried out and similar relationships elicited among aminoacyl-tRNA's of other animal systems and tissues (Yang *et al.*, 1969; Goldman *et al.*, 1969).

Several studies have recently been concerned with the characteristics of tRNA in human leukemic cells. Gallo and Pestka (1970) grew in suitable cultures normal human lymphoblasts collected from healthy volunteers and lymphoblasts obtained from a patient with ALL. The various aminoacyl-tRNA's were prepared, and comparisons were made between the aminoacyl-tRNA profiles of normal and leukemic lymphoblasts. The results indicated that there were at least 56 species of tRNA from both the normal and leukemic cells. In most instances, the aminoacyl-tRNA of the leukemic cell gave elution profiles very similar to, if not identical with, the elution profile of normal cells. However, there were small but reproducible differences between the normal and leukemic cells with regard to leucyl-, seryl-, threonyl-, and prolyl-tRNA. The most pronounced differences were obtained for tyrosyl-tRNA and glutaminyl-tRNA. Nishimura and Weinstein (1969) found that normal mammalian tissue contained two isoaccepting phenylalanine tRNA's.

Preparations of tRNA obtained from the spleens of 12 patients with various leukemias and lymphomas were found to contain, in addition, a third accepting phenylalanine transfer RNA (Mittelman, 1971).

4. Activity of tRNA Methylases in Leukemia

The function of these enzymes has been considered in detail by Borek and Kerr (1972). They modify the structure of preformed tRNA by introducing methyl groups into specific positions in the four main bases. The enzymes are species specific, organ specific, and even site specific for particular bases.

In general, crude preparations of tRNA methylase from neoplastic tissue have a 2- to 10-fold increase in activity, as compared with the closest normal counterpart of the abnormal tissue (Borek and Kerr, 1972). The determination of activity in blood samples of lymphocytes and polymorphonuclear leukocytes, isolated from normal blood and from the total population of white cells of leukemic patients, is shown in Table 9-7 (Tsutsui *et al.*, 1966).

These results demonstrate that the mean tRNA methylase activity of the leukocytes from 8 patients with leukemia was about 10-fold that of normal polymorphonuclear leukocytes and 30-fold that of normal lymphocytes. This increased activity was not specific for leukemia. Tissues from human carcinoma of colon and rectum showed tRNA methylase activities 4- to 10-fold the activities in normal adjacent tissues, and mammary carcinoma had 4- to 15-fold the activities in adjacent normal breast tissue. Viale (1971) has also reported a 3- to 4-fold increase

TABLE 9-7

tRNA Methylase Activity of White Cells from Normal and Leukemic Patients[a]

Group	No. of subjects or patients	Activity as cpm/mg protein	
		Mean	Range
Normal lymphocytes	10	64	20–230
Normal polymorphs	12	175	50–540
Leukemic leukocytes	8[b]	1920	670–3560

[a] Based on data of Tsutsui *et al.* (1966).

[b] This group consisted of 4 patients with acute myelogenous leukemia, one with chronic leukemia, 2 with "leukemia," and 1 with *polycythemia vera* and subsequent acute leukemia.

in methylase activity of human brain tumors as compared with normal brain tissue.

5. Other Biochemical Aspects of Nucleic Acid Metabolism in Leukemia

The methylation of transfer RNA raises the possibility that turnover of tRNA may result in substantial excretion of methylated purines and pyrimidines. We have previously considered the catabolism and urinary excretion of purines and pyrimidines in normal persons and in patients with leukemia (Sections V, E and F). It was evident from some of the studies cited in that connection (Weissman *et al.*, 1957; Adams *et al.*, 1960) that methylated purines were excreted in the urine of normal subjects. Many additional compounds of this nature have been recognized since then (Chedda, 1970).

Increased excretion of methylated purines has been reported in rats bearing thymic lymphoma and mice bearing mammary carcinoma (Mandel *et al.*, 1966) and in hamsters bearing adenovirus-12 induced tumors (McFarlane and Shaw, 1968). Mirvish *et al.* (1971) reported that Arabian and Kenyan patients with cancer had significantly elevated levels for the urinary excretion of adenine and 7-methylguanine per mg creatinine. The incorporations of ^{14}C-labeled methyl groups into DNA of leukocytes isolated from the peripheral blood of patients with AL, CLL, or CML were significantly higher than in the leukocytes of normal persons (Silber *et al.*, 1966). This relationship also held for incorporation into RNA of leukocytes from the blood of patients with AL and CLL but not for the leukocytes of CML. However, leukocytes from patients with leukomoid reaction also showed incorporations significantly higher than normal. The higher incorporations may not necessarily indicate increased methylation. Increased transport of [^{14}C]methionine across the cell membrane, a smaller pool of nonradioactive intermediates, and decreased catabolism of the added precursors may also be factors leading to higher specific activity of the DNA or RNA.

Ove *et al.* (1968) reported that DNA polymerase activity was increased in human leukemic cells. Under the conditions of the assay, purified preparations of DNA showed the following average activities in terms of micrograms of triphosphate nucleotides incorporated per milligram enzyme per hour: for normal granulocytes, 0.100; for normal bone marrow granulocytes, 0.074; and for leukocytes from chronic granulocytic leukemia, 0.731.

It may be appropriate here to summarize the changes in enzyme activities of leukemic leukocytes. As we have already seen, increases occur

in acid phosphatase (Valentine and Beck, 1951); dihydrofolate reductase (Bertino *et al.*, 1963); the first 3 enzymes involved in the *de novo* synthesis of pyrimidine, namely, aspartate carbamyltransferase, dihydroorotase, and dihydroorotic dehydrogenase (Smith *et al.*, 1960); and, as we have seen above, DNA polymerase. In contrast, reduction in the activity of other leukocyte enzymes has been reported. These include alkaline phosphatase (Beck and Valentine, 1951; Valentine *et al.*, 1957; Rosner *et al.*, 1972), lactic dehydrogenase (Rabinowitz, 1966), deoxythymidine phosphorylase (Marsh and Perry, 1964), and pyrimidine deoxyribonuclease (Gallo and Perry, 1969).

When a suspension of lymphocytes obtained from the blood of normal individuals is incubated with phytohemagglutinin (PHA) for about 36–48 hours, 60–80% of the surviving lymphocytes are transformed into blast cells characterized by their large size, much basophilic cytoplasm, and prominent nuclei. The alterations are associated with a rapid increase in the rates of RNA and protein metabolism during the first 48 hours. DNA synthesis begins at 36 hours and approaches a maximum at 72 hours when many mitotic figures are present in the cultured cells (Havemann and Rubin, 1968). In contrast, lymphocytes from patients with CLL require 120–168 hours to develop into blast cells active in RNA synthesis and DNA replication. Investigation of the patterns of tritiated uridine incorporation into the methylation of RNA and analysis of the RNA subunits showed that the sluggish response of CLL lymphocytes was associated with a defect in one of the mechanisms regulating the assembly of new ribosomes (Rubin, 1971).

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10

Neoplasms of the Bone

I. Introduction*

The subject of calcium and phosphorus metabolism is one of the most important in the area of human cancer. Primary bone tumors constitute a small proportion of newly reported malignant neoplasms as, for example, 0.33% in 1967 for New York State (Cancer Control and Registry Report, 1969). It has been estimated that essentially the same proportion, 0.29%, would occur in the United States in 1973 (Silverberg and Holleb, 1973). The skeletal system is also involved in approximately 25% of cases of metastatic spread from tumors primary at other sites (Abrams *et al.*, 1950). Some neoplasms, such as carcinoma of the breast in the female and prostate in the male, are characterized by particularly high incidences of bone involvement, namely, 70–90% of cases (Elkin and Mueller, 1965; Vest, 1954). These two neoplasms accounted for approximately 17% of all neoplasms reported in New York State, exclusive of New York City, for the year 1967 (Cancer Control and Registry Report, 1969).

* The following abbreviations are used most commonly in the present chapter: AMP = adenosine monophosphate (adenylic acid); B = Bodansky (unit); BFR = bone formation rate; BRR = bone resorption rate; C_{Cr} = clearance of creatinine; C_{In} = clearance of inulin (glomerular filtration rate); C_P = phosphate clearance; E = exchangeable calcium pool; IPTH = immunoreactive parathyroid hormone; KA = King-Armstrong (unit); PTH = parathyroid hormone; T_m = transport maximum; Tm_{PAH} = tubular excretory maximum; TRCa = tubular reabsorption of calcium; TRP = tubular reabsorption of phosphate.

The primary bone tumors and metastases from tumors at other sites directly encroach upon the substance of the bone and lead, in varying degrees, to alterations in calcium and phosphorus metabolism. But this metabolism has long been known to be affected also by adenoma and carcinoma of the parathyroid gland (Albright and Reifenstein, 1948; Rapoport *et al.*, 1960; Lemann and Donatelli, 1964). In addition, during the past decade, there have been increasing numbers of reports on hypercalcemia in malignant disease which, in these cases, are characterized by absence of roentgenographic, biopsy, or autopsy evidence of bone metastases or bone destruction (Loebel and Walkoff, 1962; Goldberg *et al.*, 1964; Svane, 1964; Lafferty, 1966; Muggia and Heinemann, 1970). These cancers will be discussed more fully later, but it may be noted here that their primary sites are usually in the lung, kidney, and the bladder and, less frequently, in the vulva, the ovary, the uterus, the pancreas, the liver, and as lymphosarcomas (Myers, 1956).

II. Chemical Composition and Structure of Bone

A. Introduction

There are some excellent recent collaborative monographs which treat in detail the general aspects of the biochemistry and physiology of bone

TABLE 10-1

General Chemical Composition of the Human Adult Skeleton

Component	Percent of total weight of skeleton[a]
Organic compounds	35
Water	44
Calcium	9.5
Phosphorus	4.6
Carbonate	1.14
Sodium	0.16
Potassium	0.055
Magnesium	0.10
Chloride	0.17
Sulfur	0.14

[a] Calculated from data of Shohl (1939).

and which also present newly developed methods for analysis (Whedon and Cameron, 1970; Menczell and Harell, 1971; Bourne, 1972). However, the larger portions of these monographs deal with animal tissue or with comparative measurements on man from x-ray source images (Whedon and Cameron, 1970). For basic data on the mineral composition in human bone, it still appears advisable to refer to the older studies.

B. Inorganic Components

Analyses of several human bodies have demonstrated that the skeleton constitutes about 15–17% of the total body weight (Shohl, 1939; Mitchell *et al.*, 1945; Forbes *et al.*, 1953, 1956). Table 10-1 shows the general chemical composition of the skeletal tissue. The larger part consists of water and of organic compounds. Approximately 20% consists of inorganic ions. Shohl's (1939) review permits the calculation of the ions (listed in the tabulation below) in the skeleton as fractions of the total body

Ion	Percent of total body store
Calcium	99.1
Phosphorus	79.9
Sodium	29.7
Potassium	4.3
Chloride	23.5
Magnesium	52.4

stores. Later studies confirmed these values. For example, according to Cooke (1955), the skeleton contains 99% of the total calcium in the body, 88% of the phosphate, 80% of the carbonate, 50% of the magnesium, and 35% of the sodium. Similar values were submitted by Forbes *et al.* (1953, 1956).

The skeleton contains several additional mineral constituents in trace amounts. Fluoride is present in drinking water, food, and tea. The average uptake by the bone is 25 mg/kg/year, but the final concentration may reach values as high as 3000 mg/kg (Fourman *et al.*, 1968). Manganese, zinc, copper, and iodine are present in trace amounts. Bryant and Loutit (1963–1964) have found that in the United Kingdom the concentration of stable strontium in bone rose from a value of approximately 200 μg/gm calcium at birth to a value of about 330 μg/gm calcium in adults. The concentration of ^{90}Sr in the bone, obviously dependent

upon the fall-out from atomic explosions during a particular year, was about 0.5–1.0 pg/gm calcium at birth, rose to a peak of about 3–4 pg/gm calcium at about 6 months to a year, and declined thereafter.

C. Organic Components of Bone

There appears to be no complete analysis of the organic components of human bone, but analysis of the composition of air-dried ox femur diaphysis yielded the following values, as a fraction of the weight of the total: collagen, 18.64%; mucoprotein polysaccharide, 0.245%; and other proteins, 1.2% (McLean and Urist, 1968). Essentially the same values were obtained by Herring (1972) for dried bovine cortical bone: inorganic matter, 76.04%, and organic matter, 23.96%. The composition by weight of the organic matrix was collagen, 88.5%; "resistant protein," 0.98%; chondroitin sulfate, 0.81%; bone sialoprotein, 0.80–1.15%; chondroitin protein sulfate glycoprotein, 0.31–0.44%; and other (glycoprotein, protein, etc.), 7.19–7.67%.

Approximately 90% of the organic fraction of the bone is collagen, and this protein is also present in connective tissues of the body. The collagen from these two sources constitute 25–33% of the total body protein and, therefore, approximately 6% of the body weight. According to Klein and Curtis (1964), 57% of the collagen is in the bone and another 34% in the skin. The main amino acid constituents of human bone collagen are, in amino acid residues per 1000 amino acid residues: glycine, 319; proline, 124; and 4-hydroxyproline, 100 (Eastol and Leach, 1958). The presence of hydroxyproline is distinctive, for this amino acid is absent from or present in only very small amounts in other proteins. We shall later consider the role of hydroxyproline as a measure of bone metabolism.

Collagen fibers in bone matrix are similar to those present in connective tissue. They are built of smaller fibrils which are 200–2500 Å wide and many microns long. Collagen is insoluble, but prolonged extraction with dilute acid yields a solution of the fundamental units, termed "tropocollagen," which are individual molecules, approximately 15 Å wide and 3000 Å long with a molecular weight of about 300,000. Each molecule is a cable of three polypeptide chains. Each chain is a tight left-handed helix, with the triple-stranded cable being twisted slightly to the right. Collagen fibrils are composed of tropocollagen molecules being joined end-to-end and side-to-side. The collagen fibers lie in a ground substance which consists mostly of mucoprotein, or proteins combined with aminopolysaccharides (Fourman *et al.*, 1968). The aminopolysaccharides, it will be recalled, are linear polymers of glucuronic acid and

a hexosamine, either *N*-acetyl-D-glucosamine or *N*-acetyl-D-galactosamine. Their molecular weights range from about 50,000 to 150,000.

D. Structure of Bone Mineral

Although the crystal structure and chemical composition have been investigated for many years, Richelle and Onkelinx (1969) consider present concepts only an approximation of reality. It has been generally held that the bone salt is probably a hydroxyapatite with the structure, $[Ca_3(PO_4)_2]_3 \cdot Ca(OH)_2$, existing in crystalline form. The smallest repeating unit of the crystal lattice, as revealed by x-ray diffraction, has the dimensions of $9.4 \times 9.4 \times 6.9$ Å. The crystals themselves lie on and in the collagen fibers and measure approximately $40 \times 250 \times 600$ Å (Fourman *et al.*, 1968). The composition of the bone salt may be modified by the ions substituted at the surface of the crystals. Ions like fluoride, chloride, or carbonate can be substituted for phosphate and hydroxide ions, and divalent ions like magnesium, strontium, or barium can be substituted for calcium. On the other hand, Eanes *et al.* (1967) have suggested that bone mineral is composed of a poorly crystallized apatite and a noncrystalline or amphorous calcium phosphate. Richelle and Onkelinx (1969) have discussed recent advances in the formulation of the crystalline structure, the manner in which carbonate was combined, and the concepts of equilibrium between bone mineral and its bathing fluids.

E. Calcification

Microscopic examination of a thin ground section of the shaft of a long bone shows that it is largely composed of calcified interstitial substance, bone matrix, deposited in layers, or *lamellae*, 3–7 μm thick. Uniformly spaced throughout this interstitial substance are cavities, called "lacunae," each occupied by a bone cell or osteocyte. These osteocytes are connected with each other by an extensive network of minute canals which may well furnish avenues for exchange between the cells and the nearest perivascular space. In actively growing bone, the cellular component is much more prominent, and three kinds of bone cells are distinguishable: osteoblasts, osteocytes, and osteoclasts.

The osteoblasts, approximately 20–30 μm in their dimensions, are associated with the formation of osseous tissue and are invariably present at the advancing surfaces of developing or growing bones. The function of the osteoblast is to lay down the bone matrix by forming both collagen and ground substance. Histochemical methods have revealed the pres-

ence of enzymes of the citric acid cycle, of glycolysis, and of the functional pentose shunts (Vaughan, 1970; Pritchard, 1972). Alkaline phosphatase is present in particularly high concentration. As has been pointed out earlier in this volume (Chapter 3, Section III,B,2), in the absence of diseases of the hepatobiliary tract, the height of the serum alkaline phosphatase activity reflects the intensity of the cellular activities mobilized in laying down osteoid tissue and evoked in response to a disturbance between orderly deposition and resorption of bone. It has not been possible to assign a more definite role to the phosphatase of the osteoblast in the process of deposition of bone salts. McLean and Urist (1968) have reviewed critically the various enzyme mechanisms that have been implicated in the process of calcification.

It has been suggested that the young osteocyte continues the accretion of the bone salts, while the mature hypertrophic osteocyte presides over salt removal through changes induced in the organic matrix (Vaughan, 1970). The third type of osteogenic cell is the osteoclast. The typical osteoclast is a large multinucleated cell present on or near bone surfaces in the process of resorption. Several alternate functions have been proposed for this cell. It has been held that resorption of bone is accomplished directly, and that the calcified ground substance undergoes no preparatory change. Reynold's studies (1968) on an *in vitro* system has raised again the question of whether calcium is first removed and the different parts of the matrix are then digested. Another line of thought, expounded by Jaffe (1972), holds that decalcification occurs first and that osteoclasts then proliferate to phagocytose the decalcified bone matrix. Vaughan (1970) noted that in recent years it has been generally held that removal of both matrix and mineral occurs simultaneously.

III. Calcium, Magnesium, and Phosphorus Metabolism in the Normal Individual

A. Calcium

As we have already noted, over 99% of the body's calcium and approximately 80–90% of the body's phosphorus are to be found in the skeleton. The amounts of these elements necessary to maintain the adult skeleton in equilibrium were the subject of many studies from the period 1910–1940. These values were obtained by measuring the total output of calcium or phosphorus at various intakes of these elements and determining the point of equilibrium between the intake and output of the element in question. For example, in 1941, Sherman summarized the

results of 97 studies in the literature on calcium and calculated these on the basis of a 70-kg man. The value for the daily intake necessary for equilibrium was 0.45 ± 0.12 (SD) gm. Similarly, for 95 studies with phosphorus, the value was 0.88 ± 0.17 (SD) gm.

The data from some of these studies may be formulated as a regression equation showing the relationship between the total output (urinary and fecal excretion) and various intakes. The data of Steggerda and Mitchell (1939) on 7 male adults, 24–39 years of age, yielded the equation,

$$Y = 2.5 + 0.71X$$

where X was the calcium intake and Y was the total calcium excretion, both expressed as milligrams per kilogram of body weight per day. The point at which calcium excretion is equal to the intake is 8.6 ± 0.3 mg/kg/day or about 0.60 gm/day for a 70-kg man, well within the range given by Sherman (1941).

The cellular mechanisms involved in the absorption of calcium from the intestinal tract have received considerable attention (Wasserman and Taylor, 1969). Most studies have utilized conventional balance techniques with or without tracer or the measurement of the disappearance of radiocalcium from the gastrointestinal tract after oral administration or from a ligated loop of intestine. During the past decade, the everted gut technique has been employed. It is beyond the scope of this volume to consider the various mechanisms in any detail, but there is much evidence for the existence of a calcium pump in intestinal tissue (Wasserman and Taylor, 1969). With average calcium intakes, approximately 30% of ingested calcium is transferred from lumen to blood in the human adult. Absorption is influenced by several physiological and nutritional factors such as the overall nutritional state, age, reproduction status, the absolute levels of calcium intake, vitamin D intake, and the presence of dietary agents that can form insoluble complexes with calcium. Certain disease states such as steatorrhea, vitamin D–resistant rickets, and diseases of the parathyroid also influence the absorption.

Calcium, excreted into the intestine through the gastric juice and bile, is known as the endogenous fecal excretion and has been estimated in experiments in which ^{47}Ca was employed. With this technique, Nordin (1959) found a mean daily value of 170 mg for this excretion in a series of 7 subjects.

In order to determine the amount of calcium that actually passed through the metabolic processes, Bauer *et al.* (1929) studied balances on very low intakes of calcium, namely, 0.33 gm per 3-day period. The average urinary excretion during this period was 0.19 gm, and the average fecal excretion 0.60 gm, with a negative balance of 0.46 gm. Knapp

(1947) observed that at various calcium intakes the logarithm of the urinary calcium, expressed as percent of the calcium intake, was, in general and except for the first year of life, inversely proportional to the logarithm of the daily calcium intake, expressed as milligrams per kilogram of body weight. For example, at a low daily intake of 2 mg/kg or about 140 mg for a 70-kg adult, the urinary excretion averaged about 90% of the intake, or about 125 mg/day. At very high intakes, as, for example, 75 mg/kg, or about 5 gm per 70-kg adult, the urinary excretion was about 5% of the intake, or 250 mg/day.

The proportions of calcium excreted in the urine and feces can be illustrated further by the work of Deitrick *et al.* (1948) who studied the effect of immobilization on a few young men. The average daily calcium intake during the last 3–4 weeks of a 6–8-week control period ranged from 0.85 to 0.92 gm in the 4 subjects. The total daily calcium excretion ranged from 0.72 to 0.84 gm, with the urinary calcium constituting 7–25% of the total.

As will be noted in greater detail later, the concentration of calcium in normal serum ranges from about 9 to 11 mg per 100 ml. The serum calcium exists in three forms, as ionic calcium, as calcium bound to protein, and as calcium bound to organic ions such as citrate. The ionic and organic-ion bound calcium constitutes about 50% of the total serum calcium, and it is these forms that are filtered through the glomeruli. It is estimated that in adults 93–99.5% of this filtered calcium is reabsorbed through the tubules, and the remainder constitutes the calcium found in the urine. As we have seen, this ranges from about 140 mg/day at low intakes of calcium to about 250 mg/day at high intakes. Only about 20% of this urinary calcium is in the ionic form and the rest is covalently bound with organic ions such as citrate, or electrostatically bound with multivalent anions such as sulfate (McLean and Urist, 1968). Calcium is also excreted through the skin at an average rate of 50 mg/day (Dolphin and Eve, 1963).

B. Phosphorus

Phosphorus is ingested as various inorganic and organic phosphate compounds. Studies of the overall phosphorus metabolism in normal persons (Robertson, 1941; Ackermann and Toro, 1953; Bogdonoff *et al.*, 1953) lead to a regression equation:

$$Y = 4.0 + 0.74X$$

where Y is the total excretion and X the intake, both expressed as milligrams of phosphorus per kilogram per day. The point at which phos-

phorus excretion was equal to intake or, in other words, the point of zero balance, was 15.4 ± 0.5 mg/kg/day, or about 1.09 gm/day for a 70-kg adult male. The value is in the higher portion of the range, 0.88 ± 0.17 gm, given by Sherman (1941).

Although there is considerable variation in the proportions of urinary and fecal phosphorus, in general approximately 50–70% of the total excreted phosphorus appears in the urine. For example, in the 4 normal young adults studied by Deitrick *et al.* (1948), where the average dietary intake of phosphorus ranged from 1.49 to 1.66 gm/day, the urinary excretions constituted 52, 62, 68, and 68% of the total phosphorus excreted. In their extensive series of studies on growth in childhood, Macy (1942) found that, on the average, 14% of ingested phosphorus was retained, 55% was excreted in the urine, and 31% was excreted in the feces. Of the total excreted phosphorus, an average of about 65% appeared in the urine and about 35% in the feces. It may be seen that, whereas most of excreted calcium appears in the feces, the reverse is true of phosphorus.

Although about 80% of the body's phosphorus is present in the skeleton, a considerable proportion, or about 10–15% of the body's store, is in the muscle. Since the calcium–phosphorus ratio in bone is equal to 2.23, and the nitrogen–phosphorus ratio in muscle is about 14.7, it has been possible to determine by appropriate calculations from nitrogen, calcium, and phosphorus metabolic balances the extent to which the bone and muscle may alter their chemical composition on a stated regimen. This method has been employed by Reifenstein *et al.* (1945).

The clearance of a substance such as inulin or mannitol whose concentration in plasma is identical with that in the glomerular filtrate and which is neither reabsorbed nor secreted by the tubular epithelium represents the rate of glomerular filtration. In the human male, this is about 125 ml/minute per 1.73 m^2 of surface area. Since the clearance of phosphate is less than inulin clearance, it follows that phosphate is reabsorbed, probably in the proximal tubules (Vaughan, 1970). The parameter, T_m, expresses the maximum ability of the kidney tubules either to reabsorb or secrete a given material. We shall later consider the extent to which the T_m for phosphate may be altered in parathyroid disease.

C. Magnesium

Approximately 50% of the body's magnesium is in the skeleton. The daily magnesium requirement, determined from balance studies on a series of 11 normal adult females and 7 normal males, is approximately 0.25–0.30

mEq/kg body weight (Jones *et al.*, 1967), or approximately 250 mg in a 70-kg individual.

Animal studies indicate that magnesium is absorbed mainly in the small intestine. The time course of appearance of orally administered labeled ^{28}Mg in plasma indicates a similar mechanism in man. However, absorption may occur through the colon as evidenced by the development of hypermagnesemia after rectal enemas (Wacker and Parisi, 1968). In their study of the amount of magnesium intake required to keep adult men in balance, Jones *et al.* (1967) found that 32–55% of the total magnesium excreted was present in the urine. Employing radioactively labeled, orally administered ^{28}Mg, Graham *et al.* (1960) found that, in a series of 10 patients and 3 normal individuals, the absorption averaged 76% on a dietary intake of 1.9 mEq of magnesium per day, 44% on a higher intake of 20 mEq/day, and 24% on a very high intake of 47 mEq/day. Magnesium is excreted by glomerular filtration and is almost completely reabsorbed in the tubules (Vaughan, 1970).

D. Concentrations of Serum Calcium, Phosphorus, and Magnesium in the Normal Individual

The concentrations of calcium and inorganic phosphate in the serum may serve as valuable indicators of altered calcium and phosphorus metabolism in neoplastic diseases of the bone. The determinations of the concentrations of these components in normal individuals have been the subject of many investigations for over 50 years. These various studies have been previously reviewed (Bodansky and Bodansky, 1952), and it is beyond the scope of this volume to discuss the details of the techniques which have been used in these determinations or, except for several instances, to evaluate the results statistically. Suffice it to note that mean values for serum calcium have ranged from 9.1 mg per 100 ml, obtained by Snyder and Katzenelbogen (1942) in a series of 12 adults, to that of 10.6 mg per 100 ml, obtained by Mull and Bill (1933) in a series of 207 women. In considering deviations of serum calcium from the normal, it is preferable to use as a base the normal range obtained by the particular investigator in his laboratory.

The mean values from various studies for the concentration of serum inorganic phosphate, expressed as phosphorus, range from 3.1 mg per 100 ml to 3.8 mg per 100 ml in adults and are about 1.0–1.5 mg per 100 ml higher in infants and children (Bodansky and Bodansky, 1952). In 1965, Alcock *et al.* summarized the results of 18 separate studies on the concentration of magnesium in human plasma or serum that had been reported during the period 1947 to 1963

and that had been based on a variety of methods such as phosphate precipitation, photometry with titan yellow, fluorimetry, flame emission, and atomic absorption. These studies represented a total of over 900 determinations. The mean values ranged from 1.57 to 2.05 mEq/liter, with the values of these means being approximately 1.7 mEq/liter.

E. Dynamic Aspects of Calcium Metabolism

It is apparent from the preceding discussion that calcium, phosphorus, and magnesium are subjected to a continuous, dynamic passage from the food, through the intestine to the circulation, extracellular fluid, the soft tissues, and the bone. Simultaneously, calcium from the circulation is being excreted through the intestine and the kidneys. Various models have been proposed to depict these dynamic aspects. That of Dolphin and Eve (1963) is shown in Fig. 10-1.

We may briefly consider the concepts involved in acquiring the data upon which Fig. 10-1 is based. When radioactive isotopes of calcium became available, more specific and quantitative methods of evaluating certain parameters of the dynamics of calcium metabolism began to be introduced (Bauer *et al.*, 1957; Bronner and Harris, 1956–1957; Heaney and Wheadon, 1958; Lafferty and Pearson, 1963). Following the injection of labeled ^{47}Ca, a plot of the logarithm of the specific activity of the serum against time yields a triphasic curve (Bauer *et al.*, 1957; Lafferty and Pearson, 1963). The first phase is curvilinear, lasts about 2–3 days, and reflects the mixing of the isotope in the extracellular fluid and the exchangeable calcium space. The logarithm of the

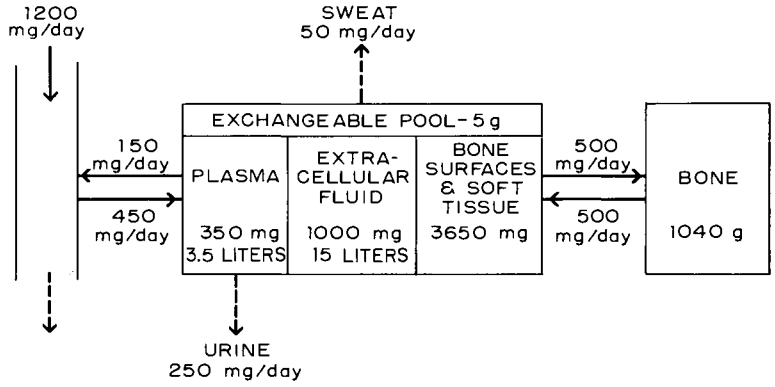


Fig. 10-1 Compartment model for calcium metabolism in human adults. From Dolphin and Eve (1963). Reproduced by permission of The Institute of Physics.

specific activity then decreases in a linear fashion with a slope, designable as k, and represents the fractional removal rate of calcium. At a time, usually 4–7 days after the injection of the radioactive calcium, a break in the slope occurs, and the specific activity then begins to decline with a different slope, k'.

The exchangeable calcium pool, E, can be calculated from the second phase of the curve by means of the following equation (Lafferty and Pearson, 1963; Bauer *et al.*, 1957):

$$100\% = ES_b(t) + kE\int_0^t S_b(t)\,dt \tag{10-1}$$

where 100% represents the dose of ^{47}Ca given, E is the exchangeable calcium pool and $S_b(t)$ is the serum specific activity of ^{47}Ca at the time, t, expressed as a percentage of dose per gram of serum calcium. Equation (10-1) can be expressed explicitly in terms of E as follows:

$$E = \frac{100\%}{S_b(t) + k\int_0^t S_b(t)\,dt} \tag{10-2}$$

Since the specific activity of the calcium excreted by the gut and kidneys each day is the same as the serum specific activity, the percentage of excretion from 0 to t may be expressed as follows:

$$\%\ \text{Excretion} = U\int_0^t S_b(t)\,dt \tag{10-3}$$

where U represents the grams of calcium excreted in both urine and stool and, as before, $S_b(t)$ represents the serum specific activity of ^{47}Ca at the time, t.

The specific activity of the serum, $S_b(t)$, can be determined experimentally. The term, $\int_0^t S_b(t)\,dt$, represents the area covered by the blood serum activity during the interval 0 to t days, and can be readily calculated from the semilogarithmic plot. The value of E, the exchangeable calcium space, can thus be calculated from Eq. (10-2). The value for U can be obtained from Eq. (10-3).

Since k, E, and U can thus be solved for, the accretion, or "bone formation" rate, A, in grams per day can be determined readily from Bauer's equation (Bauer *et al.*, 1957):

$$A = kE - U \tag{10-4}$$

where, as noted, E is the readily exchangeable calcium and U is the sum of urinary and fecal excretions. The amount of calcium present in extraosseous tissues is only about 1% of that in the body and varies only slightly in a given patient. Accordingly, changes in calcium balance chiefly reflect

the balance between bone resorption and bone formation. The total skeletal resorption, R, may be calculated as follows:

$$R = A - \text{calcium balance} \qquad (10\text{-}5)$$

In order to compare patients with different weights, the accretion and absorption rates are expressed per kilogram of body weight. Lafferty and Pearson (1963) have taken the degree of obesity into account, and have expressed these rates per kilogram of ideal body weight based on height.

Table 10-2 shows rounded values for several parameters of calcium dynamics in the adult 70-kg male. These may be further illustrated by reference to several specific studies. Thus, in the series of 10 "control" patients who had various primary malignancies without evidence of skeletal involvement, the values for the exchangeable calcium pool or fraction (E) averaged 5.28 gm, or 77.9 mg/kg (Bauer *et al.*, 1957). Other mean values for the exchangeable fraction (E) obtainable from the literature were 5.86 gm (Lafferty and Pearson, 1963) and 4.94 gm (Rinsler *et al.*, 1965). The following mean values for the accretion (A) or bone formation rate (BFR) may be culled from the literature: 492 mg/day (Bauer *et al.*, 1957), 527 mg/day (Lafferty and Pearson, 1963), and 598 mg/day (Rinsler *et al.*, 1965).

The rate of bone resorption (BRR) represents the rate at which bone is resorbed or lysed and enters the exchangeable calcium pool. We have already noted that it is equal to the accretion (bone formation) rate *minus* the balance. For example, if an individual has an accretion rate

TABLE 10-2

Values of Parameters for Dynamic Aspects of Calcium Metabolism[a]

Parameter	Values
Daily dietary intake of calcium (mg/day)	1200
Daily absorption of calcium (mg/day)	450
Total calcium in plasma (mg)	350
Diffusible calcium in bone (mg)	250
Exchangeable calcium pool (E) (gm)	5
Accretion rate in bone (A, BFR)[b] (mg/day)	500
Calcium content of skeleton (gm)	1040

[a] Based on data of Dolphin and Eve (1963). Reproduced by permission of the Institute of Physics, Great Britain.

[b] BFR (bone formation rate) is a synonym for A.

of 538 mg/day and a balance of +23 mg/day, then the resorption rate would be 515 mg/day. When the mean values for the bone resorption rates of groups are compared, it is preferable to express values for the balances and accretion rates per kilogram of body weight and obtain the values for bone resorption rates in similar terms. To illustrate, Lafferty and Pearson (1963) obtained the following values for the bone resorption rates in 3 normal persons: 8.4, 8.9, and 7.1 mg/kg per 24 hours.

Several other parameters are relevant in the study of calcium and phosphorus metabolism. Following the intravenous injection of ^{47}Ca, the quantity of endogenous calcium excreted in the feces can be calculated with the aid of the following equation (Bronner and Harris, 1956–1957):

$$En_f = \frac{A_f}{SA_s}$$

where A_f is the total amount of ^{47}Ca recovered in the feces by time, t, after injection; SA_s, is the mean specific activity of the serum between time 0 and time, t; and En_f is the total endogenous calcium excreted in the feces by time, t. The subtraction of the endogenous calcium from the fecal calcium yields the amount of unabsorbed dietary calcium. The latter subtracted from the dietary calcium intake is equal to the amount of calcium absorbed. The normal values for endogenous fecal calcium, expressed as a fraction of the total fecal calcium, have been found, in one study, to average 7.7% (Bronner and Harris, 1956–1957) and 24% in another (Lafferty and Pearson, 1963).

The renal clearances of phosphorus and calcium also play an important role in evaluating calcium and phosphorus metabolism in diseases of the bone. The reader will recall that the term "clearance" denotes the removal of a substance from the blood through the kidneys and is defined as the least volume of blood or plasma which contains all of a particular substance excreted in the urine in 1 minute. The clearance can be calculated from the equation:

$$C = \frac{U \times V}{P}$$

where U is equal to the concentration of the substance in the urine, V is the urine volume in milliliters per minute, P is the concentration of the substance in the plasma, and C is equal to the clearance in milliliters per minute. Substances whose concentration in the plasma is identical with that in the glomerular filtrate and which are neither reabsorbed nor secreted by the tubular epithelium can serve as measures

of glomerular filtration. Inulin, mannitol, and creatinine fulfill these criteria, and all yield clearance values of about 125 ml/minute per 1.73 m^2 of surface area in human males and females, 20–40 years of age (Wesson, 1969). If the clearance of a substance is less than that of inulin or creatinine and if the substance is not bound to plasma protein, then it follows that some of it must have been reabsorbed by the tubules. The reabsorption or secretion of a substance may also be described by T_m, the transport maximum. This is the maximum ability of the kidneys either to reabsorb or secrete a given material. For example, the T_m value of glucose is 300 mg/minute. If the plasma concentration is raised to 400 mg per 100 ml, the filtered load would be 400 mg per 100 ml multiplied by the clearance rate of 125 ml/minute or 500 mg glucose per minute; of this, 300 mg/minute would be reabsorbed as indicated by the T_m value, and 200 mg/minute would be excreted in the urine.

The normal concentration of inorganic phosphate in the plasma of the human adult, expressed as phosphorus, averages about 3.6 mg or 0.119 mmoles per 100 ml. The serum phosphate has usually been considered freely diffusible, although more recent reports indicate that a small fraction, not more than 25%, may be bound (Wesson, 1969). The normal clearance of phosphate, C_P, at usual daily intakes of 900–1200 mg phosphorus averages about 15 ml/minute. Since the glomerular filtration rate is about 125 ml/minute, then the fraction of phosphorus reabsorbed by the tubular (% TRP) would be $110/125 \times 100$, or 88%. This general formulation agrees well with specific calculations on 2 normal individuals and 1 diabetic patient made by Lafferty and Pearson (1963).

Calculation of the normal calcium clearance is somewhat more complex since approximately 50% of the serum calcium is bound to protein and not filtrable. This can be taken into account in calculations. At the usual dietary intake of about 700–800 mg/day the calcium clearance (C_{Ca}) in normal individuals is about 1.0 ml/minute. The fraction of tubular reabsorbed calcium (TRCa) amounts to over 99%.

F. Factors Involved in Regulation of Calcium and Phosphorus

The regulation of calcium and phosphorus metabolism is subject to a variety of influences; derangements of this regulation are usually, though not inevitably, manifested in departure from the normal levels of calcium and phosphorus in the serum and in the skeleton. The mechanisms which are involved in this homeostasis are manifold and include chiefly: (a) the character of the dietary intake; (b) intestinal absorption;

(c) renal function; (d) parathyroid hormone; (e) calcitonin; (f) vitamin D; (g) cyclic AMP; and (h) to a lesser extent, vitamin A, vitamin C, growth hormone, thyroid hormone, adrenocortical hormone, and estrogens (Vaughan, 1970; Barnicot and Datta, 1972; Bourne, 1972). Citrate and the pH level also play important roles. There is an interplay between some of these factors which have been studied in great detail, many at the molecular level. It is beyond the scope of this volume to review this literature, except to mention relevant features in connection with our consideration of the various neoplastic diseases involving bone.

Attempts to incorporate the factors which we have mentioned into a generalized mechanism of calcium homeostasis have been made by several investigators (Vaughan, 1970). Rasmussen (1971) has recently reviewed the literature in this connection. Arriving at conclusions somewhat different from those previously held and employing data obtained from isolated tissue culture cells, isolated renal tubules and bone marrow, and isolated thymocytes, he has offered a theoretical model depicting the mechanism by which parathyroid hormone (PTH), calcitonin and phosphate regulate the concentration of Ca^{2+} intracellulary in the cytosol (Fig. 10-2).

It may be seen that Ca^{2+} enters the cell by a passive leak and is pumped back out by an active process. Within the cell, the calcium exists free in the cytosol or may be accumulated by an active process into subcellular organelles. Within these organelles, the calcium exists chiefly in a bound form from which, however, it can be readily mobilized back into the cytosol.

Two effects ensue when PTH interacts with the cell membrane. The passive entry of calcium into the cell is increased, and the membrane-bound adenyl cyclase is activated. This activation and the subsequent increase in intracellular cyclic 3′,5′-AMP causes a shift of intracellular calcium into the cytosol. Calcitonin acts by stimulating exit of calcium from the cell and, therefore, tends to counteract the actions of both PTH and cyclic AMP. As may also be seen from Fig. 10-2, phosphate tends to increase the entry of calcium into the cell and into bound intracellular calcium.

The relationships that we have been discussing apply chiefly to the cells of extraskeletal tissues. In the kidney and intestine, the cells that are responsive to PTH are epithelial cells. In contrast, the origin of cells of bone is mesodermal and no epithelial membrane covers the surface of the bone. Whereas it was formerly held that mineral exchange between blood and bone was rather direct and did not involve cellular exchange systems, more recent studies indicate a greater metabolic role for the various bone cells (Cameron *et al.*, 1967; Baud, 1968; Rasmussen,

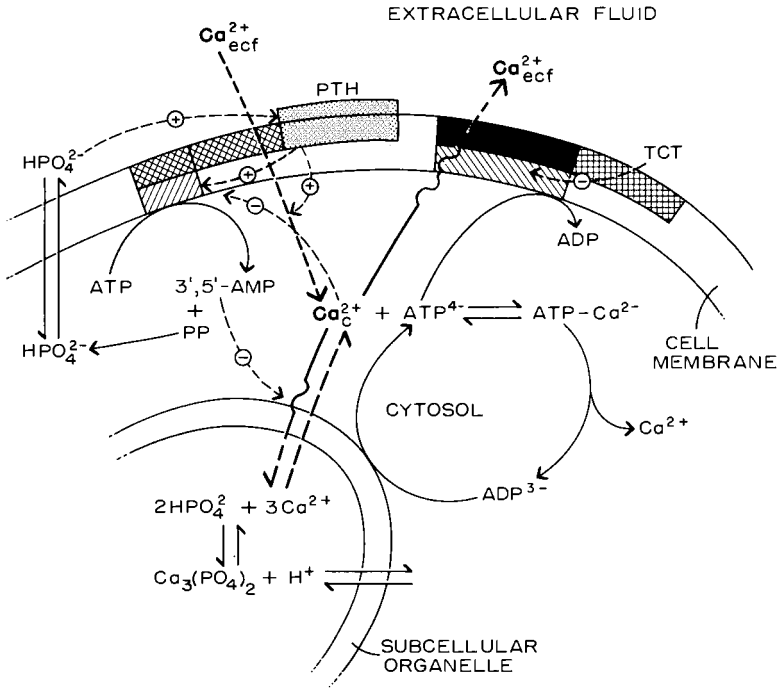


Fig. 10-2 Model depicting mechanism by which PTH, calcitonin, and phosphate regulate $(Ca^{2+})_{cytosol}$. See text for further details. From Rasmussen (1971). Reproduced by permission of Dun and Donnelley Publishing Corporation, New York.

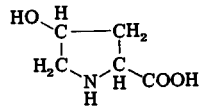
1971). There are three different areas of bone and related bone cells which are involved in calcium homeostasis. These are (a) the active and resting osteoblasts which cover most of the bone surfaces and which form an effective membrane separating the bone extracellular fluid from the general extracellular fluid space, (b) the osteocytes which form an extensive syncitium, and (c) the osteoclasts which appear at sites of surface resorption.

As a result of his review of many experiments in the literature on the physiological effects of infusing PTH, calcitonin, phosphate, and pyrophosphate in thyroparathyroidectomized animals, Rasmussen (1971) has drawn similarities between *in vivo* effects and the cellular model. The initial effect of infusing PTH is to increase the uptake of calcium into bone cells as well as extraskeletal cells. This results in an initial, transitory hypocalcemia. The increased concentration of calcium in the bone cells is a part of a complex of intracellular messages which stimulates the process of bone resorption and leads to hypercalcemia. When the infusion of PTH is stopped, plasma calcium continues

to increase because calcium, which had accumulated in the cells, returns to the extracellular fluid and serum. Associated with these effects are alterations in urinary calcium, phosphorus, and hydroxyproline.

G. Metabolism of Hydroxyproline

We pointed out earlier in this chapter that collagen is the only mammalian protein that contains appreciable amounts of hydroxyproline, and that approximately 57% of the body's collagen is in the bone. Accordingly, measurement of this amino acid in urine and blood can give us some measure of the metabolism of bone in health and disease. The structure of this amino acid, as obtained from collagen, is



and is more formally designated as 4-hydroxypyrrolidine-2-carboxylic acid or 4-hydroxyproline. In 1955, Westall first noted the presence of hydroxyproline in normal urine and found that this amino acid was present, not in the free form, but practically completely in peptide combination. At least 12 different peptides of hydroxyproline have been isolated from human urine. About 90–95% of these peptides can be accounted for: about 50–60% as prolinyl hydroxyproline and 15% each of the 2 tripeptides, glutamylhydroxyprolylglutamic acid and glycylprolyl hydroxyproline (Kibrick *et al.*, 1962; Meilman *et al.*, 1963; LeRoy, 1967).

Several types of procedures have been developed for the assay of hydroxyproline. These include colorimetric, chromatographic, isotope dilution, and radioactive methods (LeRoy, 1967). Hydrolysis of the peptides is first necessary to free hydroxyproline in each of these procedures. For example, Prockop and Udenfriend (1960) hydrolyzed the urine peptides by autoclaving the urine at 124°C in a sealed tube with concentrated HCl for 3 hours. Tissue homogenates or proteins were autoclaved for 24 hours.

In 1967, LeRoy reviewed the available values for urinary excretion of hydroxyproline in normal adults. These are shown in Table 10-3. In most of these reports, the sex was not stated, and the average value was 28 mg per 24 hours. There was no significant difference between the values for females and males. The average values, when expressed as milligrams per square meter of surface area per 24 hours, were naturally lower, namely, 17 mg for males and 16 mg for females, but, again not significantly different from each other. LeRoy (1967) also summarized the values for children and adolescents.

TABLE 10-3

Urinary Hydroxyproline in Normal Adults[a]

Subjects	No. of subjects	Urinary hydroxyproline (mg/24 hr)	
		Average	Range
Adults (mixed)	524	28	9–70
Males	49	33	17–59
Females	37	27	11–50

[a] From LeRoy (1967). Reproduced by permission of Academic Press.

Hydroxyproline is present in human plasma in three forms: free, peptide, and protein-bound. Their average concentrations are 1.5, 0.6, and 8.0 μg/ml, respectively (LeRoy, 1967). The values obtained by Laitinen *et al.* (1966) for free serum hydroxyproline were essentially the same: averages of 1.05 μg/ml in 38 subjects, 18–20 years of age; 1.07 μg/ml in 34 subjects, 22–40 years of age; and 1.01 μg/ml in 23 subjects, 41–76 years of age. The values ranged from 0.63 to 1.52 μg/ml. There was no difference between the sexes and, as can be seen from the above values, no variation with age. Approximately 80% of the plasma hydroxyproline is protein-bound. This material is nondialyzable, precipitable with the usual protein precipitants, and, as determined by Sephadex G-200 gel filtration, has a molecular weight of at least 200,000. LeRoy (1967) observed that parallel serum values were 10–20% lower than those of the plasma and that this difference may have resulted from variable losses in the methods applied to serum.

IV. Parathyroid Adenomas and Carcinoma

A. Introduction

On the basis of the considerations we have presented (Section III,E), it may be expected that adenomas or carcinomas of the parathyroid gland would result in excessive production of PTH and, hence, affect the metabolism of calcium and phosphorus of bone. Indeed, in the early era of the study of this condition, these effects were paramount, and the skeleton often presented evidence of bent, broadened, extremely porotic,

and fractured bones. This was the syndrome known as "osteitis fibrosa cystica generalisata."

However, during the past 40 years, an impressive decrease has occurred in the fraction of patients exhibiting far advanced disease of the bone and its accompanying metabolic effects and, instead, changes in the renal system have been more pronounced. This reversal was already evident in the series of 64 cases from the Massachusetts General Hospital reported by Albright and Reifenstein (1948). In this series, the incidence of bone without renal involvement was only 17%, as compared with an incidence of 59% in Norris' (1947) series. The converse situation held for the incidence of renal disease without bone involvement: 44% in the series of Albright and Reifenstein (1948) and only 5% in that of Norris (1947). More recent series (Cope, 1960; Black, 1961; Lloyd, 1968) show a continuation of this trend, with bone changes alone being present in 10–25% of cases, whereas renal changes alone being observed in 50–65% of cases (Table 10-4). The exact basis for this reversal has not been elucidated. Earlier diagnosis and treatment, change in dietary level of calcium intake, and increased awareness of renal involvement have been submitted as possible explanations.

The parathyroid glands are usually 4 in number and are found on the posterior aspect of the thyroid gland. The total weight of the 4 glands in the adult is about 120 mg, and each of the glands is about 3–6 mm in length, 2–4 mm in width, and 0.5–2 mm in thickness. The normal parathyroid gland consists mainly of fat cells, chief cells, and the oxyphil cells. The latter appear at puberty and show increase with age (Roth,

TABLE 10-4

Incidence of Bone and Renal Disease in Primary Hyperparathyroidism

	Norris' series (1947)		Albright and Reifenstein's series (1948)		Lloyd's series (1968)	
Disease	No.	Percentage	No.	Percentage	No.	Percentage
Bone but not renal	191	59	11	17	28	20
Bone and renal	101	3.1	24	38	10	7
Renal but not bone	17	5	28	44	81	58
Neither bone nor renal	5	1.5	1	1	19	13
No record	8	2.5	0	0	0	0
	322		64		138	

1971). The chief cell is about 6–8 μm in diameter with a rather large nucleus, 4–5 μm in diameter. The cytoplasm usually has a somewhat vacuolated appearance so that the cell is spoken of as a "clear" cell. Some of the chief cells are larger, about 8–10 μm, and the cytoplasm is more vacuolated; such cells are spoken of as "water clear" or *wasserhelle* cells.

The reported cases of primary hyperparathyroidism probably number in the thousands, and it has been estimated that 1 case occurs per 10,000 persons per year (Aurbach and Potts, 1964). Primary hyperparathyroidism was originally defined as that condition in which the parathyroid glands elaborate an excessive amount of their characteristic hormone. The development of a specific radioimmunoassay by Berson and his associates (1963) provided a means of actually confirming the presence of excessive secretion of PTH in the circulation. Potts and Deftos (1974) reported that the concentration of hormone in the serum of most patients with primary hyperparathyroidism was in the range of 2–10 ng/ml, as contrasted with concentrations less than 0.6 ng/ml in normal persons.

Parathyroid hormone may be secreted by a single or multiple adenoma, by hypertrophy of the parathyroid tissue, or by a carcinoma of one or more of the 4 glands. Aurbach and Potts (1964) estimated the frequency of the various pathological types as follows: single adenoma, 80–90%; double adenoma, 5%; *wasserhelle* hyperplasia, 5–7%; and chief cell hyperplasia, 1–4%. The incidence of carcinoma will be discussed later in this chapter (Section IV,D).

B. Effect on Calcium and Phosphorus Metabolism

1. General Features

The usual biochemical findings obtained in early studies of hyperparathyroidism were increased urinary excretion of phosphate and calcium, elevated concentration of serum calcium, decreased level of serum phosphorus, and elevated serum alkaline phosphatase activity. These features are well illustrated by a case of osteitis fibrosa generalisata and nephrocalcinosis studied by Albright and Reifenstein (1948).

The patient was a male, 33 years of age, who had complained of pain in his legs, weakness, and loss of weight. The diagnosis of hyperparathyroidism was made when roentgenographic studies revealed fine calcium deposits in the kidney pyramids. No information about a skeletal survey was given. The biochemical findings were serum calcium, 17.0 mg per 100 ml; serum phosphorus, 3.0 mg per 100 ml; serum alkaline phosphatase, 22 Bodansky (B) units; and immediate preoperative excre-

tions of calcium and phosphorus, 0.54 gm per 24 hours and 0.75 gm, respectively. At operation, a single parathyroid tumor was removed. Within 1 to 2 days, the urinary calcium and phosphorus excretions dropped rapidly to subnormal levels, less than 0.03 gm/day. The serum calcium decreased from its highly elevated preoperative level of 17 mg per 100 ml to a normal level of 10 mg per 100 ml within 2 days, and in the next few days to a tetany-producing level of 7–8 mg per 100 ml. The serum phosphorus fell to a level of about 2.0 mg per 100 ml within 2 days, then began to rise. The serum phosphatase rose to about 30 B units in the course of about 10 days.

Bone biopsies revealed the basis for these changes. At the time of operation, the bone surfaces were about equally divided between bone-forming and bone-resorbing surfaces, with many osteoclasts on the bone-resorbing surfaces and many osteoblasts on the bone-forming surfaces. There was no increase in the width of the osteoid seams, indicating that the matrix which was being deposited by the osteoblasts was being immediately calcified. In the biopsy taken 8 days after the operation, the bone-resorbing surfaces had disappeared; all surfaces were covered with osteoblasts and the osteoid seams were wide. These findings indicated that healing was taking place and that calcium and phosphorus in the body fluids were being diverted to and being deposited in the bone.

As time progressed, the biochemical parameters began to revert to normal levels. The serum calcium was low for about 50 days, then began to climb to a level of 10 mg per 100 ml. The serum phosphatase activity began to decrease 12 days postoperatively, and reached, at about 100 days postoperatively, a stable, though slightly elevated, level of about 6 B units. The serum phosphorus began to climb 2 days postoperatively and reached a normal level of about 3.0 mg per 100 ml 16 days postoperatively, then increased toward elevated normals of about 5.0 mg per 100 ml during the next 40–50 days, when it began to decrease toward normal levels, attaining these at about 90 days postoperatively. In a bone biopsy taken at 119 days postoperatively, the findings had reverted almost completely to the normal state of affairs.

In the case noted above and in a number of other situations, there appeared to exist a reciprocal relationship between the concentration of calcium and phosphorus in the serum. These observations were the basis for Albright's original view that the product, Ca $\times$ P, in the serum was constant, a thesis that appeared to be supported by the physicochemical principles governing the precipitation of calcium phosphate. However, many exceptions to these observations have subsequently been noted and Albright's view is no longer held (Vaughan, 1970).

Baylor *et al.* (1950) reported that the intravenous infusion of calcium in man causes a decrease in phosphorus excretion, presumably by inhibiting endogenous secretion of PTH and thereby increasing the tubular reabsorption of phosphorus in the kidneys. The potentiality of this phenomenon as a diagnostic test has been explored by a number of investigators, and the results of Horwith *et al.* (1966) may be cited in illustration. In 11 normal subjects, the infusion of 500 ml of a 1% calcium gluconate over 4 hours led to a decrease in urinary phosphorus excretion ranging from 5 to 50% and averaging 32%. Nine of the 11 normal individuals, or 82%, had decreases greater than 20%. Of 38 patients with primary hyperparathyroidism, only 6, or 16%, had decreases greater than this level.

2. Serum Calcium and Phosphorus

The concentration of serum calcium is practically always, but not invariably, elevated in primary hyperparathyroidism. However, in those cases where a single determination is normal, the average of repeated determinations almost invariably tends to be high (W. P. L. Myers, personal communication, 1974). In view of the different values and ranges for serum calcium in normal individuals which, as we pointed out earlier in this chapter, have been obtained by different investigators, we may consider the normal value, for our present purposes, to lie between 9 and 11 mg per 100 ml.

Of 156 cases of hyperparathyroidism recorded in the literature and reviewed by Gutman *et al.* in 1936, the serum calcium level was determined in 114. In 109 cases, or 96%, the level was higher than 11 mg per 100 ml, and in 91 cases, the level was higher than 12 mg per 100 ml. In 35 cases studied by Albright and Reifenstein (1948), the concentrations ranged from 8.9 to 17.6 mg per 100 ml. Thirty-four, or 97%, had concentrations of serum calcium higher than 11 mg per 100 ml; 26% had concentrations between 12.1 and 13.0 mg per 100 ml; and 48% had concentrations higher than 13.0 mg per 100 ml.

Although patients with primary hyperparathyroidism usually present themselves with chronic symptoms referable to the urinary tract, skeleton or gastrointestinal tract, some patients may first be seen with acute and progressive symptoms attributable chiefly to markedly elevated concentrations of serum calcium. As in other types of hypercalcemia, these symptoms include muscular weakness, anorexia, lethargy, difficulty in swallowing, nausea, vomiting, constipation, thirst, polyuria, and nonspecific abdominal pain. Lemann and Donatelli (1964) collected 42 such cases from the literature and added 4 of their own. Of 38 cases in which the serum calcium was measured, 28, or 74%, had concentrations of 15

to 19.8 mg per 100 ml, and the remaining 26% had values 20 mg per 100 ml or higher. A value of 24 mg per 100 ml was noted in one of their own cases.

In spite of the high association between hypercalcemia and hyperparathyroidism, the older literature indicates the occasional occurrence of normal serum calcium levels (Gutman *et al.*, 1936; Albright and Reifenstein, 1948). More recently, a number of well-documented cases with this situation have been reported (Wills *et al.*, 1969). In most of these cases, the patients exhibited renal calculi as the major clinical feature of their parathyroid hyperfunction. However, Frame *et al.* (1970) recently reported the following case of osteitis fibrosa with normocalcemia.

The patient was a 62-year-old woman who had suffered several fractures and whose skeletal roentgenograms showed generalized bony rarefaction. The serum calcium levels were normal, namely, 10.0 and 9.4 on two occasions. The serum phosphorus levels were somewhat low, 2.1, 2.6, and 3.0, on three occasions, and the serum alkaline phosphatase levels were substantially elevated to 15 and 19 B units. After 9 years in a nursing home, the patient was admitted again because of a spontaneous fracture of her right femoral shaft. The following biochemical values were obtained: serum calcium, 8.3 and 9.2 mg per 100 ml, and serum phosphorus, 3.0 mg per 100 ml. The serum alkaline phosphatase activity was unusually high, namely, 73 and 80 B units, on two occasions. Roentgenograms showed the expected fractures, generalized bony rarefaction, but no cystic changes. The patient died shortly after admission and autopsy revealed a parathyroid adenoma, generalized osteitis fibrosa, but not renal calculi.

In repeated surveys of patients in a general diagnostic clinic for the occurrence of hypercalcemia, Boonstra and Jackson (1971) have noted an incidence of 50 cases of hyperparathyroidism in 50,330 individual patients seen over a 10-year period. These surveys revealed an increased incidence of hyperparathyroidism in several families. Indeed, familial hyperparathyroidism has been described by several groups of investigators. Cutler *et al.* (1964) reported the occurrence of 7 proved and 4 probable cases of the condition in a single kindred involving 55 individuals.

We have already indicated that the concentration of serum phosphorus is often decreased in hyperparathyroidism. However, the incidence of these decreases is not as high as the incidence of serum calcium elevations. On the basis of the normal values obtained by different investigators and presented earlier in this chapter, we may regard the normal range of serum phosphorus concentrations to lie between 2.5 and 4.5 mg per 100 ml. Gutman *et al.* (1936) found a level less than 2.5 mg per 100 ml in 35, or only 44%, of 79 cases in which the determination

was done. Other studies have shown a similiar incidence (Hellström and Wahlgren, 1944; Burk, 1948).

3. Excretion of Calcium and Phosphorus

We have already quoted the studies of Albright and Reifenstein (1948) to the effect that the excretions of calcium and phosphorus are increased in patients with hyperparathyroidism and are diminished after parathyroidectomy. More comprehensive studies confirming these effects are available in the recent literature. Of particular interest are the results of Transbøl *et al.* (1970). The daily urinary excretion of calcium ranged from 36 to 565 mg and averaged 301 mg in 49 patients with primary hyperparathyroidism. The dietary intake was generally in the range of 700–800 mg/day. If we consider 250 mg calcium per day as the upper limit of normal, 31 patients, or 63%, showed an abnormally higher excretion. Using a somewhat different criterion, Transbøl *et al.* (1970) stated that 57% of his hyperparathyroid patients showed a high excretion. A series of 19 patients with nonparathyroid hypercalcemia showed a more extensive hypercalciuria with a mean value of 537 mg/day, significantly higher than that of the parathyroid group.

Parathyroid hormone acts upon the renal tubules by decreasing the reabsorption of phosphate and increasing that of glucose and calcium. Transbøl *et al.* (1970) found that the former could not be applied for diagnostic purposes. Thus, they observed that the ratio of urinary phosphorus excretion to creatinine clearance (C_{Cr}) (phosphate excretion index) was elevated in 26 of 41 patients with hyperparathyroidism at C_{Cr} of less than 50 ml/minute and in all of 7 patients with C_{Cr} higher than this. These incidences did not differ significantly from those in patients with nonparathyroid hypercalcemia in which the phosphate excretion index was increased in 4 of 7 patients at C_{Cr} values less than 50 ml/minute and in all of 12 patients at C_{Cr} values higher than this.

The tubular reabsorption of calcium (TR_{Ca}%) was found to be increased in 49 out of a series of 56 patients with hyperparathyroidism, as compared with an increase in only 2 of 19 patients with nonparathyroid hypercalcemia. The mean values for the hyperparathyroid group was 97.2%, significantly higher than the mean value, 90.4% for nonparathyroid hypercalcemia patients.

4. Serum Alkaline Phosphatase Activity

Alterations of serum alkaline phosphatase activity in human cancer were discussed generally in Chapter 3. The tabulation on hyperparathy-

roidism listed there is based on the data of Gutman and his associates (1936) who found that the serum alkaline phosphatase activity was increased in every one of 28 cases in which the determination was done. Twenty-nine percent of the patients had moderate elevations of serum alkaline phosphatase activity, namely, from 4 to 12 B units, as compared with a normal range of 1.4–3.8 B units; the remaining 71% had values above 12 B units. There are other early studies as, for example, that of Hellström and Wahlgren (1944) who showed that the serum alkaline phosphatase was elevated in practically every patient with hyperparathyroidism. On the other hand, recent studies tend to show a much lower incidence of hyperphosphatasemia. For example, Transbøl *et al.* (1970) reported that only 45% of a series of 55 patients with hyperparathyroidism had elevated serum alkaline phosphatase activities. Albright and Reifenstein (1948) had noted that, if a patient has marked bone disease as shown by x-ray and the serum alkaline phosphatase activity is not elevated, hyperparathyroidism may be ruled out as a cause of the bone disease.

Perhaps the lesser incidence of hyperphosphatasemia in hyperparathyroidism in more recent studies may be explained by the increasing fraction of patients with renal lesions. As a result of a detailed study of 138 cases with hyperparathyroidism, Lloyd (1968) has submitted the thesis that there are 2 main types of parathyroid tumor. One grows rapidly, is highly active, and causes overt bone disease. The second type grows slowly, has low activity, and leads to the formation of kidney stones. The mean value for plasma alkaline phosphatase activity for 38 patients with the first type, that is, in those with skeletal manifestations of hyperparathyroidism, was 40.1 ± 23.2 (SD) King-Armstrong (KA) units, as contrasted with a value of 8.11 ± 3.0 (SD) KA units for 80 patients with kidney stones and no bone disease (Table 10-5). The normal value is 7.8 ± 2.2 (SD) KA units. It would, therefore, appear that serum alkaline phosphatase activity tends to be normal in the slow growing, low activity type of parathyroid tumor without bone involvement. Table 10-5 also shows statistical evaluation of several other parameters of the proposed 2 types of hyperparathyroidism.

5. Skeletal Calcium and Phosphorus

The excessive urinary excretion of calcium and phosphorus in hyperparathyroidism, particularly in those cases which would appear to fall into type 1, should involve a loss of calcium and phosphorus from the skeleton. As we have already noted, Albright and Reifenstein (1948) analyzed a number of such cases. However, direct methods for determin-

TABLE 10-5

Comparison of Mean Values of Parameters in 2 Types of Hyperparathyroidism[a]

Parameter	Type 1 Patients with overt bone disease, without kidney stones		Type 2 Patients with kidney stones and without overt bone disease	
	No.	Mean ± standard deviation	No.	Mean ± standard deviation
Tumor weight-gm	29	5.90 ± 5.9	57	1.05 ± 0.9[b]
Plasma calcium (mg per 100 ml)	38	13.34 ± 2.4	81	11.74 ± 1.1[b]
Duration of symptoms (years)	34	3.56 ± 4.8	77	6.88 ± 7.2[b]
Plasma alkaline phosphatase (KA units)	38	40.13 ± 23.2	80	8.11 ± 3.0[c]

[a] Based on data of Lloyd (1968). Reproduced by permission of Williams & Wilkins.

[b] Significantly different from values for type 1.

[c] Since the value of plasma alkaline phosphatase was the criterion of classification, the significance of difference between the mean values for type 1 and 2 was not estimated. The mean normal value in adults by the King-Armstrong method is 7.8 ± 2.2 (SD) units (Chapter 3).

ing changes in the skeletal composition are limited. Radiographic techniques are not sufficiently sensitive. Analyses of biopsy samples of bones by histological, chemical, and microradiographic techniques, though useful, do not naturally apply to changes for the total skeletal mass (Riggs *et al.*, 1965).

Earlier in this chapter (Section III,E), we considered certain dynamic aspects of calcium metabolism. Lafferty and Pearson (1963) found the bone resorption rate (BRR) and bone formation rate (BFR) in one cases of primary hyperparathyroidism lower than the range in 3 controls, but a second case showed values for these parameters that were normal or slightly elevated.

A more sensitive and direct measure of the total skeletal mass appears to be the recently developed method of total body neutron activation (Chamberlain *et al.*, 1968; Cohn *et al.*, 1973). Of a series of 8 patients with untreated primary hyperparathyroidism, the total body calcium was within the normal range in 2 patients, and ranged from 81.6 to 88.2% of the normal value in the remaining 6. Thus, these results indicated that demineralization of the skeleton occurred in most hyperparathyroid patients. Those who showed no demineralization might possibly have

fallen into the type 2 group which we have previously discussed. It is of interest that 3 cases of idiopathic hypoparathyroidism had total body calcium contents ranging from 110 to 172% of the normal.

C. Other Metabolic Effects

1. Magnesium Metabolism

Substantial evidence, both experimental and clinical, suggests that hyperparathyroidism affects magnesium metabolism. Agna and Goldsmith (1958) reported 3 cases who before parathyroidectomy exhibited hypomagnesia and neuromuscular irritability. The preoperative concentrations of serum magnesium were 1.0, 0.2, and 0.8 mg per 100 ml, as contrasted with a normal value of 2.13 ± 0.13 mg per 100 ml obtained in the same laboratory. The symptoms were not corrected by parathyroidectomy in 2 of the patients, but subsided in association with magnesium sulfate therapy, and the serum magnesium levels in these 2 patients ultimately returned to normal levels. In 6 cases of primary hyperparathyroidism and in 1 case of pluriglandular syndrome, the preoperative serum magnesium levels were close to the normal range except in 1 patient who had been vomiting excessively (Hanna *et al.*, 1961). The preoperative magnesium balances were negative in every case. Parathyroidectomy resulted in a lowering of the plasma magnesium level and in the production of positive magnesium balances with a decrease in urinary magnesium excretion. These findings indicated a retention of magnesium by the tissues, most probably by the bone.

There appears to be a discrepancy with regard to pre- and postoperative serum magenisum levels between Agna and Goldsmith (1958) and Hanna *et al.* (1961). However, these investigators did not differentiate among their patients with respect to renal or skeletal involvement. In a detailed study of the magnesium metabolism in 16 patients with primary hyperparathyroidism, Heaton and Pyrah (1963) found that the serum magnesium concentrations were always within normal limits, the mean value being 2.02 mg per 100 ml, practically the same as their value of 2.00 ± 0.15 for a series of 176 normal individuals. Thirteen of their patients were of the renal stone-forming type, and parathyroidectomy in these had no effect on the serum magnesium level. However, in 3 patients who also had skeletal decalcification as determined radiologically, the serum magnesium concentration began to fall to subnormal levels on the second postoperative day and reached a minimum about

the sixth day. Magnesium balances were determined in 9 patients with hyperparathyroidism. Excluding 1 patient with skeletal decalcification, Heaton and Pyrah (1963) found no consistent metabolic disturbance. Preoperatively, there were small positive balances in 3 patients, negative balances in 2 patients, and 4 patients were in an equilibrium state. There were no consistent changes in one direction or the other after parathyroidectomy.

2. Collagen Metabolism and Hydroxyproline Excretion

It will be recalled (Section III,G) that the average daily urinary excretion of hydroxyproline in normal adults ranges from 9 to 70 mg and averages 28 mg. Bonadonna *et al.* (1966) found essentially the same value, 24.8 ± 7.0 (SD) mg, in a group of normal subjects. In cancer, the mean excretions per 24 hours and their standard deviations, expressed as milligrams, were, in 18 patients with cancer metastatic to bones and hypercalcemia, 82 ± 44; in 26 patients with cancer metastatic to the bone without hypercalcemia, 86 ± 42; and in 19 patients with widespread tumors without bone lesions or hypercalcemia, 40 ± 23. In summarizing the literature, LeRoy (1967) found that, in 22 patients with primary hyperparathyroidism, the excretions were higher than normal, ranging from 12 to 477 mg and averaging 99 mg/day. However, these elevations represent increased metabolism of collagen and are observed in other diseases of the bone and connective tissue. Thus, the urinary excretions, expressed as milligrams per day, showed, in Paget's disease, a range of 20–998 and an average of 202; in metastatic bone disease, a range of 10–340 and an average of 79; and in Marfan's syndrome, a range of 44–416 and an average of 161.

Nonetheless, there appears to be a more specific relationship between parathyroid function and collagen metabolism. The injection of parathyroid extract in normal human subjects and in patients with hypoparathyroidism was shown to increase the urinary excretion of hydroxyproline. An infusion of calcium gluconate decreases the urinary excretion of hydroxyproline in normal subjects, presumably by suppressing the release of PTH (Keiser *et al.*, 1964). Removal of parathyroid adenomas causes definite and sometimes marked decreases in the total and free urinary hydroxyproline and free serum hydroxyproline (Laitinen *et al.*, 1966; Ney *et al.*, 1966). This is illustrated in Fig. 10-3. Even when the preoperative excretions of hydroxyproline are in the normal range, parathyroidectomy results in a marked, albeit delayed, decrease in urinary excretion (Ney *et al.*, 1966).

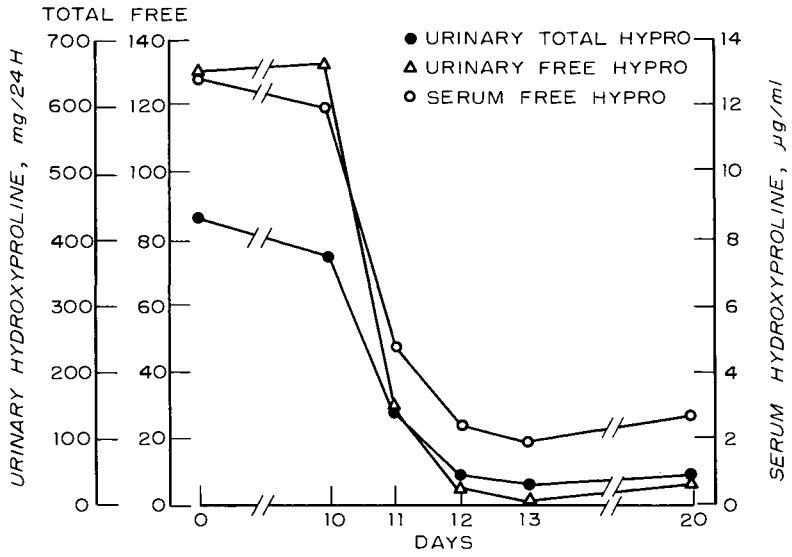


Fig. 10-3 Effect of the removal of parathyroid adenoma on the hydroxyproline values in the urine and serum. The operation was performed on the tenth day after 2 control determinations of hydroxyproline. From Laitinen *et al.* (1966). Reproduced by permission of Acta Medica Scandinavica.

3. Urinary Excretion of Adenosine 3′,5′-Monophosphate (Cyclic AMP)

We have already described the role of cyclic AMP in the cellular regulation of calcium (Section III,F). In 1962, Butcher and Sutherland reported the presence of cyclic AMP in human urine. It was soon found that the urinary excretion of this compound increased in the rat in response to the infusion of PTH (Chase and Aurbach, 1967). Kaminsky *et al.* (1970) have found that, under basal conditions, normal adults have plasma concentrations of cyclic AMP ranging from 10 to 25 nmoles/ml and excrete from 1.5 to 5 μmoles of cyclic AMP per gm urinary creatinine. Infusions of large doses of PTH led to substantial increases in urinary cyclic AMP, that is, up to about 10 μmoles/gm creatinine. The plasma cyclic AMP was increased only modestly.

Nine patients with primary hyperparathyroidism were studied by Kaminsky *et al.* (1970), and surgical removal of the adenomas was invariably followed by a decrease in the excretion of urinary cyclic AMP. For example, in 1 patient, the excretion decreased from a preoperative value of 11.7 μmoles/day to a postoperative value of 4.7 μmoles/day. The urinary creatinine was the same, 1.4 gm/day. In normal subjects,

approximately one-half to two-thirds of the urinary cyclic AMP originates from the plasma by glomerular filtration, and the remainder is produced by the kidney. In primary hyperparathyroidism, the renal fraction is higher.

D. Carcinoma of the Parathyroid

Carcinoma of the parathyroid gland is quite rare. In 1961, Pollack *et al.* reviewed the literature, defining as carcinoma a parathyroid tumor which metastasizes or is locally invasive and which causes hyperparathyroidism. They considered 36 cases in the literature as meeting that criterion and added 2 cases of their own. Ellis *et al.* (1971) estimated that approximately 50 cases had been reported by 1970. The initial serum calcium concentrations in the series reviewed by Pollack *et al.* (1961) ranged from 10.2 to 21 mg per 100 ml, with a mean of 15.9 ± 2.9 (SD) mg per 100 ml. The other biochemical findings are also similar to those found in hyperparathyroidism resulting from adenoma.

The site of production of PTH in a patient with parathyroid carcinoma has been studied by Zisman *et al.* (1968). The patient had had 5 masses, on the left side of the mandible removed during a period of 4 years, at the end of which he began to develop easy fatigability, generalized muscular pain, and malaise. The masses were not considered as being related to the action of a parathyroid adenoma. Several months later, a palpable mass appeared adjacent to the lower pole of the right lobe of the thyroid; he had a serum calcium of 13.6 mg per 100 ml, and cystic changes in the bones were evident. Operation was performed and a tumor of the right inferior parathyroid gland was removed. There was no evidence of local invasion.

Approximately 1 year later, symptoms recurred. The serum calcium was 16.0 mg per 100 ml and the serum phosphorus 2.4 mg per 100 ml. Roentgenographic examination revealed generalized demineralization of the bones, areas of cystic rarefaction of the femur and ileum, and scattered densities throughout both lung fields. Surgical exploration of the parathyroid and of the left thorax was carried out, and it was then decided to remove the remaining parathyroids and as much metastatic tissue as possible from the lung. During the first thoracotomy on the left side, a blood sample obtained from the pulmonary vein showed, by radioimmunoassay, a concentration of 3.91 ng parathyroid hormone per ml, as compared with concentrations of 1.18 and 1.07 ng/ml in the peripheral blood and pulmonary artery, respectively. Blood samples during a second thoracotomy on the right side to remove residual metastatic tissue showed that the concentration of parathormone in the pul-

monary vein was 1.79 ng/ml, somewhat lower than at the first operation. The concentrations in the peripheral blood and pulmonary vein were 1.00 and 1.02 ng/ml, respectively.

The concentration of the hormone in the pulmonary metastatic tissue was 63.0 μg/gm of tissue. The gradients across the pulmonary bed provided strong evidence that the pulmonary metastases were producing and releasing PTH. Lowering of blood calcium, induced by EDTA, caused no further release of PTH. These findings suggested that the metastatic parathyroid tissue was an autonomous source of PTH.

V. Primary Neoplasms of the Bone

A. Introduction

The classification of primary tumors of the bone has been the subject of much consideration and some revision since the first proposal by the American College of Surgeons in 1928. Jaffe (1958) and later Lichtenstein (1965) have both proposed their own systems. Lichtenstein, for example, classifies tumors according to type of tissue or cellular origin, such as cartilage-cell or cartilage-forming connective tissue derivation, osteoblastic derivation, hematopoietic origin, nerve origin, fat cell origin, or any of several other tissue origins. Provision is made for malignant as well as benign counterparts. In contrast, Jaffe (1958) favored a clinicopathological designation in which the clinical findings, the x-ray, and the dominant histological pattern all played a part.

Of 3987 cases of bone tumor reviewed by Dahlin (1967) at the Mayo Clinic and classified according to Lichtenstein's criteria, 1025, or 25.7%, were benign. The distribution of some of the major types of *benign* tumors as a fraction of the total benign tumors were osteochondroma, 45.2%; chondroma, 11.3%; benign giant cell tumor, 15.2%; osteoid osteoma, 10%; and hemangioma, 4.5%. Of the 2962 cases of *malignant* bone neoplasms, myelomas constituted 43.0%; osteogenic sarcoma 21.0%; reticulum cell sarcomas, 6.6%; primary chondrosarcoma, 10.0%; and Ewing's tumor, 7.0%. It must be realized that different hospitals in differently located geographical areas will attract different types of patients. The series of 790 cases of *benign* bone neoplasms reported by Coley (1960) at Memorial Hospital in New York showed the following distribution: osteochondroma, 33.4% chondroma, 16.7%; giant cell tumor, 13.6%; and hemangioma, 1.5%. Of the 1534 cases of primary *malignant* bone neoplasms listed by Coley (1960), myelomas constituted 17.0%; osteogenic sarcoma, 39.3%; reticulum cell sarcoma, 3.2%; chondrosarcoma, 17.9%; and Ewing's sarcoma, 14.5%.

B. Benign Bone Tumors

The benign types of bone tumors have received relatively little biochemical attention. Little is known concerning the detail of the chemical or molecular mechanisms which may be involved in the formation and growth of these benign tumors. Employing Kay's method (1930), Franseen and McLean (1935) determined the alkaline phosphatase activity of a number of benign and malignant bone tumors. The values for 8 samples of various areas of normal bone ranged from 0.1 to 2.4 and averaged 1.1 Kay units. The benign tumors showed slight elevations of activity. The mean values for 5 cartilage tumors (osteochondromas and chondromas) averaged 3.6 Kay units and the values for two giant cell tumors were 5.0 and 12.9 units.

Obviously, these determinations represent few cases. Using histochemical as well as quantitative chemical methods, Jeffree and Price (1965) studied alkaline and acid phosphatase, nonspecific esterase, and β-glucuronidase in a series of bone tumors and allied lesions. Giant cell tumors were characterized by high levels of acid phosphatase and intense staining for this enzyme, nonspecific esterase, and β-glucuronidase in the osteoclasts. But these cells were almost entirely lacking in alkaline phosphatase. Indeed, high levels of alkaline phosphatase were not found in giant cell lesions, except in relation to osteogenic matrix. Consequently, when the whole tissue was taken for quantitative enzyme determination, substantial alkaline phosphatase activity could be obtained. A series of 7 cartilaginous tumors (4 chondromas and 3 chondrosarcomas) exhibited low levels of all 4 enzymes.

These tissue findings account for the normal or only slightly elevated serum alkaline phosphatase activities that are seen in patients with benign bone tumors. A. Bodansky and Jaffe (1934), establishing 2.6 ± 0.6 B units as the normal range and 3.8 units as the upper limit of normal for serum alkaline phosphatase, obtained the following values for patients with benign bone tumors: recurring osteochondroma, 4.5 units, and giant cell tumors, 2.9, 3.3, and 4.4 units. Woodard and Higinbotham (1937) reported a more extensive series of benign tumors. This included 14 patients with chondromas, 11 with giant cell tumors, 3 multiple cartilaginous exostoses, and 1 hemangioma. The alkaline serum phosphatase activity was within the normal range in practically all of these cases.

C. Osteogenic Sarcoma

It will be recalled that osteogenic sarcoma is the most common type of malignant bone tumor according to the Memorial Hospital series,

constituting approximately 40% of all malignant tumors (Coley, 1960). In Dahlin's series (1967), it is the second most common type of malignant tumor. The tumor occurs most frequently at the lower end of the femur, then the upper ends of the tibia and humerus. According to Lichtenstein (1965), the essential histopathological features are the presence of a frankly sarcomatous stroma and the direct formation of tumor osteoid and bone by this malignant tissue. Osteogenic sarcoma may be grouped into the osteolytic and osteoblastic types. The former tends to spread rapidly, eroding and destroying spongiosa and cortex in its path and showing little or no reactive bone formation by host tissue. In the osteoblastic type, the formation of new bone is more dense and extensive and there is no or relatively little evidence of bone destruction.

Osteogenic sarcoma may also arise in somatic soft tissues. In 1971, Allan and Soule reported 26 such cases of extraosseous osteogenic sarcomas, making the amount reported in the English literature through 1968 a total of 94. The sites included tissues in the upper and lower extremities, the retroperitoneum, and the abdomen.

The early biochemical work on osteogenic sarcoma consisted in correlating the tissue and blood serum alkaline phosphatase. Franseen and McLean (1935) presented 9 cases of osteoblastic osteogenic sarcoma in which the activity of tumor phosphatase ranged from 11 to 200 times that of the normal bone phosphatase activity and the serum alkaline phosphatase was 20–40 times the normal phosphatase activity. Jeffree and Price (1965) described histochemical and microchemical analyses of the tumor tissue in 10 cases. The tissue was generally characterized by high levels of alkaline phosphatase, with rich staining for this enzyme in the tumor cells. Less marked elevations of serum alkaline phosphatase have been reported by others (Gutman *et al.*, 1936; Bodansky and Jaffe, 1934). The osteolytic type of osteogenic sarcomas in which bone destruction is a prominent feature has only moderate elevations of tissue alkaline phosphatase activity (Franseen and McLean, 1935). Gutman *et al.* (1936) presented values for 2 cases in which the serum alkaline phosphatase activity was normal or only slightly elevated: 3.4 and 5.6 B units.

The level of serum alkaline phosphatase activity may also serve as an indication of the progression of disease. In a report on multiple osteogenic sarcomata, Amstutz (1969) noted that an alkaline phosphatase activity of 59 B units fell to 26 units after removal of tumor by hip disarticulation. With the appearance of new sites of osteogenic sarcoma, the enzyme level rose again, this time to a value of 430 units 5 months after admission and 1 month before death.

There do not appear to have been any studies on the overall metabo-

lism of calcium or phosphorus in patients with osteogenic sarcoma, nor any specific studies on the release of calcium from the bone, its absorption from the intestine or tubular reabsorption. It would appear, however, that these processes are not sufficiently deranged to affect the levels of calcium and phosphorus in the serum. Values for these parameters published by Bodansky and Jaffe (1934) and by Gutman and his associates (1936) were within the normal range, and these findings have been subsequently confirmed by many others.

D. Chondrosarcoma

This tumor is to be differentiated from osteogenic sarcoma by the salient pathological feature that the basic proliferating tissue is cartilaginous throughout, although occasionally the malignant cells produce an osteoid lacework or osteoid *trabeculae* that tend to classify the neoplasm as an osteogenic sarcoma (Dahlin, 1967). Chondrosarcoma usually has a slow clinical evolution and, unlike osteogenic sarcoma, metastases are relatively rare and often late in appearance.

Patients with this tumor have received relatively little biochemical study. The serum alkaline phosphatase activity may be moderately elevated. Another biochemical feature of interest is the report by Marcove and Francis (1963) that 12 of 14 patients with chondrosarcoma, or 86% exhibited decreased glucose tolerance. Although the series is small, the incidence appears higher than that reported for general cancer populations, a subject which was discussed in some detail in Chapter 1. The explanation for this phenomenon is not clear, but according to Marcove (Personal communication, 1972) the finding itself appears to have been useful in the differential diagnosis of osteogenic sarcoma from chrondrosarcoma.

E. Multiple Myeloma

1. Introduction

Multiple myeloma is one of the most common malignant bone tumors. As was noted earlier, it constituted 43% of all such tumors in Dahlin's (1967) series and 17% of those reviewed by Coley (1960) at Memorial Hospital. As was pointed out in Chapter 5, multiple myeloma is chiefly a malignancy of plasma cells and closely related cells. It results in local tumors, particularly in bone, as well as in disseminated infiltration of the bone marrow. Clinically and radiologically, the condition may remain localized to one bone for some time, but it is more common to observe

initially lesions in several bones. The nodules are multicentric, rather than metastatic from one primary focus. The clinical symptomatology was noted in the earlier chapter, and detailed attention was given to the nature of the serum and urinary proteins found in this disease as well as to their effect on renal function. Marked osteolytic destruction of spongy and cortical bone frequently characterizes the growth of multiple myeloma in the medullary cavity, and it is the biochemical aspects of this effect that we shall consider in the present chapter.

2. Serum Calcium and Phosphorus

The concentration of serum calcium is frequently elevated in multiple myeloma. In 1936, Gutman and his associates reviewed the biochemical findings in 72 cases of multiple myeloma and added 6 of their own. Of 66 patients for whom one or more numerical values for the serum calcium were available, 50 patients, or 76%, had a serum calcium concentration of 11.0 mg per 100 ml or higher at some stage in the course of their illness; 34 patients, or 52%, had a serum calcium higher than 13.0 mg per 100 ml; and 25 patients, or 38% had a serum calcium concentration of 15.0 mg per 100 ml or higher. Values as high as 20 mg per 100 ml were observed by Schittenhelm (1929) and by Jores (1931). More recent reports have shown similar ranges of elevations (Adams *et al.*, 1949). The serum calcium may vary considerably in the same patient as, for example, between 10.4 and 20.2 mg per 100 ml in the case reported by Schittenhelm (1929).

The concentration of serum phosphorus is within the normal range except when renal impairment has occurred. Thus, of 6 cases described by Gutman *et al.* (1936), 4 had normal concentrations of inorganic phosphate ranging from 2.9 to 3.9 mg per 100 ml and the concentrations of nonprotein nitrogen were within the normal range or slightly elevated in these cases. In contrast, in 2 cases in which the nonprotein nitrogen concentrations were 125 and 80 mg per 100 ml, the concentrations of phosphorus were 4.8 and 6.0 mg per 100 ml, respectively. In 36 cases studied by Bayrd and Heck (1947), the serum phosphorus concentration ranged from 0.8 to 14.3 mg per 100 ml and in 8 of these 36 cases, or 22%, the concentrations of phosphorus were higher than 4.2 mg per 100 ml.

3. Serum Alkaline Phosphatase

The bone lesion in multiple myeloma is essentially destructive and provokes little or no new bone formation. Consequently, as has been

noted in Chapter 3 (Section III,B,2), even moderate rises in the serum alkaline phosphatase activity are infrequent and substantial rises are rare (Bayrd and Heck, 1947; Adams *et al.*, 1949; Snapper, 1949). For example, Bayrd and Heck (1947) found that this enzyme activity was greater than 3.6 B units in only 3 cases, or 12% of a total of 24 cases in which the determination was made.

4. Calcium and Phosphorus Metabolism

Excessive excretion of calcium and phosphorus does not appear to occur in patients with multiple myeloma during normocalcemic periods (Aub and Farquharson, 1932; Hunter, 1935). However, patients with elevated serum calcium levels exhibited elevated excretions. For example, in 3 patients with multiple myeloma and serum calcium levels ranging from 13.4 to 16.1 mg per 100 ml on basal intakes of 0.30 mg per 3-day metabolic period, fecal excretions of calcium ranged from 0.40 to 0.80 gm and the urinary excretions of calcium from 0.40 to 0.75 gm per 3-day period, values substantially higher than the controls and than those of fecal calcium, 0.30–0.60 gm, and of urinary calcium 0.20–0.30 gm, for the normocalcemic patients with multiple myeloma.

The interrelationship between the level of serum calcium and the metabolism of calcium has more recently been studied by Lazor and Rosenberg (1964) in a 65-year-old woman with multiple myeloma who passed from a normocalcemic to a hypercalcemic state (Table 10-6).

TABLE 10-6

Parameters of Calcium Metabolism in Normocalcemic and Hypercalcemic Phases of a Patient with Multiple Myeloma[a]

Parameter	Values of parameters	
	During normocalcemic phase	During hypercalcemic phase
Calcium intake (mg/day)	200	215
Fecal calcium excretion (mg/day)	340	500
Urinary calcium excretion (mg/day)	75	325
Calcium balance (mg/kg/day)	−3.4	−10.3
Exchangeable calcium pool (*E*) (gm)	4.5	9.4
Bone formation rate (BFR) (mg/kg/day)	13.6	28.7
Bone resorption rate (BRB) (mg/kg/day)	17.0	39.0

[a] Based on data of Lazor and Rosenberg (1964).

Approximately 6 weeks after the completion of a first study in a normocalcemic phase, which is not shown in Table 10-6, the patient became hypercalcemic. Following treatment with prednisone for 14 days, the dietary intakes of calcium and phosphorus were reduced to 200 and 800 mg/day. The patient became normocalcemic, and a 20-day metabolic study was begun. Immediately following the end of this metabolic period, the serum calcium began to rise, attaining a level of 15.1 mg per 100 ml, and a second study was instituted. Table 10-6 shows clearly the increases in fecal and urinary calcium, the negative balance, the exchangeable calcium pool, and the bone formation (accretion) rate. The bone reabsorption rate (BRR) was 39.0 mg/kg/day, more than twice that of the preceding period. These values indicated that the elevation of serum calcium and the enlargement of pool size resulted from accelerated bone dissolution.

The bone formation (accretion) rate in a group of 14 patients with multiple myeloma was 35.7 ± 11.4 (SD) mg Ca/hour, significantly higher ($p = 0.05$) than the mean value, 24.8 ± 7.7 mg Ca /hour for a group of 6 control patients (Rinsler *et al.*, 1965). Since no serum calcium levels were given, it is not possible to draw from these data a relationship between the magnitude of the bone accretion rate and the existence of hypercalcemia. However, there was no correlation between urinary calcium excretion and the bone accretion. This finding suggested that, in contrast to the results of Lazor and Rosenberg (1964), new bone formation in multiple myeloma is not dependent on the rate of bone destruction.

5. Renal Function in Multiple Myeloma

In Chapter 5, we referred briefly to renal impairment in multiple myeloma. Several early reviews (Geschichter and Copeland, 1936; Bayrd and Heck, 1947; Adams *et al.*, 1949) showed that renal function, as judged by proteinuria, phenolsulfonphthalein clearance, urea clearance, or elevation of blood nonprotein nitrogen or of blood urea, was impaired in a substantial percentage of patients with multiple myeloma, sometimes as high as 60% (Geschichter and Copeland, 1936). More recently, Martinez-Maldonado *et al.* (1971) classified in considerable detail the various types of renal pathology to be found in multiple myeloma and presented the biochemical findings in a series of 47 male patients. Proteinuria, as determined by "Dipstix," was present in 83% of the cases. Quantitative measurements revealed that 42% of the cases had excretions greater than 1 gm/day. Bence Jones protein, determined by the heat precipitation method, was present in 23% of the cases. This is in agree-

ment with the range of incidences, 15–50%, reported by other investigators (Brownell, 1955; Carson *et al.*, 1955; Combined Staff and Clinic Conference, 1957; Dammacco and Waldenström, 1968). The incidence appears to be related to the immunological type of the protein: 20% in patients with A-myeloma, 35% in G-myeloma, and over 90% in those with D-myeloma (Fahey *et al.*, 1968). The nature of the Bence Jones proteins was discussed in greater detail in Chapter 5.

Adams *et al.* (1949) reported that 26, or 46%, of a series of 57 patients with multiple myeloma had elevated blood nonprotein nitrogen values. Defining renal insufficiency as the presence of a blood urea nitrogen greater than 30 mg per 100 ml and/or a creatinine greater than 1.5 mg per 100 ml, Martinez-Maldonado *et al.* (1971) found values greater than these to be present during the course of disease in 53% of their series of 47 patients with multiple myeloma. Five patients had blood urea nitrogen values ranging from 128 to 242 mg per 100 ml, and these had correspondingly elevated blood creatinine concentrations. Autopsy was performed in 22 of these cases. The following distribution of renal pathology was observed: myeloma kidney, 11; amyloidosis, 2; acute suppurative pyelonephritis, 1; and plasma cell infiltration, 1. Other forms of renal involvement were nephrocalcinosis and glomerulitis.

Clearance tests have not contributed much additional diagnostic information. Adams *et al.* (1949) found that phenolsulfonphthalein excretion and/or urea clearance were decreased in 32, or 57%, of 56 cases with multiple myeloma. For example, Armstrong (1950) performed 18 renal clearances in 15 patients with multiple myeloma who had no evidence of other diseases. Eight patients had glomerular filtration rates (C_{In}) less than 70% of normal, and of these 8, only 3 had a decreased tubular excretory maximum (Tm_{PAH}) of 70% or less. Four patients had glomerular filtration rates of 40% or less, and of these, 3 had decreased values for tubular excretory maximum of 50% or less of normal.

VI. Skeletal Metastases

A. Introduction

The bone is a common site for metastases in cancer. In an analysis of the incidence of 1000 consecutively autopsied cases of carcinoma, Abrams *et al.* (1950) found that the skeleton was involved in 272 cases. Table 10-7 shows that the skeleton is most frequently affected in carcinoma of the breast (73%), carcinoma of the prostate (65%), and carcinoma of the lung (33%). However, these incidences may depend upon

TABLE 10-7

Frequency of Involvement of the Skeleton by Carcinomas at Various Sites[a]

Primary site of carcinoma	No. of cases	Frequency of involvement of bone (%)
Breast	167	73
Prostate	104	65
Lung	160	33
Stomach	119	11
Rectum	87	13
Ovary	64	9
Kidney	34	24
Pancreas	32	13

[a] These values are based on the data of Abrams *et al.* (1950) except for the prostate which is based on the data of Elkin and Mueller (1954).

the thoroughness with which necroscopies are performed. Jaffe (1958) has stated that when a careful autopsy has been carried out, skeletal lesions are found in approximately 85% of women dying of breast cancer.

The frequency of skeletal metastases may be estimated during life by roentgenographic means or by the scintigram. Galasko (1972) recently reported that 86 of 127 patients with advanced mammary cancer, or 68%, developed radiological evidence of mammary cancer. Of the last 50 cases in this series, 25, or 50%, had such evidence when they first presented with advanced mammary cancer. Scintigrams obtained in these patients within a few days of radiological examination showed a much higher incidence of lesions, namely, 42 of 50, or 84%.

B. Calcium and Phosphorus Metabolism in Skeletal Metastatic Carcinoma of the Breast

As we have seen, the incidence of skeletal metastases in carcinoma of the breast is approximately 70–80%. Somewhat more than 90% of all such lesions are osteolytic by roentgenographic examination (Garland *et al.*, 1950). It may be readily understood, therefore, as Pearson and his associates (1954) showed, that in most instances of rapid growth of metastatic breast carcinoma, the effects in the skeleton would be manifested by increased excretion of urinary calcium and that, in a number of these instances, hypercalcemia would also become evident.

Hypercalcemia occurs in about 10–20% of patients with cancer of the breast and skeletal metastases (Griboff *et al.*, 1954; Galasko, 1972). Table 10-8 shows the distribution of serum calcium levels in a Memorial Hospital series of 746 patients with carcinoma of the breast, 445 of whom had bone metastases (Woodard, 1953). It may be seen that, in accordance with other estimates, approximately 10% of patients with skeletal metastases had elevated serum calcium levels above 12 mg per 100 ml. Patients with carcinoma of the breast, but without skeletal metastases, had a distribution of serum calcium levels similar to that of the normal group. Patients with metastatic carcinoma of the prostate have been included in Table 10-8 for convenience, but will be discussed later.

As was pointed out in Chapter 3, an inverse relationship exists between alterations in serum calcium concentration and changes in activity of serum alkaline phosphatase activity. The possible mechanisms underlying such a relationship were considered.

Hypocalcemia in patients with metastatic breast cancer is an extremely rare occurrence, but 3 such cases have been reported by Hall *et al.* (1966). The symptoms, which included weakness, muscular twitching, anorexia, nausea, and vomiting developed in all 3 cases. Slurred speech, drowsiness, disorientation, and coma were present in 2 cases. A positive Chvostek sign was elicited in all 3 patients. The serum calcium and phosphorus levels were, respectively, 3.9 and 9.0 mg per 100 ml in the first case, 6.7 and 3.5 mg per 100 ml in the second, and 6.0 and 5.2

TABLE 10-8

Distribution of Serum Calcium Levels in Patients with Carcinoma of the Breast or Prostate Metastatic to Bone[a]

Group	No. of patients	Range	Percent of patients having serum calcium (in mg/100 ml) of:		
			<12.0	12.1–13.0	>13.1
Normal	185	9.6–12.7	98.4	1.6	0.0
Carcinoma of the breast without bone metastases	201	9.4–12.9	99.0	1.0	0.0
Carcinoma of the breast with bone metastases	445	8.9–20.7	90.6	5.1	4.3
Carcinoma of the prostate with bone metastases	101	8.8–12.2	99.0	1.0	0.0

[a] Calculated from the data of Woodard (1953).

mg per 100 ml in the third. All 3 patients responded promptly to the intravenous administration of calcium lactate. Several possibilities may be considered to account for the hypocalcemia. In contrast to the common feature of osteolytic metastases in patients with breast cancer, these 3 patients had diffuse osteoblastic lesions. Although, as we shall presently see for prostatic carcinoma, osteoblastic disease is characterized almost always by normal serum calcium levels, it is possible that in these 3 cases the uptake of calcium by bone might have been responsible. Permission for autopsy was obtained in 2 patients, in 1 of whom no parathyroid glands could be found, whereas in the other patient all 4 were normal.

The overall calcium and phosphorus balances in patients with carcinoma of the breast and skeletal metastases were first studied by Laszlo and his associates (1952) and by Pearson *et al.* (1954). Table 10-9 shows that marked osteolysis in metastatic cancer of the breast was characterized metabolically by excessive urinary excretions of calcium and phosphorus, inversion of the urinary-to-fecal calcium ratio, and negative calcium and phosphorus balances. However, not all of the patients

TABLE 10-9

Calcium and Phosphorus Metabolism in Patients with Breast Cancer and Osteolytic Skeletal Metastases[a]

Parameter	Control[b] (mg/24 hours)	Average of 6 patients with metastases[c] (mg/24 hours)
Calcium		
Intake	142	136
Urinary excretion	40	354
Fecal excretion	126	210
Balance	−24	−428
Phosphorus		
Intake	658	486
Urinary excretion	237	385
Fecal excretion	74	185
Balance	+357	−84

[a] Based on data of Laszlo *et al.* (1952).

[b] Average values for 2 6-day metabolic periods on control patient (see text).

[c] Average values for a total of 18 6-day metabolic periods on 6 patients.

with carcinoma of the breast and skeletal metastases who were studied by Laszlo *et al.* (1952) showed this metabolic pattern. As will be pointed out presently, skeletal metastases may at times be in a quiescent state and, hence, not encroach upon the bony tissue.

These metabolic aspects were explored more fully by Pearson *et al.* (1954) who made the assumption that, in general, a normal person on a low calcium diet, namely, 200 mg/day, excretes 50 mg of calcium per day in the urine and 200 mg in the feces and is in 50 mg of negative calcium balance. The destruction of 1 gm of bone resulting from growth of skeletal metastases would result in the liberation of 100 mg of calcium, its excretion through the kidney, and the increase of the negative calcium balance to 150 mg. Hypercalcemia was usually found to appear when the urinary calcium excretion exceeded 500 mg/day in the presence of unimpaired renal function. These considerations were applied to the study of 6 premenopausal women with osseous breast metastases during

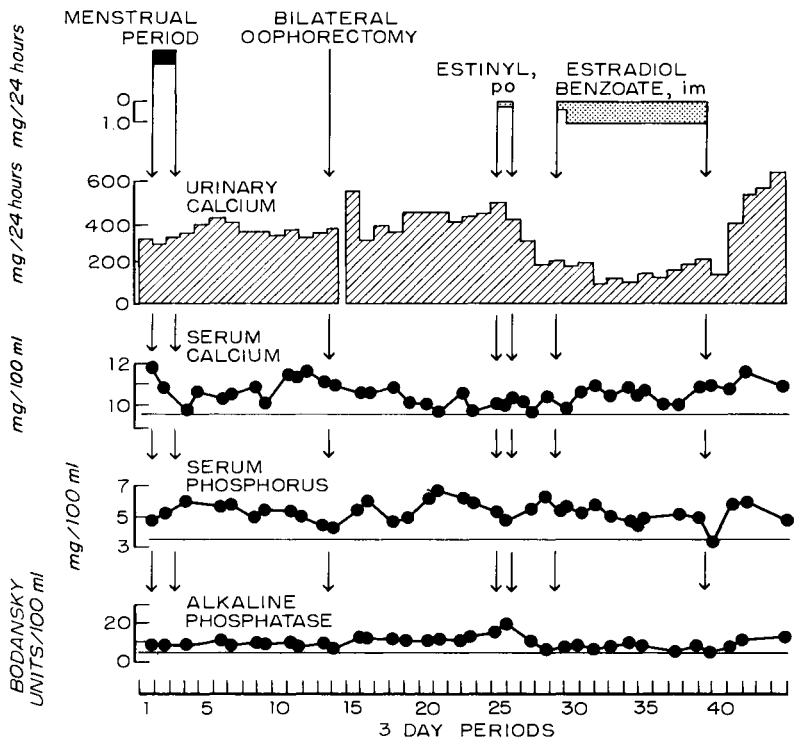


Fig. 10-4 Changes in urinary calcium, serum calcium, serum phosphorus, and serum alkaline phosphatase in a patient with osteolytic metastases. From Pearson *et al.* (1954). Reproduced by permission of the American Medical Association.

a menstrual cycle, after surgical oophorectomy and during administration of ovarian hormones. Sequential studies of one such patient are shown in Fig. 10-4. It is important to note that the urinary excretion of calcium does not proceed at a constant level, but fluctuates, depending upon the growth and regression of the metastatic tumor in the skeleton. This pattern of urinary calcium excretion and its association with the level of serum calcium has been repeatedly confirmed (Bodansky, 1954).

A more precise criterion of calcium kinetics was obtained by calculating the values for exchangeable calcium and the accretion rate (Rinsler *et al.*, 1965). Table 10-10 shows that the mean value for the bone accretion rate of patients with carcinoma of the breast without bone metastases was not significantly lower than the control value. Nor was the mean accretion rate for patients with carcinoma of the breast and bone metastases, regardless of the activity of these metastases, significantly higher than the control. However, some of these patients had a daily urinary excretion less than 300 mg, and presumably their skeletal metastases were quiescent. When these patients were excluded, the remaining 7 had an accretion rate significantly higher than the control value.

C. Carcinoma of the Prostate

The vast majority of metastatic bone lesions resulting from carcinoma of the prostate are osteoblastic as judged both roentgenologically and

TABLE 10-10

Rates of Bone Accretion and Urinary Excretion of Calcium in Patients with Carcinoma of the Breast[a]

Group	No. of patients	Mean bone accretion rate (mg/hour ± SD)	Mean urinary excretion (mg/24 hours ± SD)
Control	6	24.8 ± 7.7	207.0 ± 51.0
Carcinoma of breast without metastases	6	16.7 ± 10.2	259.3 ± 63.2
Carcinoma of breast with metastases			
a. All cases	14	45.2 ± 27.9	277.4 ± 162.8
b. Cases with urinary excretion >300 mg/day	7	50.3 ± 24.6[b]	409.9 ± 82.3

[a] Based on data of Rinsler *et al.* (1965). Reproduced by permission of the British Medical Association.

[b] Significantly higher than control.

upon microscopic examination of autopsy material (Elkin and Mueller, 1954). The serum calcium is well within the normal limits. In 101 cases, Woodard (1953) found this parameter to range from 8.8 to 12.2 mg per 100 ml and to average 10.37 mg per 100 ml. Only one value lay between 12.0 and 13.0 mg per 100 ml. In a comparable series of 185 normal persons, the serum calcium ranged from 9.6 to 12.7 mg per 100 ml and averaged 10.71 mg per 100 ml.

The serum alkaline phosphatase activity in patients with prostatic carcinoma and skeletal metastases has been discussed previously in Chapter 3. It may be noted briefly here that in Woodard's series (1953) 89% of a series of 110 patients had values above 5.1 B units, as contrasted with only 2.4% in a normal population. The activities ranged from 3.4 to 147.2 and averaged 20.7 B units.

Few calcium and phosphorus metabolic studies appear to have been carried out in patients with carcinoma of the prostate. For example, in a study by Schilling and Laszlo (1950) on a 69-year-old male, the urinary calcium excretion was moderately elevated, and the calcium balance was negative prior to therapy. The serum calcium, phosphorus, and alkaline phosphatase values remained within normal limits. The serum acid phosphatase rose from 51 to 199 KA units; the normal value is 7.8 ± 2.2 KA units. It may be seen, therefore, that, contrary to the situation in the osteolytic phases of carcinoma of the breast, the urinary excretion of calcium was much lower, and the calcium balance was much less negative (Laszlo *et al.*, 1952).

With the administration of diethylstilbestrol, the urinary calcium decreased precipitously, but there was little effect on the phosphorus excretion. As was described in Chapter 3, decrease in serum acid phosphatase activity may occur upon treatment with estrogens. In the patient described above, the serum acid phosphatase activity decreased continuously to a value of 13.6 KA units by the thirtieth day of therapy. The serum alkaline phosphatase activity increased gradually to a maximum of 9.1 B units. The calcium balance became positive. The serum calcium decreased to a low level of 7.8 mg per 100 ml, but the serum phosphorus showed no fluctuation. These results indicate that estrogen mediated the deposition of calcium in the bone.

D. Other Neoplasms Metastatic to the Bone

As has already been noted, other tumors may invade or metastasize to the bone. In the series of Abrams *et al.* (1950), the incidences of metastases to the bone from carcinoma of the lung were 33%; from carcinoma of the kidney, 24%; from carcinoma of the rectum or the pancreas,

13%; and less for those from other organs. The effects on the serum calcium concentration and alkaline phosphatase activity depend on the extent to which the skeletal reaction tends to be osteolytic or osteoblastic. In a series of 144 cases of miscellaneous cancers metastatic to bone without hepatic involvement, the serum calcium determination was performed in 135 instances and ranged from 8.9 to 17.6 mg per 100 ml, with 8.9% having values greater than 12.1 mg per 100 ml and 6.7% having values greater than 13.1 mg per 100 ml. The serum alkaline phosphatase activity ranged up to 58.3 B units, with 46% of the cases having values greater than 5.1 units (Woodard, 1953).

On rare occasions, hypocalcemia may occur not only in association with osteoblastic metastases in breast cancer (Hall *et al.*, 1966) but also in connection with other types of tumor. Persistent hypocalcemia, with serum calcium concentrations ranging between 7.4 and 8.2 mg per 100 ml, was observed by Sackner *et al.* (1960) in a 56-year-old male with adenocarcinoma of the lung and extensive osteoblastic metastases. The serum alkaline phosphatase activity was slightly elevated, ranging between 5.3 and 8.6 B units. The patients excreted 26 mg of calcium in 24 hours in response to the intravenous administration of 1 gm of calcium as calcium gluconate. This was in accord with the marked degree of retention in patients with carcinoma of the prostate and osteoblastic metastases. Schilling and Laszlo (1951) found that, following the administration of 446 mg of calcium as a salt, the 24-hour urinary excretion in patients with osteoblastic metastases was 50 ± 14 mg, as compared with a value of 302 ± 24 mg in normal persons.

VII. Hypercalcemia in Neoplastic Disease without Evidence of Bone Metastases (Ectopic Hyperparathyroidism, Pseudohyperparathyroidism)

A. Introduction

In 1936, Gutman *et al.* first reported the occurrence of hypercalcemia and hypophosphatemia in a 57-year-old patient with bronchogenic carcinoma without skeletal metastases, as determined by roentgenologic and postmortem examination. The parathyroids were found to be normal at autopsy. By 1966, Lafferty had assembled 50 cases from the literature which met at least one of the following criteria: (a) the absence of both roentgenographic and postmortem evidence of metastases to bone and the failure to find a parathyroid adenoma at surgical exploration

of the neck or detailed postmortem examination of the neck or mediastinum, (b) a significant reduction of serum calcium following resection of the neoplasm, and (c) a positive parathormone immunoassay of a neoplasm extract. Thirty-two cases met the first criterion, 20 cases the second, and 7 the third. This syndrome was designated as "pseudohyperparathyroidism" by Fry (1962) and by Snedecor and Baker (1964), but ectopic hyperparathyroidism has become a designation of wider use.

This condition has been stated to account for approximately 15% of all cases of hypercalcemia (Lafferty, 1966). In his 1960 Memorial Hospital series, Myers found no roentgenologic evidence of skeletal metastases in 56 cases, or 13%, of the 430 cases of hypercalcemia. It is possible that this series may have included cases with skeletal metastases too small to be detected roentgenographically. The distribution of primary sites was breast, 14; lung, 5; kidney, 3; cervix, 4; lymphoma, 10; leukemia, 5; and miscellaneous, 15 (Myers, 1960). The distribution of the primary tumor in Lafferty's series (1966) was as follows: hypernephroma, 15; bronchogenic carcinoma, 14; ovarian tumor, 4; bladder carcinoma, 3; and pancreatic carcinoma, 2. The remainder included single cases of carcinomas of the renal pelvis, penis, vulva, uterus, esophagus, bronchial rest, and colon, as well as single cases of hemangiosarcoma of the liver, of cholangiosarcoma, primary hepatoma, lymphosarcoma, and Hodgkin's disease. Since Lafferty's series in 1966, additional cases of pseudohyperparathyroidism have continued to be reported as, for example, the report of Ballard and Marcus (1970) in a patient with chronic myelogenous leukemia and that of Ferenczy *et al.* (1971) in a patient with ovarian mesonephroma.

B. Serum Calcium, Phosphorus, and Chloride

Table 10-11 shows the distribution of serum calcium and phosphorus levels in those patients of Lafferty's series (1966) for whom quantitative determinations were available. Forty-five of 47 patients, or 96%, had serum calcium levels higher than 12.1 mg per 100 ml. If the values of 3.6 ± 0.42 mg per 100 ml is taken as the normal mean value for phosphorus, then 12 of 45 patients, or 27%, had values of less than or equal to 2.8 mg per 100 ml, the lower limit of normal.

In 1964, Wills and McGowan found that the serum chloride level was above 102 mEq/liter in each of 32 cases of primary hyperparathyroidism, whereas concentrations of 102 mEq/liter or lower were present in 28 cases of hypercalcemia associated with other conditions such as metastatic bone disease, sarcoidosis, vitamin D intoxication, and multiple

TABLE 10-11

Serum Calcium and Phosphorus Levels in Patients with Pseudohyperparathyroidism[a]

Serum calcium mg per 100 ml		Serum phosphorus mg per 100 ml	
Range	No. of patients	Range	No. of patients
11.1–13.0	5	1.6–2.0	3
13.1–15.0	13	2.1–2.5	4
15.1–17.0	13	2.6–3.0	12
17.1–19.0	8	3.1–3.5	14
19.1–21.0	7	3.6–4.0	7
21.1–23.0	1	4.1–4.5	3
—	—	4.6–5.0	2

[a] Values arranged from data collected from the literature by Lafferty (1966). Where several calcium determinations were recorded for a patient, the highest one was used.

myeloma. These results were confirmed by Lafferty (1966) in studies in his own laboratory. Ten of 12 patients with primary hyperparathyroidism had serum chloride levels above 102 mEq/liter, whereas only one of 21 cases of hypercalcemia secondary to metastatic bone diseases exceeded this level. In a group of 10 cases of pseudohyperparathyroidism (8 probable and 2 proven), 7 had serum chloride levels below 102 mEq/liter and 2 below 90 mEq/liter.

These findings have limited diagnostic value in differentiating between hyperparathyroidism as a result of a parathyroid adenoma from pseudohyperparathyroidism. If it is assumed that, in the latter entity, the neoplastic tissue secretes a parathyroid or parathyroid-like hormone, one must also postulate the production of other hormonal substances by the neoplastic tissue which counteracts the hypochloremic effects of parathormone.

C. Calcium and Phosphorus Metabolism; Dynamics of Calcium Metabolism

These aspects are well illustrated in a 61-year-old male who had developed hypercalcemia and hypophosphatemia secondary to a bronchogenic carcinoma in the left chest (Lafferty and Pearson, 1963). Skeletal survey revealed no metatases. The patient was studied metabolically during a 9-day period and was then subjected to thoracotomy. The tumor was

found to be inoperable and 3 days later, neck exploration was performed. A left upper parathyroid gland was removed. The gland was found to be normal, as were the remaining three at subsequent autopsy about 7 months later. Radiation therapy was instituted a week after operation, and continued for $1\frac{1}{2}$ months. A second 9-day metabolic study was performed 2 months after cessation of radiotherapy; ^{47}Ca was given intravenously on the morning of the first day of each balance period to determine calcium dynamics.

After treatment, the serum calcium decreased, the serum phosphorus rose to normal levels, and the calcium and phosphorus balances became less negative. The bone accretion rate which had been about 4 times the normal rate decreased by about 60% after therapy. The bone resorption rate before therapy was higher than the bone accretion rate and, although it also decreased markedly following therapy, it still remained higher than the bone accretion rate.

Several other parameters were of interest. On essentially the same dietary intake before and after treatment, the fecal calcium rose after irradiation. The endogenous fecal calcium remained the same, and the fraction of calcium absorbed decreased considerably from 43% before treatment to 15% after treatment. The percentage of tubular reabsorption of filtered phosphorus (TRP) which, as we have noted previously (Section IV,B,3), is about 80–90% in the normal individual, was 70% before treatment and rose to 79% after treatment.

D. Mechanisms of Ectopic Hyperparathyroidism

1. Introduction

Hypercalcemia in neoplastic disease results whenever an increase in bone resorption is not counteracted by appropriate increases in bone accretion and calcium excretion (Pearson, 1968; Muggia and Heinemann, 1970). The basis for this imbalance and hypercalcemia is readily understood when there is widespread destruction of bone, as in the relatively common osteolytic metastases of breast cancer.

Several mechanisms have been postulated to account for those instances when the bone is not affected, as determined either by roentgenologic or autopsy evidence (Muggia and Heinemann, 1970). These mechanisms include one or more of the following possibilities: (a) the elaboration of a polypeptide hormone, either similar to or identical with parathormone, by tumor tissue; (b) the elaboration of a substance with vitamin D-like action; (c) hypercitremia leading to increased binding of calcium and increased parathyroid activity; and (d) other, as yet,

undefined factors related to the host, the tumor, or to treatment which may facilitate the development of hypercalcemia by modifying the accretion, the resorption, or the excretion of calcium.

2. Parathyroid-like Hormone

As early as 1923, Klemperer suggested that, in some carcinomas, hypersecretion of PTH by an enlarged gland might be associated with bone resorption. For approximately the next 40 years, the clinical similarity of hyperparathyroidism to cases of cancer with hypercalcemia but without evidence of skeletal metastases led to errors in diagnosis, followed by operative procedures that failed to reveal the existence of adenoma or carcinoma of the parathyroid glands. Autopsy examination of other cases in this group showed that the parathyroid glands were, with rare exceptions, normal.

In 1948, Albright and Reifenstein raised the question of whether carcinomas at sites other than the parathyroid gland could, in and of themselves, produce a parathyroid-like substance and thereby induce hypercalcemia and the other sequalae of this state. Evidence to this end was submitted in 1964 by Goldberg *et al.* A patient with renal adenocarcinoma and hypercalcemia that fluctuated between 11.4 and 18.6 mg per 100 ml was explored surgically. The parathyroid glands were normal, and there were no evidences of mediastinal masses. Extracts of the primary renal adenocarcinoma and of pulmonary metastases were prepared, purified, and assayed for PTH immunologically by quantitative complement fixation. Rabbit antisera prepared against purified human PTH reacted positively with preparations of the primary renal tumor, the lung metastases, and the parathyroid gland of the patient, but did not react or reacted poorly with similar extracts of the patient's kidney or lung tissue adjacent to the metastases.

In 1963, Berson *et al.* submitted a sensitive radioimmunoassay for the quantitative determination of bovine and human parathyroid hormone (hPTH). Although values for activity in tumors producing pseudohyperparathyroidism (Sherwood *et al.*, 1967) and in serum (Reiss, 1968; Reiss Canterbury, 1968) have been reported, other investigators were barely able to measure normal serum levels of the hormone, found a large overlap between the values in normal and hyperparathyroid sera, or were unable to detect hPTH in sera of patients with known hyperparathyroidism (Arnaud *et al.*, 1971). In general, these difficulties have been the result of lack of sufficient quantities of crude hPTH for antibody production, a lack of homogeneous hPTH for use as a reference standard, unavailability of radioiodinated preparations, and incompleteness of cross

reaction between the hPTH and the bovine parathyroid hormone (bPTH) which is readily available in large enough quantities for extraction and purification of the hormone.

Arnaud and his associates (1971) have attempted to circumvent these difficulties by using porcine parathyroid hormone (pPTH) as antigen, with the hope that the cross reaction between porcine and human PTH's might be better than that between the bovine and human hormones. The accompanying studies led to the development of an unusually sensitive radioimmunoassay for hPTH in serum. The assay was standardized with a serum obtained from a patient with parathyroid cancer, so that the concentrations of immunoreactive parathyroid hormone (IPTH) were expressed in terms of microliter equivalents of this serum per milliliter of serum (μl Eq/ml) of the patient being tested.

Some of the results obtained by Arnaud *et al.* (1971) with this procedure may be briefly noted. Serum IPTH was undetectable in 10 totally parathyroidectomized patients. In a series of 51 normal subjects, ranging in age from 13 to 76 years, serum IPTH did not differ significantly with age or sex, was measurable in 94% of the sera tested, and ranged between undetectable amounts and 38 μl Eq/ml. In a group of 54 patients with parathyroid tumors, as proved later at surgery, the serum IPTH averaged 392 ± 129 (SE) μl Eq/ml and ranged from 25 to 6500 μl Eq/ml (Riggs *et al.*, 1971). A significant correlation held between serum IPTH and the weight of the tumor. When the serum IPTH was plotted against the serum calcium in these cases, a significant positive correlation was also obtained; the coefficient was 0.384 with $p < 0.02$.

Employing this assay procedure in a series of 18 patients with pseudohyperparathyroidism, Riggs *et al.* (1971) found that the serum ITPH ranged from undetectable amounts to 110 μl Eq/ml and averaged 25 ± 9 (SE) μl Eq/ml. This mean value was significantly lower than that for the group of patients with primary hyperparathyroidism. These 2 groups could also be differentiated in other ways. A plot of serum IPTH against serum calcium and linear discriminant analysis showed that, at any serum calcium value, serum IPTH was lower in the pseudohyperparathyroid (ectopic) group. There was an overlap of only 2 patients with ectopic and one with primary hyperparathyroidism (Fig. 10-5). For example, it might be confidently stated that, at a serum calcium of 14 mg per 100 ml, a serum of IPTH less than 40 μl Eq/ml would indicate a case of ectopic rather than primary hyperparathyroidism. It was also possible to differentiate the 2 groups immunologically.

The radioimmunoassay used by Arnaud *et al.* (1971) involves preincubation at 4°C for 3 days of various concentrations of a standard, highly purified PTH preparation or unknown serum with a guinea pig antiserum prepared to dried, defatted porcine parathyroid glands. Bovine PTH-^{131}I

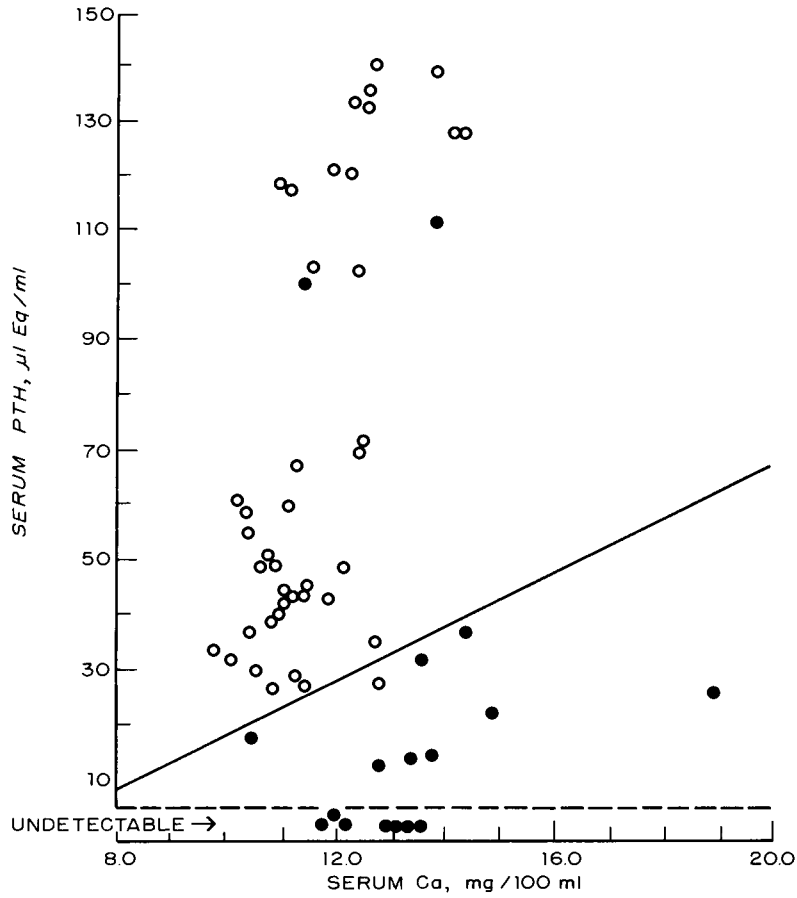


Fig. 10-5 Relationship between serum IPTH (assayed using antiserum GP-1 M) and serum calcium in primary (○) and in ectopic (●) hyperparathyroidism. Only patients with primary hyperparathyroidism with serum IPTH values less than 150 μl Eq/ml are represented although the actual range extended to 6500 μl Eq/ml. Serum IPTH is given as equivalent concentration of standard hyperparathyroid serum. From Riggs *et al.* (1971). Reproduced by permission of the American Society for Clinical Investigation.

is added in a standard diluent and incubation is continued for another 3 days at 4°C. Dextran-coated charcoal is then mixed with the incubation mixture and this is centrifuged at 3000 rpm. The supernate contains the antibody-bound PTH-^{131}I and the residue has the free PTH-^{131}I. When the log of dilution of the serum is plotted against the corresponding bound to free (B/F) PTH-^{131}I ratio, a line is obtained which is straight except at the very low and very high concentrations. For sera from patients with primary hyperparathyroidism, the curves were all

parallel to each other and to the curve obtained with standard hyperparathyroid serum, and the slopes of the straight line portion of these curves had the same value. In patients with ectopic hyperparathyroidism and sera with sufficient IPTH activity to permit the development of a dilutional curve, the slopes of the straight line portions were similar to each other, but significantly lower than that for patients with primary hyperparathyroidism (Riggs *et al.*, 1971). This finding indicates that the PTH-like material in the serum of patients with ectopic hyperparathyroidism is immunologically different from the PTH in the serum of patients with primary hyperparathyroidism.

At the present time, it would therefore appear that certain malignant neoplasms are capable of producing a substance which does not seem to be PTH, but which is very similar to it and is capable of inducing many of the effects of PTH, albeit to a different extent. Scholz *et al.* (1973) recently reported from Arnaud's laboratory a case of Hodgkin's disease with hypercalcemia, active renal stone disease, and subperiosteal resorption. The 4 parathyroid glands were identified surgically as normal. Three of these were removed and three-fourths of the remaining gland was resected, but the hypercalcemia persisted. This, then, appeared to be a very unusual case, and perhaps indeed the first reported case, of the association of ectopic hyperparathyroidism with renal and skeletal changes. That this was indeed a case of ectopic hyperparathyroidism rather than of occult primary hyperparathyroidism was substantiated by the determination of the serum ITPH level as 27 μl Eq/ml, well below the upper limit of normal, 40 μl Eq/ml obtained in their laboratory.

The determination of the primary structure of bPTH and, to a lesser extent, of pPTH has been achieved in recent years (Potts *et al.*, 1966, 1971). Brewer *et al.* (1972) have described the amino acid sequence of the first 34 residues of hPTH and have shown that it differs from the bPTH by 6 residues and from the pPTH by 5 residues. Some attempts have been made to describe the chemical differences between the circulating PTH present in primary hyperparathyroidism and the parathyroid-like material present in the circulation of patients with ectopic hyperparathyroidism. Benson *et al.* (1974) have recently reported that plasma IPTH in ectopic hyperparathyroidism has a decreased quantity of biologically inactive COOH-terminal fragments, as compared with plasma from patients with primary hyperparathyroidism.

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11

Neoplasms of the Pituitary Gland

I. Introduction*

Although the effects which tumors of the pituitary gland produce are of considerable biochemical and physiological interest, the incidence of such tumors is relatively low. We shall discuss the incidences in connection with the classification of these tumors and the characteristics of each type.

The normal human pituitary gland (hypophysis) is an oval structure, measuring about 10–16 mm across, 8–11 mm in its anteroposterior diameter, and 5–7 mm vertically and lying in a bony walled cavity, the sella turcica, at the base of the skull. Its weight in normal individuals ranges from 0.4 to 1.1 gm, with an average of 0.5–0.6 gm in the male and 0.6–0.7 gm in the female. The anterior lobe, also known as the adenohypophysis, comprises about 70% of the total weight of the gland and consists almost completely of the pars distalis. A small area,

* The following abbreviations are used most commonly in the present chapter: ACTH = adrenocorticotropic hormone (adrenocorticotropin); FSH = follicle-stimulating hormone; GFR = glomerular filtration rate; GH = growth hormone; HGH = human growth hormone; 17-KS = 17-ketosteroids; LH (ICSH) = luteinizing hormone (interstitial cell-stimulating hormone); MSH = melanocyte-stimulating hormone; 17-OHCS = 17-hydroxycorticosteroids; PBI = protein-bound iodine; TSH = thyroid-stimulating hormone.

the pars tuberalis, joins it with the optic chiasma of the brain and another area, the pars intermedia, abuts on the posterior lobe or neurohypophysis. The latter, which forms as an outgrowth from the brain, consists of a neural lobe that is connected to the brain by a stalk or infindibulum (Grollman, 1964).

The formerly well-established histological classification of the anterior pituitary cells into 3 types—acidophil, basophil, and chromophobe (Rasmussen, 1929)—is inadequate to account for the independent secretions of 6 known hormones and possibly more. As a result of recent application of histochemical, immunofluorescent, and electron microscopic techniques, chiefly to the rodent pituitary, another classification of cell types has been proposed (Daughaday, 1968): (a) the somatotropic cell type containing large acidophilic, dense, round secretory granules and a high concentration of growth hormone in these granules; (b) lactotropic cells associated with prolactin secretion; (c) thyrotropic cells, having a polygonal shape, a small nucleus, basophilic staining, and secretory granules associated with the secretion of thyrotropin; (d) gonadotropic cells, which are of two types—small cells with relatively scant cytoplasm associated with the secretion of luteinizing hormone (LH or ICSH) and larger, rounded cells associated with the secretion of follicle-stimulating hormone (FSH); and (e) corticotropic cells, probably basophilic, concerned with the secretion of corticotropin.

Obviously, the estimation of the frequency of similar pituitary cell types in healthy human beings is difficult because these must be determined in individuals who have met sudden accidental deaths. Using the older classification, Sommers (1968) has obtained the following percentage distribution of cell types in males of a group of 372 adults who died of various diseases: acidophils, 34.8 ± 8.2; basophils, 21.7 ± 6.8; amphophils, 19.7 ± 6.5; chromophobes, 22.8 ± 3.9; hypertrophic amphophils, 1.4 ± 0.8; and hyaline basophils, 0.06 ± 0.3. The distribution for women was essentially the same. It is of interest to compare these values with the average values obtained by Rasmussen in 1929: acidophils, 37%; basophils, 11%; and chromophobes, 52%.

Dingman (1971) has recently summarized current concepts of pituitary function. The FSH promotes maturation of the graafian follicle and stimulates estrogen secretion in women and development of the spermatic tubules in men. Luteinizing hormone (LH), also known as interstitial cell-stimulating hormone (ICSH), initiates ovulation and progesterone secretion in women and stimulates testosterone secretion of the interstitial Leydig cells of the testicle. Growth hormone (GH), or somatotropin, promotes growth and has other important metabolic functions such as retention of nitrogen, phosphorus, sodium, potassium, and calcium

in the proportions characteristic of muscle and bone. GH also increases metabolic rate, serum free fatty acids, and serum phosphorus. Thyroid-stimulating hormone (TSH) or thyrotropin and adrenocorticotropic hormone (ACTH) regulate the secretion of thyroid and adrenocortical hormones, respectively. Prolactin stimulates secretion of milk by the developed breast.

The intermediate lobe of the pituitary produces melanocyte-stimulating hormone (MSH), which is responsible for darkening of the skin. Arginine vasopressin (AVP), which is the antidiuretic hormone, and oxytocin are secreted by the supraoptic and ventricular nuclei of the hypothalamus, respectively. Both hormones are stored in the posterior lobe of the pituitary body, and either or both is/are released in response to specific physiological stimuli.

The activity of the anterior pituitary gland is regulated in turn by the hypothalamus. This part of the brain manufactures and, under proper stimulus, releases certain polypeptide factors into the pituitary portal circulation and then into the anterior pituitary which, in turn, releases the various hormones. These thalamic-releasing factors are the corticotropin, growth hormone, thyrotropin, and luteinizing hormone, or CRF, GHRF, TRF, and LRF, respectively; CRF is also formed in the posterior pituitary.

II. Classification of Pituitary Tumors

Ideally, pituitary tumors should be classified on the basis of the cells specifically concerned with the various hormonal secretions. However, much clinical material was described when modern histochemical, immunofluorescent, and electron microscopic techniques were not available and, hence, simpler staining techniques were the basis for classification. The following is the usually accepted classification for tumors of the adenohypophysis (anterior pituitary) (Grollman, 1964; Daughaday, 1968): acidophilic tumors, basophilic tumors, chromophobe adenomas, and craniopharyngiomas. Some values on the relative incidence of these tumors are available. In a series of 595 adenomas treated surgically at the Mayo Clinic, 565 were chromophobic, 30 were eosinophilic (acidophilic), and no basophilic tumors were observed (Grollman, 1964). In Cushing's series (Henderson, 1939) of 338 pituitary tumors, chromophobe tumors accounted for 85% and acidophilic tumors for 10–14%. The general incidence of acidophilic tumor has been stated to be about one in 5,000 to 15,000 patients although, in a later study, the incidence has been set at 1:3000 hospital admissions (Gershberg *et al.*, 1957).

Basophilic tumors are much rarer. Craniopharyngiomas are the most common tumors involving the hypophysial area but, strictly speaking, they are nonpituitary tumors arising in the vicinity of the sella turcica and compress or invade the pituitary gland. Pituitary tumors may be manifested by endocrine disturbances because of interference with hypothalamic–hypophysial function and/or neurological symptoms because of impingement upon or invasion of neighboring structures.

The relative incidence of pituitary neoplasms, as judged by histology and the staining properties of the cells, has been called into question by McCormick and Halmi (1971). They contended that the vast majority of reports have depended on a technique which fails to reveal the presence and staining characteristics of the granules in the majority of pituitary tumors, except the most heavily granulated. When appropriate stains, thin sections, and adequate fixation are employed, the chromophilic identity of the cells composing the neoplasm become more identifiable. McCormick and Halmi (1971) reported that, of 145 pituitary adenomas found in about 1600 consecutive autopsies, 59% were acidophilic, 18.4% were derived from basophilic, β_1 cells, and 15.8% from mixed cells; 5.5% could not be identified and none was unequivocally chromophobic. Of 21 pituitary adenomas biopsied during removal, all 19 that could be classified were acidophilic. Only 3% of acidophilic adenomas were associated with typical acromegaly, and 7% of all β_1-cell tumors with Cushing's syndrome. Electron microscopy has also revealed true chromophobes to be uncommon or even nonexistent in human hypophyses.

III. Acidophilic Tumors: Acromegaly and Gigantism

A. Introduction

The smallest acidophilic adenomas are unencapsulated but develop a capsule as they grow larger. The granules in the tumor cells may be finer than those of the normal acidophil and are distributed about the periphery of the cell. In a review of 50 cases, Young *et al.* (1965) classified the adenomas in two classes—6 typical cases and 44 atypical cases. The typical adenomas were composed of well-granulated acidophils, whereas the atypical cases were a heterogeneous group of large pituitary tumors composed of moderately or sparsely granulated acidophils and agranular cells that were not distinguishable from chromophobes. We noted earlier in this chapter that the weight of the pituitary in normal individuals ranges from 0.4 to 1.1 gm, with an average of 0.5–0.6 gm in the male and 0.6–0.7 gm in the female. In a review of

100 cases, Gordon *et al.* (1962) were able to obtain weights in 9 cases; these ranged from 0.9 to 10.8 gm and averaged 4.1 gm. Using a biological assay, Young *et al.* (1965) found high concentrations of growth hormone in both of 2 typical tumors tested and relatively low concentrations in 3 of 4 assays of atypical tumors.

The clinical symptomatology of acromegaly was first described in some detail by Davidoff in 1926 and has been amplified in later reports (Gordon *et al.*, 1962; Young *et al.*, 1965). The disease usually starts insidiously in the third to the fifth decades. Among the most common symptoms are excessive growth of the acral parts, soft tissue growth, weight gain, hypermetabolism, hyperhidrosis, hypertrichosis, and pigmentation. This symptomatology chiefly results from excessive secretion of GH, although disturbances of other hormonal functions may be involved. These will be discussed presently. Of interest is the variability of the disease, for frequently the progress of the disease abates. This may result from a failure of the various organs to respond to continued excessive secretion of GH but, in other cases, hormonal secretion ceases because of infarction of the tumor.

When the disease starts before puberty and before the closure of the epiphyses, the impressive condition of gigantism develops. A general proportionate or symmetrical growth of the body tends to occur at first. Both the length of the body and the size of the viscera exceed the average measurements. Before our present era of excellent nutrition and crop of massive football and basketball players, heights greater than 6.5 ft were unusual in normal individuals, and those ranging in height from 6.5 to 8 ft usually suffered from prepurberal acidophilic adenomas. In 1937, Gray reported that 11 such cases of gigantism had been recorded in the literature. At approximately the same time, McFarland (1938) reported a series of 31 patients, all of whom were at least 7.5 ft in height and 50% of whom were over 8 ft tall. Two of the patients were over 9 ft tall.

The general clinical features of gigantism may be illustrated by the report of Prezio *et al.* (1961) on the "Buffalo Giant." This individual weighed 9 lb 5 oz at birth and attained heights of 5 ft 6 in. at 12 years of age, 6 ft 2 in. at 16 years, 7 ft at 20 years, and 8 ft at the age of 27 years. His parents and a sister were of normal height, although there were reports of excessive height in individuals on both the maternal and paternal sides of his family. The patient was skeletally deformed with typically acromegalic features. Neurological changes, consisting of a bilateral foot drop and peroneal atrophy, appeared secondary to peroneal nerve compression as a result of subluxation at the knee joints. Hypogonadism was definitely present as manifested by lack of develop-

ment of secondary sexual hair, small genitalia, and a complete absence of libido.

B. Levels of Human Serum GH

1. In Normal Individuals

The concentration of plasma (or serum) human growth hormone (HGH) is elevated in patients with acromegaly (Hartog *et al.*, 1964; Daughaday, 1969). In this connection, it is important to note that normally GH is secreted predominantly in short bursts, during which the plasma concentration rises abruptly (Daughaday, 1969). For example, the average level in a group of normal adults fluctuated from a mean value of about 2ng/ml or less at about 11 P.M., 1 hour before going to sleep, to a maximum mean value of approximately 27 ng/ml, about 1 hour after falling asleep, then declined within the next 3 or 4 hours to a minimum value of 2 ng/ml or less. It remained at this level throughout the waking day, except for a small rise to about 5 ng/ml at about noon. Hartog *et al.* (1964) found that 20 of 22 normal adult subjects had 14-hour fasting serum HGH levels of less than 10 ng/ml. The other 2 had concentrations of 12 and 26 ng/ml. The development of hypoglycemia, as by the intravenous injection of insulin, caused a marked rise in the concentration of serum GH to levels ranging from about 10 to 70 ng/ml. The intravenous infusion of amino acids, particularly arginine, also led to substantial rises in the concentration of serum HGH (Daughaday, 1969).

2. Serum GH Levels in Acromegaly

The fasting serum GH levels in a group of 5 acromegalics ranged from 24 to 74 ng/ml and were much higher than those in normal individuals (Hartog *et al.*, 1964). As was true for the normal individuals, the level also fluctuated during the day (Fig. 11-1) but, contrary to the occurrence of rises in plasma GH after the onset of sleep in normal persons, none appeared to occur in the acromegalics and the fluctuations during the day appeared to be much greater. Abnormally high levels of serum GH persisted during glucose tolerance tests in 4 out of 5 patients with acromegaly, only one showing a distinct fall from a fasting level of 66 ng/ml to a level of 41 ng/ml at 120 minutes (Hartog *et al.*, 1964).

Cryer and Daughaday (1969) have pointed out that the increased release of GH in response to hypoglycemia requires the integrity of

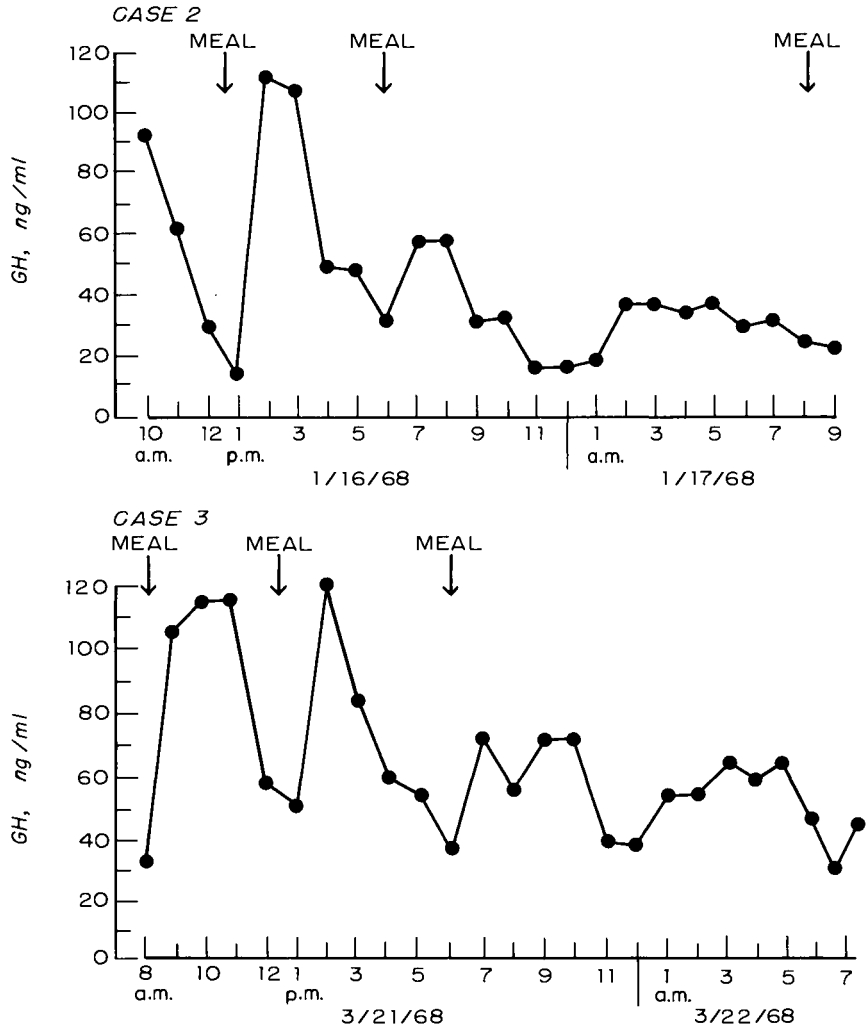

Fig. 11-1 Plasma GH concentrations obtained on 2 patients with acromegaly through a 24-hour period. From Cryer and Daughaday (1969). Reproduced by permission of Dr. M. B. Lipsett, Editor-in-Chief, *Journal of Clinical Endocrinology and Metabolism* and The Endocrine Society.

the median eminence region of the hypothalamus and the hypothalamic pituitary portal region. They, therefore, considered it probable that persistent hypothalamic activity is responsible for the fluctuations in plasma GH during the day and for the changes in response to various stimuli. The fact that these alterations in plasma GH are much greater in acro-

megalics than in normal persons could be explained by the great mass of tissue present in the tumor that secretes GH.

3. Relationship of Serum GH Level to Size of Pituitary Tumor in Acromegaly

As we have just observed, considerable variability in serum GH levels exists among different acromegalic patients. Moreover, not all show persistence of serum GH levels during glucose tolerance tests. The possibility that this variability might be related to the size of the tumor was explored by Wright *et al.* (1969). The serum GH levels were measured after an overnight fast and again at intervals of 20 minutes for 2–2½ hours after oral ingestion of 50 gm glucose. The area under the points was determined and divided by the time in hours to give the mean level of the hormone during the glucose tolerance test. The range of mean levels was 8–1860 ng/ml. The logarithms of these values were then plotted against the maximum lateral area of the pituitary fossa as determined by tomography. The regression coefficient, r, was 0.47 ($p < 0.01$). In other words, the hormone level was more responsive to oral glucose and insulin-induced hypoglycemia in patients with smaller tumors.

C. Carbohydrate Metabolism

The existence of carbohydrate metabolic disturbances in acromegaly has long been appreciated. In 1908, Borchardt reviewed 176 cases of acromegaly that had been collected up until then and reported 63, or 35.5%, to have frank diabetes and 8 more to have alimentary glycosuria. It should be realized that at that time the term "diabetes" referred chiefly to the rather constant excretion of sugar in the urine. In the classic study by Davidoff and Cushing in 1927 on 100 acromegalic patients who had been admitted to the Peter Bent Brigham Hospital during the 12½ years prior to 1926, 25 patients had glycosuria and 12 others had a fasting blood sugar which was somewhat elevated, ranging from 110 to 160 mg per 100 ml. The sugar tolerance in acromegalics was low and became normal after operative removal of the pituitary adenoma (Wright *et al.*, 1969). Insulin did not control the "diabetes" in acromegaly as well as it usually did in cases of pancreatic diabetes.

These observations have been amply confirmed and extended by subsequent workers. Thus, in a study of 100 acromegalic patients (Gordon *et al.*, 1962), 18 had glycosuria. Of the remaining 82, 29, or 35%, had decreased glucose tolerance curves, 18 had normal glucose tolerance,

and 5 others had rather flat curves. It is, of course, possible that pancreatic diabetes may occur in association with acromegaly appearing at varying intervals, up to 37 years in one report, after the onset of acromegalic symptoms. This may occur in spite of the apparent arrest of the acromegalic condition and the relief of symptoms by deep irradiation of the pituitary (Darragh and Shaw, 1951).

Patients with acromegaly frequently show evidence of insulin resistance. In the normal individual, the injection of 0.1 unit insulin per kg causes a decrease from a normal level of about 90 mg per 100 ml to a level of approximately 50 mg per 100 ml in 30 minutes with a return to normal by 90 minutes (Kupperman, 1963). In the insulin-resistant acromegalic, there is no decrease. On the other hand, in the "burned-out" acromegalic who is presumably no longer secreting GH, the injection of insulin may show the normal decrease in the concentration of blood glucose. The varying admixture of inactive and active acromegalics in groups tested for insulin resistance undoubtedly explains the variability of the results obtained by different investigators (Berg, 1937; Albright and Elrick, 1948).

More direct effects of HGH on human carbohydrate metabolism have been demonstrated by Zierler and Rabinowitz (1963). Human growth hormone was infused intra-arterially for 20 minutes in the forearm of 5 normal young men. Sampling from the artery and superficial and deep veins of the same forearm showed a sharp decrease in the arterial-venous (A-V) differences of glucose during the infusion and an increase toward a normal difference after the end of the transfusion. In other words, the concentration of GH employed reduced glucose uptake by both forearm muscle, as manifested by the arterial-deep venous (A-DV) difference, and by forearm adipose tissue, as reflected in the arterial-superficial venous (A-SV) difference. Although forearm O_2 consumption was unchanged, forearm respiratory quotient (RQ) fell from a mean of 0.75 before HGH to a mean of 0.61 at the peak effects. The increased oxidation of free fatty acid (FFA) suggested by this effect was also evidenced in the increase of A-DV difference for FFA.

In contrast, the infusion of 100 μU of insulin per kg per minute showed a marked increase in the A-V differences or uptake of glucose by both muscle and adipose tissue. The extra glucose taken up by the forearm tissue could not be accounted for, except to a very small degree, by increased lactate production or oxygen consumption. The RQ, which was about 0.7 before insulin, continued at this level, thus indicating that the forearm muscle continued to oxidize lipid chiefly. It appears clear that the extra glucose was taken up by muscle under the influence of insulin. Human growth hormone and insulin, injected simultaneously,

modified each other's effect so that the uptake of glucose rose only slightly.

Zierler and Rabinowitz (1963) reported that 6 patients with acromegaly showed a greater than normal oxygen consumption, more negative A-DV and A-SV differences in FFA than normal, and muscle uptake of potassium. When insulin was infused intra-arterially, at a rate of 100 μU per kg per minute, the FFA output was reduced.

D. Calcium-Phosphorus Metabolism

Roentgenologic evidence of skeletal rarefaction in some instances of acromegaly stimulated interest in the problem of phosphorus-calcium metabolism in this disease. Several studies in the older literature showed that, on the 3-day basal calcium intake of 0.32 mg, the excretion of calcium in the urine of acromegalics was substantially higher than in control subjects (Scriver and Bryan, 1935; Bauer and Aub, 1941). Table 11-1 summarizes a more recent and complete study by Bell and Bartter (1967) employing ^{47}Ca. All patients showed a negative calcium balance on daily calcium intakes ranging from about 300 to 500 mg. Urinary calcium was moderately increased in the 2 untreated patients and normal in the 2 treated patients. Nadarajah *et al.* (1968) reported that the 24-hour urinary excretion of calcium was higher than the upper limit of normal, 300 mg, in 47% of a group of 68 untreated patients with acromegaly.

In Chapter 10, we discussed the definitions of the terms: exchangeable calcium, E; bone accretion, A, or formation rate, BFR; and bone resorption rate, BRR. In a series of 7 normal individuals, Bell and Bartter (1967) obtained values for these parameters that were in agreement with those we noted in Chapter 10, obtained by other investigators (Bauer *et al.*, 1957; Lafferty and Pearson, 1963; Rinsler *et al.*, 1965). In all 4 acromegalic patients, the bone accretion and resorption rates were substantially elevated to 2–3-fold the normal levels, and the values were comparable to those produced in normal subjects by the administration of GH.

Whereas Bauer and Aub (1941) as well as Reifenstein *et al.* (1946) had noted that the serum calcium levels were normal and the serum phosphorus elevated in their acromegalic patients, Nadarajah *et al.* (1968) found that 12 of a series of 78 patients with untreated acromegaly, or 16%, had elevated serum calcium levels, ranging from 5.5 to 6.2 mEq/liter or 11.0 to 12.4 mg per 100 ml. Nadarajah *et al.* (1968) as well as Brown and Singer (1969) have raised the possibility that, in some acromegalic patients, excess GH secretion over a protracted period

TABLE 11-1

Parameters of Calcium Metabolism in Acromegaly[a]

Group	Fecal Ca (mg/day)	Urinary Ca (mg/day)	Ca balance (mg/day)	E^b (gm/day)	BFR[b] (mg/day)	BRR[b] (mg/day)
Normal subjects[c] (mean and range)	226 (76–348)	71 (21–147)	37 (−139–+250)	4.36 (3.48–4.90)	673 (516–848)	636 (314–867)
Acromegalic patients						
No. 1	338	146	−190	7.00	1648	1838
No. 2	226	146	−79	6.31	1425	1504
No. 3	261	344	−154	6.02	2078	2232
No. 4	336	217	−57	4.63	1898	1955

[a] Based on data of Bell and Bartter (1967).

[b] The abbreviations are for the following parameters: E, exchangeable calcium; BFR, bone formation (or accretion) rate; and BRR, bone resorption rate.

[c] This group consisted of 7 normal subjects.

may cause parathyroid hyperplasia and possibly adenomas, leading to high normal or even elevated serum calcium levels.

Serum inorganic phosphate is elevated in a substantial number of patients with acromegaly (Bauer and Aub, 1941; Reifenstein *et al.*, 1946; Bell and Bartter, 1967; Nadarajah *et al.*, 1968). For example, Nadarajah *et al.* reported that 15 of 78 acromegalics, or 20%, had elevated serum inorganic phosphate levels, that is, values above 2.6 mEq/liter. Corvilain and Abramow (1972) have recently found (Table 11-2) that the glomerular filtration rate (GFR), the maximal tubular reabsorption rate, and the reabsorption of phosphate are significantly higher in patients with untreated or "active" acromegaly than in adult controls without this disease. These data indicate that chronic oversecretion of GH alone can account for the raised tubular reabsorption capacity seen in active acromegaly. In the 12 patients with acromegaly, the serum concentration of GH, measured at 9:00 A.M. at rest and in the fasting state, ranged from 14 to 240 ng and averaged 73 ng/ml; the normal values were considered to be less than 10 ng/ml. These values for fasting acromegalics are higher than those reported by Hartog *et al.* (1964) which we considered earlier in this chapter (Section B,2).

E. Effect on Other Hormones

Davidoff (1926) listed the following incidences of clinical evidence of disturbances of other hormones in acromegaly: galactorrhea, 4%; increased libido, 38%; decreased libido, 23%; and hyperadrenocorticism and hyperthyroidism, rare. To document the possibility of hypogonadism objectively, Rosenfield *et al.* (1970) found that the plasma testosterone levels in 25 normal adult males ranged from 238 to 1001 ng per 100 ml and those in 27 normal adult females from 27 to 83 ng per 100 ml. Testosterone levels less than 238 and, indeed, less than 162 ng per 100 ml were found in 7 of the 15 males with acromegaly. The plasma testosterone levels in 10 acromegalic females ranged from 21 to 175 and averaged 69 ng per 100 ml. Only one was less than the normal range. Kjellberg *et al.* (1968) reported low plasma testosterone values in all 7 acromegalic males tested.

As we have already noted, the incidence of galactorrhea in acromegaly is low. Turkington (1972) determined the serum prolactin levels in a series of 8 patients with acromegaly, all of whom had elevated levels of serum GH but none of whom had galactorrhea. The serum prolactin level was normal, that is, below 2 ng/ml, in 7. One patient had an elevated level of 42 ng/ml. In contrast, all 9 patients with a diagnosis of chromophobe adenoma had galactorrhea and preoperative elevated

TABLE 11-2

Parameters for Renal Control of Plasma Phosphate in Patients with Untreated Acromegaly[a]

Description of group	Number	Fasting plasma phosphate (mg/100 ml)	Glomerular filtration rate (ml/minute)	Maximal tubular reabsorption (T_mPO_4) (mg/minute)	Reabsorption of phosphate $\left(\frac{T_mPO_4}{GFR} \times 100\right)$ (mg/ml × 100)
Adult controls (23–56 years)	19	3.14 ± 0.51	120 ± 18	3.69 ± 1.01	3.04 ± 0.49
Untreated or "active" acromegalics (20–75 years)	12	4.50 ± 0.50[b]	148 ± 32[b,c]	7.02 ± 1.8[b,c]	4.71 ± 0.49[b]

[a] From Corvilain and Abramow (1972).
[b] Significantly higher than the corresponding values for the adult controls.
[c] Calculated for a standard body area of 1.73 m^2.

serum prolactin levels from 65 to 980 ng/ml. Postoperatively, these levels decreased, but galactorrhea was absent from only those patients with normal serum prolactin levels. In the remaining 6 patients, intermittent galactorrhea persisted, and their serum prolactin levels remained slightly elevated.

In 1926, Davidoff observed that the adrenals are enlarged in acromegaly. Russfield *et al.* (1956) found that the weight of the combined adrenals in a series of 8 acromegalics ranged from 16 to 25 gm as compared with a weight of 12–15 gm in normals (Bodansky and Bodansky, 1952; Studzinski *et al.*, 1963). Although certain of the basophils of the anterior pituitary as well as some large chromophobe cells appear to be the source of corticotropin, there is some indication for increased activity of corticotropin in acromegaly. Forsham (1968) summarized the mechanism of the stress regulation of adrenocorticotropin secretion. A stressful stimulus reaching the cerebral cortex releases the sustained inhibition upon hypothalamic centers. Large secretory neurons then secrete corticotropin-releasing factor (CRF) into the hypophysial portal circulation and then into the anterior pituitary gland. The formed adrenocorticotropin (ACTH) accumulates in the adrenal glands and, through a series of biochemical reactions which will subsequently be described in greater detail, leads to the formation of various steroids. The chemical structure and interrelationships of the steroids will be discussed in greater detail in later chapters, particularly Chapter 12.

The rate of secretion of cortisol, as determined by the isotope dilution method, was found to be increased in patients with acromegaly (Roginsky *et al.*, 1966). The rates, expressed as milligrams secreted per day, were as follows: 18 normal subjects, range of 5.0 to 31.4 and mean of 10.9; 2 untreated female acromegalics, 34.2 and 24.0; and 10 patients who had been treated with external radiation to the pituitary region, a range of 16.8 to 41.0 and a mean of 27.2. Of 13 acromegalic patients, including the above, 5 had elevated 24-hour urinary 17-ketosteroids and 9 had elevated 17-ketogenic steroids. Nine patients were studied with respect to the effect of dexamethasone, a suppressor of adrenal function. In all of these cases, the excretions of the 17-ketosteroids and 17-ketogenic steroids were decreased substantially, in most instances to 50% of the control values or less. These data indicated that adrenal cortical hyperfunction is present in a majority of acromegalic patients.

There have been conflicting reports about the incidence of, and basis for, the occurrence of hypertension in acromegaly. Souadjian and Schirger (1967) reported the results on 46 patients with acromegaly and diastolic hypertension admitted to the Mayo Clinic during the 10-year period, 1955–1964. Sixteen of these were studied before and after

irradiation to the sella turcica. A statistically significant decrease in the diastolic blood pressure, from 112 to 97 mm Hg, closely paralleling a statistically significant decrease in serum phosphorus concentration, occurred after irradiation.

The basis for the hypertension in acromegaly has been sought in a number of factors. The enzyme, renin, is involved in renal hypertension by virtue of its capacity to split the polypeptide, hypertensin I, from hypertensinogen, a serum α_2-globulin formed by the liver. The hydrolytic liberation of a dipeptide from hypertensin I yields hypertensin II which acts directly on the adrenal gland to stimulate release of aldosterone, a steroid which plays a major role in sodium retention (White *et al.*, 1968).

Because of the increased incidence of hypertension in acromegaly, Cain *et al.* (1972) undertook to study the upright plasma renin activity (UPRA) and the aldosterone secretion rate. In a series of 13 acromegalic patients, restricted to a daily intake of 2500 ml fluid, 10 mEq sodium and 100 mEq potassium, the mean aldolsterone secretion rate (ASR) was 459 ± 55 μg/day. This value was significantly less than the mean values of 874 ± 67 μg/day in 30 normal subjects, of 897 ± 185 μg/day in 10 patients with essential hypertension, and of 822 ± 120 μg/day in 6 patients receiving glucocorticoids (Cain *et al.*, 1972). This subnormal ASR may reflect an altered metabolic clearance of aldosterone, a defect in the action of the renin–angiotensin system in stimulating aldosterone, or a block in aldosterone biosynthesis.

F. Cerebral Gigantism in Childhood

Although this condition does not represent a pituitary tumor, its relationship to our preceding discussions merits brief consideration. In 1964, Sotos *et al.* reported a group of 5 children who were characterized by excessively rapid growth in height and weight, acromegalic features, and a nonprogressive neurological disorder with mental retardation. There was, however, no evidence of pituitary tumor, as judged either by clinical manifestations such as increased intracranial pressure, abnormal cerebrospinal fluid, or enlarged sella turcica. By 1970, approximately 50 such cases had been reported and the various clinical and biochemical features were summarized by Mace and Gothin (1970) in those cases in which these were available and in 4 new cases which they presented. The presence of a period of rapid growth, advanced bone age, and absence of isosexual precocity was the triad of clinical findings most helpful in diagnosis. However, the biochemical findings were usually normal. For example, urinary 17-ketosteroid (17-KS) excretion was nor-

mal in all 4 cases reported by Mace and Gothin (1970) and in 21 cases reported in the literature in which this determination had been done. The urinary 17-hydroxycorticosteroids (17-OHCS) was normal in the 4 new cases and in 25 of 26 recorded in the literature. Impressive was the finding of normal serum GH levels in the 4 cases reported by Mace and Gothin (1970) and in all of 19 cases recorded in the literature. Similarly normal findings were obtained for the ACTH-stimulation test, urinary gonadotropins, thyroid function, glucose tolerance tests, and serum phosphorus levels. A later report (Schneider and Vassella, 1971) has not modified these findings. Although the high occurrence of normal biochemical findings would appear to be inconsistent at first glance with the clinical symptomatology, it is quite possible that most of these determinations were made when the period of rapid growth had ceased. For example, in the first cases reported by Sotos *et al.* (1964), the children grew most rapidly during the first to third years of life. Three of the 5 were not observed clinically until they were 7 to 11 years of age.

IV. Cushing's Syndrome

In 1932, Cushing presented 12 cases and later an additional 2 cases of what he believed to be a new syndrome (Cushing, 1932a,b, 1933). In 5 of the 8 cases that came to autopsy, an unsuspected pituitary adenoma was found and 3 of these were definitely basophilic. Since these early findings, much evidence has arisen that the clinical aspects of Cushing's syndrome may result not only from a pituitary adenoma which secretes ACTH, and thus affects the adrenal cortex, but also from other conditions such as aberrant regulation and release of ACTH from the pituitary; the presence of a primary tumor, benign or malignant, in the adrenal cortex; the production of ACTH-like substances from carcinomas elsewhere in the body; or even factitious production by steroid medication.

Pituitary adenoma appears to be the primary cause of Cushing's syndrome in only a fair proportion of patients with the characteristic symptoms. Plotz *et al.* (1952) reviewed 33 patients with Cushing's syndrome who were seen at the Columbia Presbyterian Medical Center from 1932 to 1951 and an additional 189 patients from the literature. Adequate histological data on both the adrenals and pituitary gland were available in 97 of these cases and showed an impressively variable combination of pituitary and adrenocortical abnormalities The five most common in their order of frequency were: (a) basophilic pituitary adenoma with

adrenal hyperplasia; (b) adrenal hyperplasia with no pituitary lesion other than Crooke's changes; (c) no pituitary lesion other than Crooke's changes with either an adrenal carcinoma or (d) unilateral adrenal hypertrophy or a unilateral benign tumor; and (e) chromophobe adenoma of the pituitary with adrenal hyperplasia. Of these 97 cases, adenomas of the pituitary were found in 47 patients, and in 31 of these the tumor was basophilic in character. Seven were chromophobic.

Salassa *et al.* (1959) reported that, of 156 patients with Cushing's syndrome whose adrenals were surgically explored at the Mayo Clinic over a 13-year period, 34 had adrenocortical tumors and 122 had hyperplastic adrenal cortices. Roentgenographic examination of the skull with particular reference to enlargement, erosion, and decalcification of the sella in the 34 patients with adrenocortical tumors failed to show any evidence of pituitary tumor. However, such examination indicated the presence of abnormalities consistent with the presence of a pituitary tumor, either before or after adrenalectomy, in 12 of the 122 patients who had adrenocortical hyperplasia.

It was of interest that, in the 7 of the 12 cases in which the pituitary tumor was examined microscopically, the lesions were those of chromophobe adenomas. Plotz *et al.* (1952) also found this type of pituitary tumor in Cushing's syndrome. These observations are certainly not consistent with the picture of basophilic adenoma originally presented by Cushing (1932a,b). The possibility exists, as McCormick and Halmi (1971) have pointed out, that the designation of a pituitary adenoma as chromophobe in character reflects inadequate fixing and staining of the tissue.

The frequencies of the more common clinical manifestations of Cushing's syndrome, whatever its genesis may be, have been listed by Plotz *et al.* (1952) in their review of 189 cases as follows: obesity, 97%; hypertension, 85%; amenorrhea, oligomenorrhea, or impotence in men, 71%; plethoric appearance, 50%; and purple striae, 71%. Their own smaller series of 33 cases shows the same or even a somewhat higher incidence of these signs and symptoms. In addition to these more common manifestations, mental symptoms, weakness and backache, acne or skin pigmentation, headache, etc., are also encountered.

Since the biochemical manifestations of Cushing's syndrome are chiefly those of adrenocortical stimulation, we shall consider these in detail in Chapter 12. We may note here briefly some of the blood biochemical values obtained by Plotz *et al.* (1952). The fasting blood sugar was above 100 mg per 100 ml in 49% of the 33 patients, and a diabetic glucose tolerance curve was elicited in 94% of 31 cases. Levels of serum calcium and phosphorus were normal in almost all instances. Serum cholesterol ranged from 147 to 460 mg per 100 ml in 28 cases and

was above 250 mg per 100 ml in 39% of the cases. However, these levels may have been a concomitant feature of the atherosclerosis present in the age group under study. As we shall note later (Chapter 12), high serum sodium and bicarbonate levels and low potassium and chloride levels are frequently found in Cushing's syndrome. In the group reviewed by Plotz *et al.* (1952), only 37% had chloride values below 100 mEq/liter and the lowest was 89 mEq/liter. The carbon dioxide content ranged from 21.2 to 46.2 mEq/liter and 64% had values above 28 mEq/liter. Four of 14 cases had sodium values above 145.0 mEq/liter and the serum potassium in 11 cases ranged from 3.2 to 5.3 mEq/liter, essentially within the normal region.

Several cases have been reported in whom bilateral adrenalectomy was performed for the treatment of Cushing's syndrome and who were later found to have ACTH-secreting chromophobe adenomas (Nelson *et al.*, 1958; Cassidy, 1960; Dingman and Lim, 1962; Cloutier *et al.*, 1966). The question had been raised whether these cases represent the later growth of a chromophobe adenoma unsuspected at the time of adrenalectomy or whether adrenalectomy acts as a stimulus to ACTH secretion and production of a pituitary adenoma (Nelson *et al.*, 1958).

V. Chromophobe Adenoma

A. Introduction

We have already noted (Section II) that chromophobe tumors comprised 85% of the 338 tumors in Cushing's series (Henderson, 1939). In contrast, of 145 pituitary adenomas found in about 1600 consecutive tumors, McCormick and Halmi (1971) could find none that were unequivocally chromophobic in nature. As we have pointed out earlier in this chapter (Section II), this discrepancy could be accounted for by differences in histological techniques.

The clinical aspects of a series of 60 patients (30 males and 30 females) with chromophobe adenoma were described in some detail by Mogensen in 1957. All of these patients were subjected to operation because of enlargement of the sella turcica and limitation of fields of vision. In general, the patients were pale in spite of a normal hemoglobin level and were slightly obese. The fat distribution in men tended to be of a feminine nature. Growth of axillary and pubic hair was absent or scanty, and the growth of pubic hair in the males was of feminine distribution. Impairment or loss of potency was present in 5 of 21 male patients questioned. Cushing's syndrome was present in one patient, and early

hirsutism developed in another. In the group of women, amenorrhea was a frequent and early manifestation and fertility semed to be lower than normal. One patient developed acromegalic features. It will be recalled that, of 122 patients with adrenocortical hyperplasia and Cushing's syndrome studied by Salassa *et al.* (1959), 12 had pituitary tumors sufficiently large to affect the sella. As a group, these 12 patients had a high incidence of cutaneous pigmentation and unusual ophthalmological difficulties.

Few quantitative biochemical results were available from Mogensen's study (1957). Serum sodium and potassium concentrations were determined preoperatively in 8 men and 12 women and normal values were present in all cases. The urinary excretion of 17-KS was low in only 4 of the 18 males and in 4 of the 19 females in whom determinations were done.

B. Cushing's Syndrome and Chromophobe Adenoma

In the last 15 years, an increasing number of reports concerning the association of Cushing's syndrome with chromophobe adenoma have appeared. Some of these, containing detailed biochemical data, will now be described briefly.

Cassidy (1960) reported 2 cases of Cushing's syndrome that were associated with large pituitary chromophobe adenomas. The first patient, a female 30 years of age, had radiation therapy without apparent effect, was operated on for removal of the tumor, and died 6 days postoperatively. Autopsy revealed a large tumor which had invaded the cavernous sinus and middle fossa. On two occasions, prior to any treatment, the urinary 17-KS were 55 and 27 mg per 24 hours, as compared with a normal value of 10 ± 5 (SD) mg per 24 hours. The corresponding values for 17-OHCS were 27 and 23 mg per 24 hours, again higher than the range of normal values, 7 ± 4 (SD) mg per 24 hours for females. Dexamethasone failed to decrease the excretion of these compounds as occurs in normal individuals.

Elevated excretions of these 2 urinary steroids were also noted in a case reported by Dingman and Lim (1962). The patient, a 29-year-old male, exhibited the typical features and findings of Cushing's syndrome, together with roentgenographic skull findings consistent with a pituitary tumor. Of the routine blood chemical findings, those of interest were calcium, 12.8 mg per 100 ml, and chloride, 95.0 mEq/liter. A plasma ACTH assay showed an elevated level of 1.2 milliunits per 100 ml.

Exploratory craniotomy revealed a large cystic intrasellar tumor mass. Approximately 60–70% of the tumor was resected and found to be a chromophobe adenoma. The slightly elevated serum calcium decreased to normal levels and the urinary 17-KS and 17-OHCS decreased. Although there was some clinical improvement in the first few weeks, recrudescence of the clinical signs suggested regeneration of the pituitary tumor and urinary steroid levels returned to prehypophysectomy levels.

C. Hyperthyroidism and Chromophobe Adenoma

The presence of hyperthyroidism in patients with chromophobe adenoma is rare and raises the question whether this is merely coincidence or a manifestation of the production of thyrotropin by the neoplasm (Jailer and Holub, 1960; Jackson, 1965; Lamberg *et al.*, 1969; Hamilton *et al.*, 1970). Although we shall discuss measures of thyroid function more fully in Chapter 13, we may briefly mention a few of these now as a guide for our present discussion. Thyroxine is transported in the plasma almost completely bound to 3 carrier proteins, namely, thyroxine-binding protein, prealbumin, and albumin. Triiodothyronine is also associated with the thyroxine-binding globulin but is less tightly bound. The normal mean values for serum total protein-bound iodine (PBI) obtained by various investigators range between 4 and 6 μg per 100 ml (Riggs, 1947; Kydd *et al.*, 1950; Bodansky, *et al.*, 1958). The level of free thyroxine is about one-thousandth of this value, namely, about 4–5 ng per 100 ml (Ingbar *et al.*, 1965; Sterling and Brenner, 1966). The inorganic iodine is normally about 0.5–1.5 μg per 100 ml (Riggs, 1947; Kydd *et al.*, 1950; Bodansky *et al.*, 1958). Triiodothyronine, 3,3′-diiodothyronine, and monoiodotyrosine are normally present in extremely small amounts. The uptake of radioactive iodine, ^{131}I, by the thyroid during a stated period after the ingestion or injection of inorganic iodide containing this isotope has been used as a measure of the function of this gland. The mean 24-hour uptake ranges from 15 to 30%, and averages about 25%. The measurement of the serum protein-bound radioactive iodine ($PB^{131}I$), expressed as percent of the administered dose, also serves as an indicator of thyroidal hyperfunction.

The occurrence of hyperthyroidism in patients with pituitary chromophobe adenoma has been described by Jackson (1965) and by Hamilton *et al.* (1970). Figure 11-2 shows the clinical course and sequential values for several biochemical measures of thyroid function in a patient with chromophobe adenoma (Hamilton *et al.*, 1970). Serum thyroxine, serum free thyroxine, and 24-hour uptake of ^{131}I and serum thyrotropin were

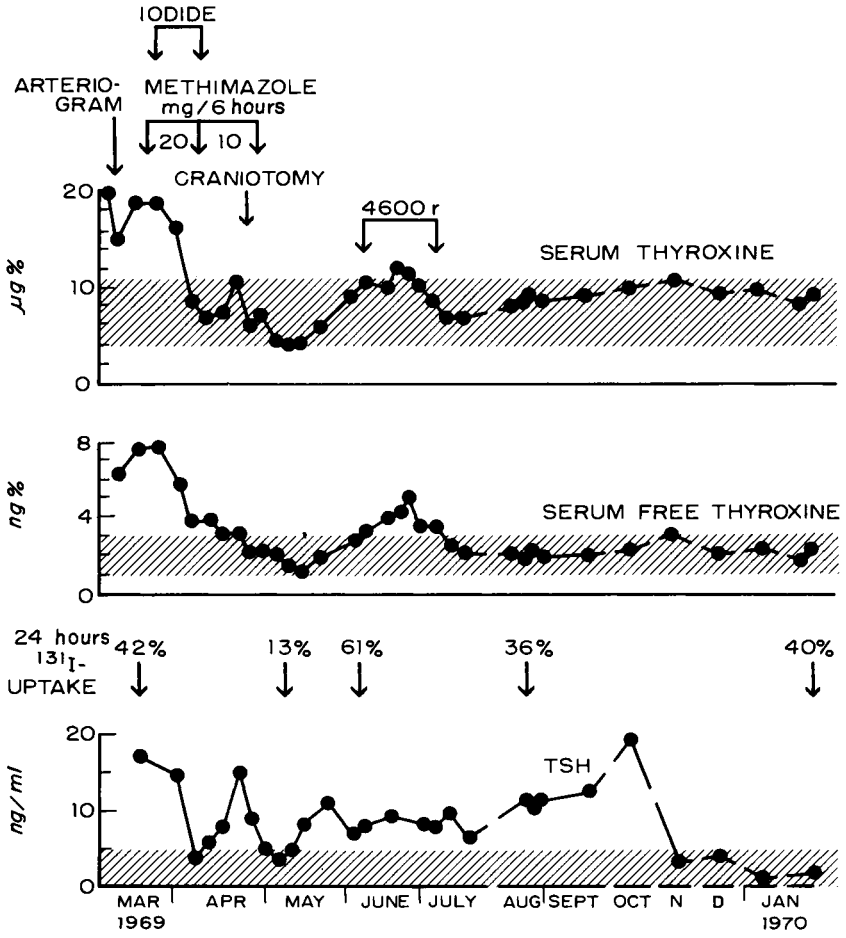

Fig. 11-2 Clinical course and sequential changes in biochemical parameters in a patient with hyperthyroidism and a chromophobe adenoma. Hatched areas indicate normal ranges. From Hamilton *et al.* (1970). Reproduced by permission of the *New England Journal of Medicine.*

elevated at the beginning of the hospital study. Treatment with iodide and the antithyroid agent, methimazole (1-methyl-2-mercaptoimidazole) led to a decrease of these parameters to normal levels and to a clinically euthyroid state. After craniotomy, partial resection of a chromophobe adenoma and discontinuation of the antithyroid therapy, clinical and biochemical evidence of hyperthyroidism, including elevated serum thyrotropin levels, recurred. Treatment of the remaining pituitary tumor

with external radiation led to marked improvement in the visual fields, abatement of the symptoms of hyperthyroidism, and a return to normal of the various biochemical parameters, particularly the level of serum thyrotropin. It would appear that the pituitary adenoma had caused hyperthyroidism by excessive production of thyrotropin.

D. Aldosterone Secretion and Chromophobe Adenoma

Earlier in this chapter, we considered studies on plasma renin activity and aldosterone secretion in patients with acromegaly (Cain *et al.*, 1972). Similar studies were performed by Hauger-Klevene and Cole (1970) in a series of 20 patients with chromophobe adenoma, 17 of whom had received treatment by hypophysectomy and/or x-ray. The normal aldosterone secretion rate (ASR) reported by Cain *et al.* (1972) was 874 ± 67 (SD) μg/day on a daily diet of 10 mEq sodium and 100 mEq potassium. Hauger-Klevene and Cole (1970) did not submit a definite mean value for the normal ASR, but stated it to range between 100 and 180 μg per 24 hours on a daily intake of 100 mEq sodium and 90 mEq potassium and between 200 and 350 μg per 24 hours on a daily intake of 10 mEq sodium and 90 mEq potassium per day. The mean rates in 20 patients with chromophobe adenoma were 117 ± 56 (SD) μg per 24 hours on the high sodium diet and 177 ± 64 (SD) μg per 24 hours on the low sodium diet. Nine of the 18 patients had ASR values less than 100 μg per 24 hours on the high sodium intake, and 4 of these showed increases to the normal range when placed on the low sodium diet. Following the infusion of ACTH, 5 patients exhibited increases of the ASR values to the levels observed in normal subjects. Of 7 patients on a daily intake of 100 mEq sodium, all had normal plasma renin activities (PRA) in the supine position at 8 A.M. The activity was increased in response to the upright position in 4 of these cases. Of 18 patients on a daily intake of 10 mEq sodium, 11 had low PRA levels in the supine position and 9 in the upright position. In 10 patients, PRA levels failed to increase in response to assuming the upright position.

VI. Craniopharyngiomas

Earlier in this chapter, we noted that, although craniopharyngiomas are not essentially tumors of the pituitary gland, they are the most common tumor involving the hypophysial area and may, therefore, affect

pituitary function. Arising from remnants of the craniopharyngeal duct, they are generally encapsulated, spherical or oval in shape, and, when clinically manifest, range in size from 2 to 10 cm in size. The cut surface shows the presence of one or more cysts which are the result of degeneration. When large, the tumors may compress the optic pathways, pituitary gland, and hypothalamus and extend upward into the frontal lobes or backward through the interpeduncular fossa against the brain stem (Svolos, 1969). Three benign histological types have been described: adamantinomas, cystic papillomas or simple squamous epitheliomas, and mucoid epithelial cysts or Rathke's cleft cysts (Bailey, 1933; Svolos, 1969).

The incidence of craniopharyngiomas may be illustrated by the large series of cases recently reported by various neurosurgeons as, for example, 108 cases observed in the Neurosurgical and Radiological Departments of the Serafimer Hospital, Stockholm, from 1924 to 1958 and reported by Svolos in 1969; a summary of 85 cases out of over 12,000 admissions in the Neurosurgical Department of the Radcliffe Infirmary, Oxford, between the years 1938 and 1970 and reported by Bartlett in 1971. It has been estimated that there are about 150 new cases of craniopharyngioma in England and Wales each year (Bartlett, 1971). However, as compared with intracranial tumors as a whole, craniopharyngiomas represent only a small proportion, 2.37%, of almost 16,000 reported cases (Svolos, 1969). The clinical problems involved in diagnosis and treatment have recently been summarized (Editorial, 1972).

The symptoms, signs, and biochemical manifestations produced by craniopharyngiomas vary partly with the age of onset and partly with the size, location, and direction of spread of the tumor. The chief symptoms and signs are increased intracranial pressure, involvement of the optic pathways and pituitary hypofunction and hypothalamic dysfunction. It is this last group of signs that is of interest here. In a summary of 257 cases from the literature including 108 from his own institution, Svolos (1969) observed impaired growth in 48 cases, hypogonadism in 121, diabetes insipidus in 59, and obesity in 61. However, few specific biochemical or hormonal determinations had been carried out with respect to the 108 cases from his own institution. The serum PBI was low, between 2.1 and 3.8 μg per 100 ml, in 5 of 8 patients, and normal, between 4.7 and 7.2 μg per 100 ml, in the remaining 3. The water test of Soffer and Gabrilove (1952) was carried out in 24 patients and diuresis was found to be impaired in 13. Determinations of urinary 17-KS and 17-ketogenic steroids were made in 1 male 11 years of age and 4 females 15, 7, 10, and 24 years old. They were stated to be below normal in all instances.

VII. Pituitary Tumors and Hypopituitarism

The results which we have just discussed indicate the existence of hypopituitarism rather than hyperpituitarism. It has been pointed out that, as a chromophobe adenoma enlarges, normal pituitary tissue is compressed and the production of hormones is reduced. Nabarro (1972) has stated that the rate of production of the pituitary hormones generally fails in decreasing order as follows: gonadotropin, GH, TSH, and ACTH.

The development of hypopituitarism is not only the result of the presence and growth of chromophobe adenoma but may also occur in other types such as the acidophilic pituitary tumor. Epstein *et al.* (1971) have described the rare condition of pituitary apoplexy which is characterized by a rather sudden onset of headache, loss of vision, and alteration in the level of consciousness. It appears to result from hemorrhagic necrosis of a pituitary adenoma and probably arises as the result of neoplastic cells outstripping the vascular supply and producing ischemic necrosis. Although the process of necrosis makes it difficult to identify the histology of the pituitary tumor, Brougham *et al.* in 1950 reviewed the cases of pituitary apoplexy then available and concluded that, of 10 histologically identifiable necrotic tumors, 6 were acidophilic. Of the 5 cases reported by Epstein *et al.* (1971), the necrotic pituitary tumor cells were suggestively recognizable in only 2 instances, both of chromophobe adenoma.

The hypopituitary state of the patient with pituitary apoplexy may be illustrated by the results of various hormonal tests in one of the patients studied by Epstein *et al.* (1971). Whereas normally, the administration of metyrapone results in a doubling at least of urinary 17-OHCS or 17-ketogenic steroids, no such effect was observed in this patient. The urinary follicle-stimulating hormone (FSH) was less than 6 mouse units, as compared with a normal range of 6–48 units. The insulin test was terminated because of severe hypoglycemia and growth hormone was not detected during the test. The ^{131}I uptake was 42% at 24 hours and was, therefore, normal. Thus, in this case, the patient had a deficiency of pituitary-adrenal reserve and of gonadotropic and growth hormones.

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12

Neoplasms of the Adrenal Cortex

I. Introduction*

Although neoplasms of the adrenal cortex are relatively rare, they are of compelling biochemical interest. Hyperactivity of the adrenal cortex results in three conditions, namely, Cushing's syndrome, adrenogenital syndrome, and primary aldosteronism or Conn's syndrome. Each of these conditions can arise from hyperplasia or a tumor of the adrenal cortex although, as we shall note in Chapter 16, secretion of ACTH from extra-adrenal sources may also lead to hyperactivity. The particular type of adrenocortical hyperplasia is usually associated with a characteristic constellation of corticosteroid elaboration and clinical symptomatology. However, sometimes, particularly in adrenal carcinoma, the dividing line is not sharp. For example, of 38 cases of adrenocortical carcinoma reported by Lipsett *et al.* (1963), 4 patients had Cushing's syndrome, 4 women and 3 children had the virilization syndrome, 10 had no endocrine syndrome, but the remaining 17 patients had both Cushing's syndrome and virilization.

* The following abbreviations are used most commonly in the present chapter: ACTH = adrenocorticotropic hormone (adrenocorticotropin); APA = aldosterone-producing adenoma; CRF = corticotropin-releasing factor; DHEA = dehydroepiandrosterone; DOCA = deoxycorticosterone acetate; GRHA = glucocorticoid remediable aldosteronism; IHA = idiopathic aldosteronism; Ind HA = indeterminate aldosteronism; 17-KGS = 17-ketogenic steroids; 17-KS = ketosteroids; 11-OHCS = 11-hydroxycorticosteroids; 17-OHCS = 17-hydroxycorticosteroids; THB = tetrahydrocorticosterone; THDOC = tetrahydroxycorticosterone; THS = tetrahydrodeoxycortisol.

In summarizing the statistics in three large, different cancer registeries, Hutter and Kayhoe (1966) estimated the incidence of carcinomas of the adrenal cortex as about two people per one million of population. These neoplasms accounted for less than 0.2% of the deaths from cancer in a series reported by Steiner (1954). In reviewing the literature between 1940 and 1949 and adding the experience at the University of California, Rapaport *et al.* (1952) reported a total of 276 cases of adrenocortical tumor, including 188 cases of cancer. More recently, in connection with a study on the association of hypertension with adrenal cortical adenomas, Russell *et al.* (1972) noted that 35,000 autopsies performed at Johns Hopkins Hospital between 1889 and 1966 provided 690 cases of adrenal cortical adenomas or an incidence of 1.97%.

The relative incidences of the various types of adrenocortical neoplasms depend to some degree upon the interests of the institution from which they are reported. For example, the distribution in the 38 cases of adrenocortical carcinoma studied by Lipsett *et al.* (1963), and to which we have just referred, may be compared with that in 91 cases of adrenocortical carcinoma reported by Hutter and Kayhoe (1966): Cushing's syndrome, 59%; virilization, 19%; both Cushing's syndrome and virilization, 4%; feminization, 12%; other, 4%; and none, 4%. Further values on reported incidences will be given later in this chapter in connection with the discussion of the various types.

II. Steroids of the Adrenal Cortex

A. Structure

Approximately 44 steroid hormones had been extracted from human and animal adrenal glands by about 1970 (Forsham, 1968; Symington, 1969). The concentration of any of these is less than 20 μg/gm (Symington, 1969) and, since larger amounts are present in the circulation or excreted into the urine, it follows that the adrenal cortex must synthesize these from precursors stored in the gland or brought to the gland by blood. Our knowledge of the nature of the steroids has been obtained not only by extraction studies but also more importantly in other ways, namely, by *in vitro* perfusion of isolated human or animal adrenals, incubation of adrenal slices or homogenates with radioactive precursors, cannulation of the adrenal vein during operative procedures, *in vivo* study of the hormone content of adrenal venous blood, and use of isotope techniques in humans to determine the amount and nature of steroids produced *in vivo* by the adrenal cortex.

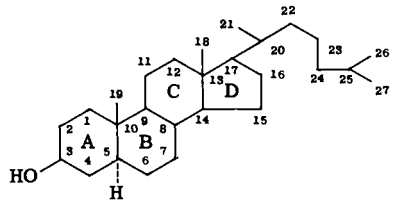
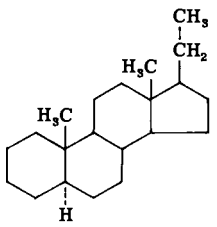


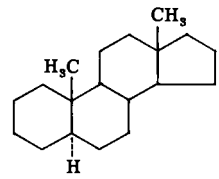
Fig. 12-1 Numbering system used for the steroid nucleus as exemplified by the formula for cholestanol.

These can be divided into 3 groups: the C_{21} steroids, the C_{19} steroids, and the C_{18} steroids. We present in Fig. 12-1 the numbering system used for the steroid nucleus as exemplified by the structure for cholestanol. To aid in the structural representation of the various steroids, it should be noted that there are nine centers of asymmetry in the molecule; these are at carbon atoms 3, 5, 8, 9, 10, 13, 14, 17, and 20. Where we have occasion to present such structures, we shall follow the convention of indicating substituents that are β-oriented (to the front) by a solid bond and those that are α-oriented (to the back) by a dashed line. When the hydrogen atom on carbon-5 and the methyl group on carbon-10 are on opposite sides of the plane of the molecule, the molecule is in the *allo* configuration. This also holds with respect to the hydroxyl group on carbon-3 and the methyl group on carbon-10.

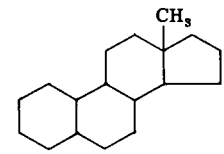
Each adrenal steroid has been designated by more than one name, frequently by a variety of names. One of these indicates the structure and is known as the systematic name, although this too may be expressed in more than one way. The C_{21}, the C_{19}, and the C_{18} steroids are based on the following theoretical structures, respectively:



5α-Pregnane



5α-Androstane



Estrane

Thus cortisol, which is a C_{21} steroid and which is also known as 17α-hydroxycorticosterone or hydrocortisone, has the systematic name 4-pregnen-11β,17α,21-triol-3,20-dione or 11β,17α,21-trihydroxy-pregnen-4-ene-3,20-dione, which designate its structure in terms of 5α-pregnane

(Symington, 1969; "Steroids," 1974). The C_{18} steroid, commonly known as estriol, has a systematic name of 1,3,5(10)-estratrien-3,16α,17β-triol which designates that there are 3 double bonds, namely, at C-1, C-3 and between C-3 and C-10 and, in addition, 3 hydroxyl groups. The most frequently used common name is very often designated as the preferred trivial name. Table 12-1 lists the trivial and systematic names of the adrenocortical and related steroids with which we shall be dealing.

B. Regulatory Mechanisms in Secretion

The regulatory mechanisms involved in the secretion of the adrenal corticosteroids as the result of adrenocorticotropin (ACTH) may be described briefly as follows. Stressful impulses reaching the cerebral cortex release the inhibition which the reticular formation or the limbic system ordinarily exerts upon hypothalamic centers in and around the tuberoinfindibular nucleus and the median eminence. Large secretory neurons then secrete corticotropin-releasing factor (CRF), which can pass down the hypophysial portal vessels into the anterior pituitary (Forsham, 1968; Symington, 1969). Adrenocorticotropin is then secreted, enters the general circulation, and acts upon the cortex to produce the various corticosteroids. Of these, only cortisone and cortisol appear to exert a feedback effect on the hypothalamic centers and thus also regulate the secretion of ACTH. This "servomechanism" tends to maintain the constancy of circulating plasma cortisol within relatively narrow limits, provided that no stressful situation intrudes (Forsham, 1968).

C. Normal Biosynthesis of Adrenocortical Steroids

An understanding of the biosynthesis and metabolism of the adrenocortical steroids is necessary to understand the derangements in Cushing's syndrome and, indeed, in other neoplasms of the adrenal cortex. The immediate precursor in this biosynthesis is cholesterol which, in turn, is synthesized from acetate or enters the body through dietary means. The liver is the major site of cholesterol synthesis, but such synthesis also occurs in other tissues as, for example, the intestine, skin, nervous tissue, aorta, the adrenal cortex, ovary, and testis. The two major pathways of cholesterol catabolism are its conversion to bile acid and, as we have indicated, to the C_{21} and C_{19} steroids.

The first steps in the formation of C_{21} steroids are the action of hy-

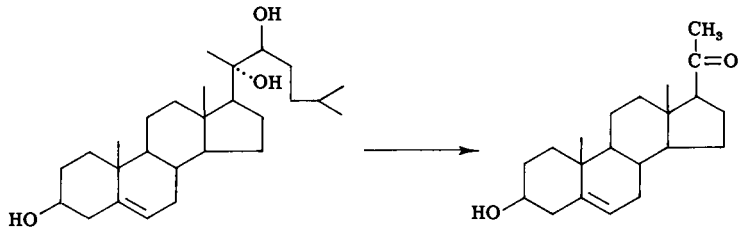
TABLE 12-1

Trivial and Systematic Names of Some Adrenocortical and Related Steroids[a]

Trivial	Systematic
Aldosterone	11β,21-Dihydroxy-18-aldo-pregn-4-ene-3,20-dione
Allotetrahydrocortisol	3α,11β,17,21-Tetrahydroxy-5α-pregnan-20-one
Androstenediol	5-Androstene-3,17β-diol
Androstenedione	Androst-4-ene-3,17-dione
Androsterone	3α-Hydroxy-5α-androstan-17-one
Corticosterone	11β,21-Dihydroxypregn-4-ene-3,20-dione
Cortisol	4-Pregnen-11β,17α,21-triol-3,20-dione
Cortisone	17,21-Dihydroxypregn-4-ene-3,11,20-trione
Cortol	5β-Pregnane-3α,11β,17,20α,21-pentol
Cortolone	3α,17,20α,21-Tetrahydroxy-5β-pregnan-11-one
Dehydroepiandrosterone	3β-Hydroxyandrost-5-en-17-one
11-Deoxycortisol	4-Pregnen-17α,21-diol-3,20-dione
Deoxycorticosterone	21-Hydroxypregn-4-ene-3,20-dione
11-Deoxycortisol	17,21-Dihydroxypregn-4-ene-3,20-dione
Dexamethasone	1,4-Pregnadiene-9α-fluoro-16α-methyl-11β,17α,21-triol-3,20-dione
Estradiol	Estra-1,3,5(10)-triene-3,17β-diol
Estriol	1,3,5(10)-Estratrien-3,16α,17β-triol
Estrone	3-Hydroxyestra-1,3,5(10)-trien-17-one
Etiocholanolone	3α-Hydroxy-5β-androstan-17-one
11β-Hydroxyetiocholanolone	3α,11β-Dihydroxy-5β-androstan-17-one
17α-Hydroxypregnenolone	3β,17-Dihydroxypregn-5-en-20-one
17α-Hydroxyprogesterone	4-Pregnen-17α-ol-3,20-dione
11-Ketoetiocholanolone	3α-Hydroxy-5β-androstane-11,17-dione
Prednisone	17,21-Dihydroxypregna-1,4-diene-3,11,20-trione
Pregnanediol	5β-Pregnane-3α,20α-diol
Pregnanetriol	5β-Pregnane-3α,17,20α-triol
Pregnenolone	3β-Hydroxy-5-pregnen-20-one
Progesterone	Pregn-4-ene-3,20-dione
Testosterone	17β-Hydroxy-4-androsten-3-one
Tetrahydrocortisol	3α,11β,17,21-Tetrahydroxy-5β-pregnan-20-one
Tetrahydrocortisone	3α,17,21-Trihydroxy-5β-pregnane-11,20-dione
Tetrahydro-11-deoxycortisol	3α,17,21-Trihydroxy-5β-pregnan-20-one
Tetrahydrodeoxycorticosterone	3α,21-Dihydroxy-5β-pregnan-20-one

[a] The systematic names are those listed by Symington (1969) or in "Steroids" (1974).

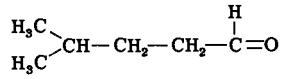
droxylases on the side chain of the cholesterol molecule to place a hydroxyl group on C-22 and a second hydroxyl group in the α-position on C-20. The resulting dihydroxycholesterol is then acted upon by steroid C-20 to C-22 desmolase to break off the side chain and yield pregnenolone and isocaproic aldehyde (White *et al.*, 1973):



20α, 22-Dihydroxycholesterol

Pregnenolone

+



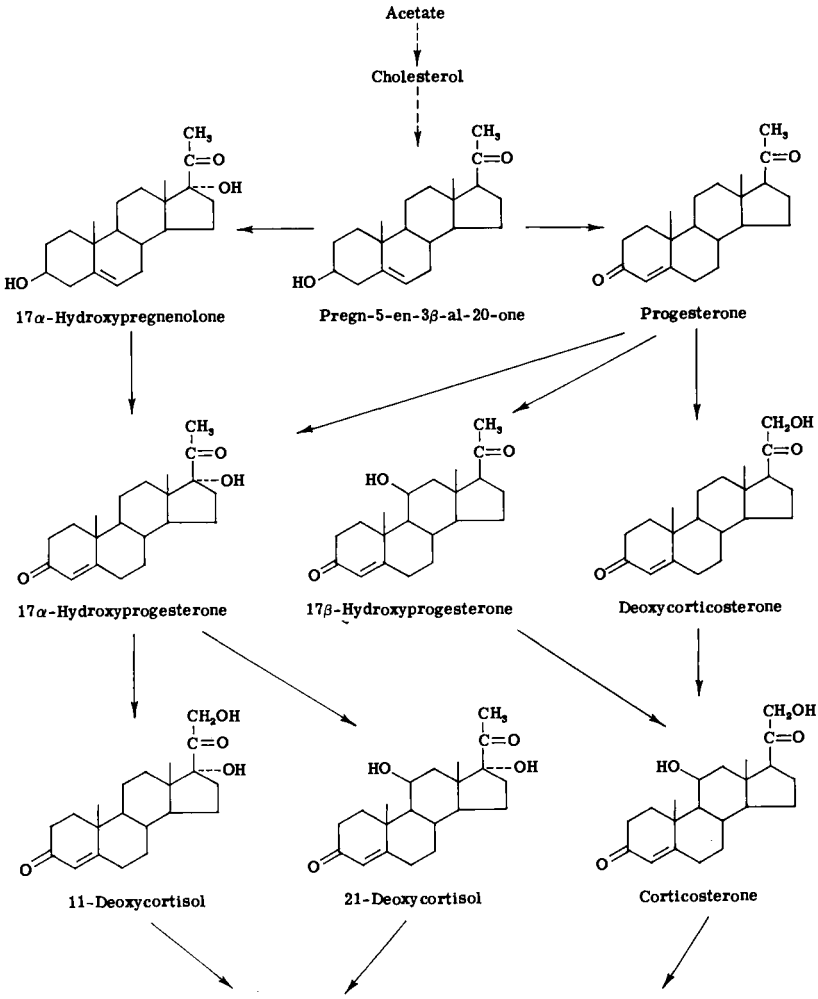
Isocaproic aldehyde

Figure 12-2 shows the further transformation of pregnenolone to the 3 main hormones of the C_{21} group, namely, cortisol, corticosterone, and aldosterone. All have a keto group at C-3 and a double bond, Δ^4, between C-4 and C-5. Cortisol and corticosterione have a hydroxyl group in the β-configuration on C-11 and a —CO—CH_2—OH attached to C-17. In addition, cortisol has a hydroxyl group attached to C-17. In aldosterone, an aldehyde group replaces the C-18 methyl group of corticosterone. There are many other possible biotransformations of C_{21} steroids not shown in Fig. 12-1 (Rosenfeld *et al.*, 1967). For example, the hydroxyl group on C-11 of cortisol may be reversibly oxidized through the mediation of a DPN- or TPN-dependent dehydrogenase system to cortisone. Cortisol and cortisone may each be reduced at C-3 and C-20 to yield tetrahydro and allotetrahydro compounds and further to yield cortoles and cortolones, respectively.

D. Normal Metabolism of Adrenocortical Steroids

The greater portion of the steroids produced by the adrenals are metabolized in the liver and appear in the blood or urine. The metabolism of the C_{21} compounds formed by the adrenal may be summarized as follows: Approximately 20 metabolites of cortisol and the 11-oxy congener, cortisone, with which it is in equilibrium have been detected in the urine and blood. The major metabolites result from reduction and conjugation reactions. The reduction reactions consist of the following phases: (a) saturation of ring A in cortisol and reduction of the 3-keto group to form tetrahydrocortisol and allotetrahydrocortisol, de-

pending on the configuration of the H atom at C-5; (b) reduction at C-20, to form cortol. A parallel metabolic process occurs with cortisone, the 11-oxy congener of cortisol; the resulting C_{21} compounds, tetrahydrocortisone, allotetrahydrocortisone, and cortolone, are excreted in the urine. Indeed, tetrahydrocortisone and tetrahydrocortisol make up the bulk of the material estimated in urine as the Porter-Silber chromogens. More generally, the Porter-Silber chromogens include cortisol, cortisone, 11-deoxycortisol, and their metabolites; indeed, any corticosteroid with 20,21α-ketol and 17-hydroxyl groups. This class of compounds is designated as 17,21-dihydroxy-20-ketosteroids or more simply as 17-hydroxy-



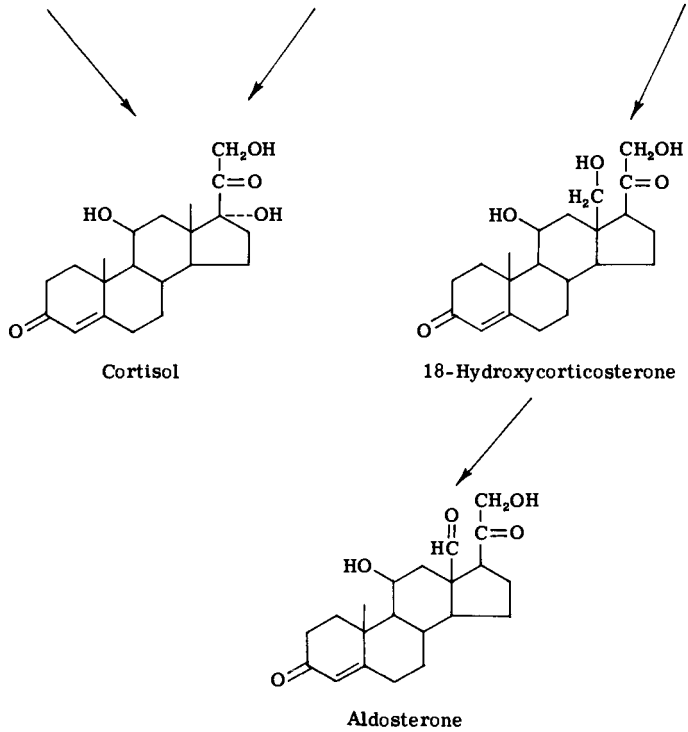
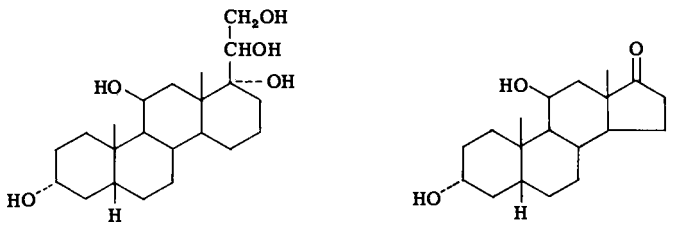


Fig. 12-2 Biosythensis of some C_{21} adrenal cortical steroids. From White *et al.* (1973). Reproduced by permission of McGraw-Hill Publishing Company.

corticosteroids (17-OHCS), and their determination in the urine affords a measure of adrenocortical secretory activity.



Another stage in the metabolism of the C_{21} steroids is the removal in several steps of the side chain from cortisol or cortisone to form a C_{19} steroid. This is illustrated above by the change of α-cortol to 11β-hydroxyetiocholanolone; similarly, 11-ketoetiocholanole is formed

from cortolone. But C_{19} steroids may also be formed from the C_{21} steroids, pregnenolone and progesterone (Forsham, 1968). Reference to Table 12-1 will enable the reader to follow the structures of the steroids. The following successive reactions, with the mediating enzyme enclosed in parentheses, illustrate the changes from pregnenolone: (a) pregnenolone to 17α-hydroxypregnenolone (17α-hydroxylase), (b) the scission of the two carbon side chains from 17α-hydroxypregnenolone to form the dehydroepiandrosterone (C17 to C20 lyase), (c) dehydroepiandrosterone to Δ^5-androstenediol (17β-hydroxysteroid dehydrogenase), (d) Δ^5-androstenediol to testosterone (3β-hydroxysteroid dehydrogenase and Δ^5-hydroxysteroid isomerase), and (e) testosterone to Δ^4-androstenedione (17β-hydroxysteroid dehydrogenase). In a similar manner, progesterone can also be converted in several steps to Δ^4-androstenedione and the latter into testosterone.

Those C_{19} steroids which have a keto group at C-17 are known as 17-ketosteroids. About 75% of the urinary 17-ketosteroids consist of three compounds which are of adrenal origin. These are dehydroepiandrosterone, 11β-Δ^4-androstenedione, and Δ^5-androstenediol. Other 17-ketosteroids are formed in the testicle. In the normal male, one-third of urinary 17-ketosteroids (5 ± 5 mg/day) is of testicular origin and two-thirds (10 ± 5 mg/day) are of adrenal origin. Obviously, in the female, the source of 17-ketosteroids is almost entirely adrenal (Forsham, 1968).

Many of the steroids are excreted to some degree as conjugated forms. In the adrenocortical steroids, conjugation occurs chiefly with glucuronic acid and, to a lesser degree, with sulfuric acid at the hydroxyl group of C-21. From a practical point of view, the determination of steroid metabolites in urine requires prior hydrolysis of their conjugates.

E. Concentrations of Adrenocortical Steroids in Blood Plasma of Normal Persons

1. Cortisol

It has been recognized for some time that the concentration of various adrenal steroids in the plasma exhibit a circadian rhythm, that is, tend to fluctuate during the day but exhibit a rhythmic repetition of levels at about the same time each day (Mills, 1966). Utilizing ingenious equipment for obtaining blood samples at 20-minute intervals throughout a 24-hour period, Gallagher, Hellman and their associates (Hellman *et al.*, 1970a; Weitzman *et al.*, 1971: Rosenfeld *et al.*, 1971) have recently shown that the secretion of cortisol and dehydroisoandrosterone (dehydroepiandrosterone) is episodic and have elicited various patterns for

the levels in plasma during the 24-hour period. Figure 12-3 illustrates these patterns for plasma cortisol.

The various alterations in concentration of plasma cortisol appeared

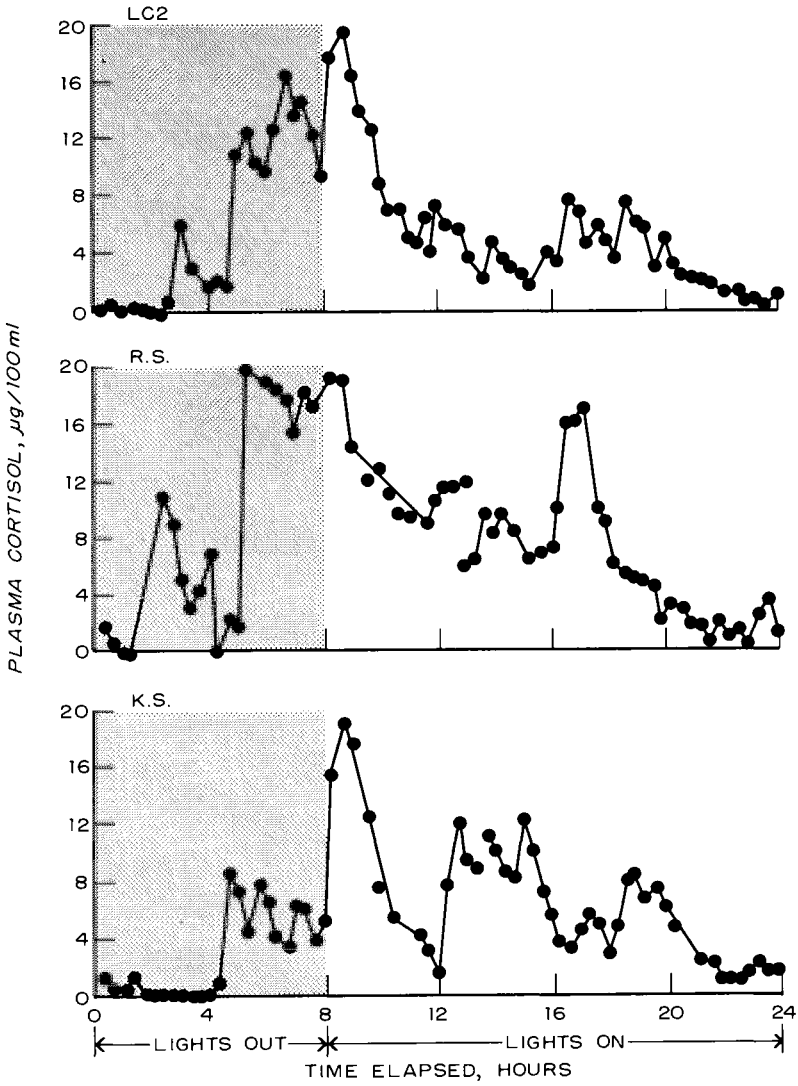


Fig. 12-3 Patterns of plasma cortisol levels in 3 normal subjects (L.C.2, R.S., and K.S.) during a 24-hour period of study. From Weitzman *et al.* (1971). Reproduced by permission of Dr. M. B. Lipsett, Editor-in-Chief, *Journal of Clinical Endocrinology and Metabolism* and The Endocrine Society.

to result from differences in frequency and duration of secretory episodes. The production or secretion rate of cortisol in milligrams per day or hour may be determined by dividing the counts per minute of [^{14}C]cortisol administered by the product of the specific activity of the cortisol metabolite in the urine and the number of days or hours required for the study. For the subjects studied by Weitzman *et al.* (1971), the mean cortisol secreted fluctuated during the day, ranging from 0 to 2.12 mg/hour. However, when the duration of the secretory activity in minutes or hours was taken into account, the secretory rate was rather constant, at about 0.04 mg/minute. In other words, the changes in output and, hence, plasma level of cortisol appeared to result from differences in the frequency and duration of the secretory episode and not from any substantial change in the secretory rate.

The question arises concerning the choice of the value that should be taken as a standard for the normal concentration of plasma cortisol. Prior to the studies we have described above, several investigators (Yates and Uhrquart, 1962; Orth *et al.*, 1967; Orth and Island, 1969) had described the 24-hour plasma cortisol concentration as an essentially smooth or progressively circadian curve. Weitzman *et al.* (1971) showed that, when the 20-minute plasma cortisol values in their studies of 6 patients were averaged for each hour, a fairly smooth curve was obtained, rising from a value of about 1 μg per 100 ml in the first hour of sleep to a maximum of about 15 μg per 100 ml in the first hour of awakening, then declining to a value of about 7 μg per 100 ml in the next 3 hours and remaining fairly constant from the eleventh to the twentieth hour of the day, when it began to decline again. Perhaps the maximum value obtained after waking should, therefore, be taken as an optimal measure of the cortisol and, indeed, of other C_{21} steroid concentrations in the plasma.

2. Plasma Blood Level of Aldosterone in Normals

Without duplicating the sedulously objective conditions employed by Weitzman *et al.* (1971) in their studies on cortisol secretion, Katz *et al.* (1972) found that aldosterone was also secreted episodically in supine man and the spurts of secretion were generally synchronous with those of cortisol which occurred during late sleep and early after arising, but not with those which occurred later in the afternoon. The plasma aldosterone concentration ranged from 23 to 294 pg/ml in one normal man and from 0 to 266 pg/ml in the second subject. The two successive 12-hour urine secretions of aldosterone were 3.46 and 3.95 μg in one subject and 3.88 and 3.44 μg in the second.

We have previously noted that aldosterone secretion and plasma renin activities are interrelated (Chapter 11, Sections III,E and V,D), and have briefly indicated the nature of this relationship. Katz *et al.* (1972) observed that plasma renin activity also fluctuated during the day in an episodic fashion and the rhythm of plasma renin activity frequently paralleled those of plasma cortisol and plasma aldosterone. The suggestion from these results that ACTH or cortisol might also stimulate renin secretion requires further proof.

The various factors involved in the control of aldosterone secretion and, hence, of the concentration of this steroid in plasma have been studied for several years. Such factors as sodium restriction, posture, acute diuresis, or hemorrhage have been considered to be mediated by the renin–angiotensin system. In addition, other factors such as potassium, ACTH, and serotonin have been demonstrated to have an effect on aldosterone secretion. A recent study may be cited to illustrate the way in which these factors exert their effect.

Williams *et al.* (1972) have shown that, on a daily intake of 10 mEq sodium per 100 mEq potassium, subjects allowed to remain supine for 82 consecutive hours had identical diurnal patterns of plasma renin activity, angiotensin II, aldosterone, cortisol, and corticosterone, with peaks at 8:00 A.M. and nadirs at 11:00 P.M. (Fig. 12-4). The mean values for the concentrations and standard errors of the mean at these two times are shown in Table 12-2. When the sodium and potassium intakes were each increased to 200 mEq/day, the plasma levels of aldosterone decreased and plasma renin activity decreased markedly to approximately one-third of their previous levels, both at the peak and nadir. At this higher concentration of sodium intake, the plasma aldosterone and renin still showed diurnal variations paralleling those of cortisol and corticosterone. When the patients were on a 10 mEq sodium per 100 mEq potassium diet, raising them from a supine to an upright position led to an increase of 150–200% in the concentrations of plasma renin activity and plasma aldosterone. These results and others obtained on diuresis induced by the administration of furosemide indicated that plasma renin activity was the dominant factor in the control of aldosterone when volume and/or dietary sodium is altered in normal man.

3. Other Adrenocortical Steroids in Plasma in Normal Persons

The older literature and even some recent literature contain a number of values for cortisol and other adrenocortical steroids in which no cognizance was taken of the circadian rhythm or the episodic character of steroid secretion (Morris and Williams, 1953; Peterson and Pierce,

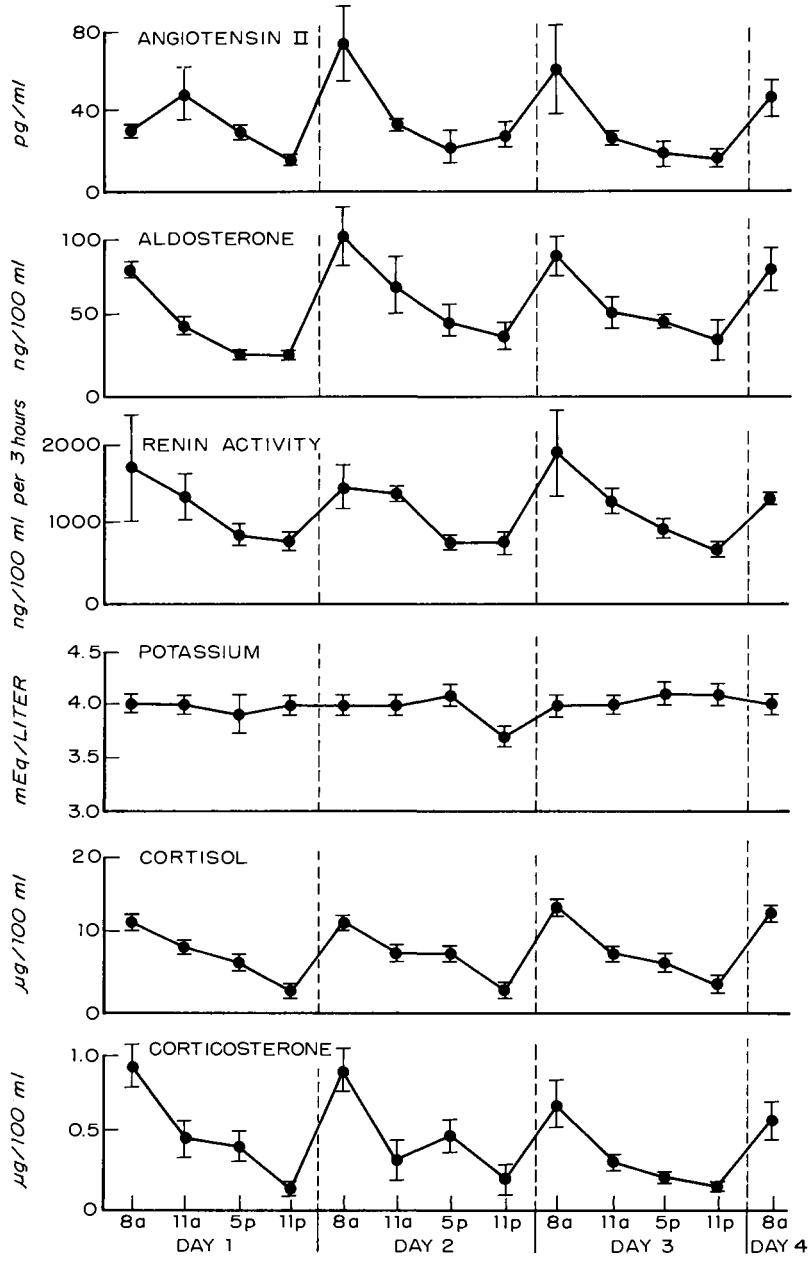


Fig. 12-4 Diurnal variation in aldosterone and other plasma components in supine normal subjects on a 10 mEq sodium per 100 mEq potassium diet. From Williams *et al.* (1972). Reproduced by permission of the American Society for Clinical Investigation, Inc.

TABLE 12-2

Plasma Peak and Nadir Concentrations of Aldosterone and Related Compounds in Normal Man[a]

Plasma component	Peak concentration (8 A.M.) mean ± SE		Concentration at nadir (11 P.M.) mean ± SE	
	10 mEq Na / 100 mEq K	200 mEq Na / 200 mEq K	10 mEq Na / 100 mEq K	200 mEq Na / 200 mEq K
Angiotensin II (ng/ml)	51 ± 9	—	24 ± 5	—
Aldosterone (ng/100 ml)	88 ± 7	28 ± 3	31 ± 5	6 ± 1
Renin activity (ng/100 ml/3 hours)	1560 ± 240	471 ± 50	707 ± 72	240 ± 28
Potassium (mEq/liter)	4.0 ± 0.1	3.9 ± 0.1	4.0 ± 0.1	3.9 ± 0.1
Cortisol (μg/100 ml)	11 ± 1	16 ± 1	2 ± 1	3 ± 1
Corticosterone (μg/100 ml)	0.77 ± 0.08	0.51 ± 0.11	0.14 ± 0.03	0.05 ± 0.01

[a] In supine condition for 82 hours at varying daily intakes of sodium. Based on data of Williams *et al.* (1972). The values are the mean and standard errors of the mean of 20 observations made on 5 subjects.

1960; Fraser and James, 1968). Other investigators have been keenly aware of this (Jubiz *et al.*, 1970; Hamanaka *et al.*, 1970) and have chosen, not necessarily the peak value, but a value close to it at 8 A.M.; still others (Newsome *et al.*, 1972; Waxman *et al.*, 1961) have also selected early morning samples. It will be recalled from Fig. 12-3 that a peak value for plasma cortisol concentration occurs at approximately 8 A.M. and a similar situation has been shown to hold for deoxycorticosterone (Hamanaka *et al.*, 1970) and for dehydroisoandrosterone (Rosenfeld *et al.*, 1971). Table 12-3 lists some of the major studies on the plasma concentration of cortisol and several other adrenocortical steroids. It may be seen that, in spite of the use of different methods, the various values for early morning samples obtained within the past few years show good agreement with each other.

Another determination that has been found to be of value as a diagnostic procedure in disease of the adrenal cortex is that of the plasma concentration and, as we shall presently note, the urinary excretion of 11-hydroxycorticosteroids (11-OHCS). This group includes the C_{21} corticosteroids with a hydroxyl group at C-11, chiefly, corticosterone and cortisol. Since the plasma concentration of corticosterone is very low, this procedure essentially measures cortisol. The method depends on the specific fluorescence of 11-OHCS in concentrated sulfuric acid

TABLE 12-3

Reported Concentrations of Adrenocortical Steroids in Human Plasma of Normals[a]

Steroid	No. of subjects	Concentration (μg/100 ml mean ± SE or range)	Reference
Cortisol	17	9.8 (3.1–20.2)	Fraser and James (1968)
	31 M	12.8 ± 0.6	Hamanaka *et al.* (1970)
	24 F	12.9 ± 0.5	Hamanaka *et al.* (1970)
	20	11.7 ± 0.8	Iturzaeta *et al.* (1970)
	6	16.0 ± 3.0	Jubiz *et al.* (1970)
	10	12.3 ± 0.8	Newsome *et al.* (1972)
Corticosterone	—	1.0 (0.8–1.8)	Peterson and Pierce (1960)
	29	0.66 (0.13–2.3)	Fraser and James (1968)
	31 M	0.3 ± 0.1	Hamanaka *et al.* (1970)
	24 F	0.4 ± 0.1	Hamanaka *et al.* (1970)
	10	0.40 ± 0.003	Newsome *et al.* (1972)
Cortisone	10	1.6 ± 0.15	Newsome *et al.* (1972)
Deoxycortisol	26	0.20 (0–1.4)	Waxman *et al.* (1961)
	6	1.0 ± 0.2	Jubiz *et al.* (1970)
	10	0.18 ± 0.01	Newsome *et al.* (1972)

[a] All of these values are based on 8 A.M. or "early morning" blood samples with the exception of Fraser and James (1968) in which the samples were drawn at random during the day. No distinction was made between male (M) and female (F) subjects in these studies, except in that of Hamanaka *et al.* (1970) which showed no sex difference.

(Mattingly, 1962). The concentrations of plasma-free 11-OHCS in a group of 52 hospital patients from which those with endocrine, liver, and renal disease had been excluded, ranged from 6.5 to 26.3 μg and averaged 14.2 μg per 100 ml (Mattingly, 1962). There was no significant sex or age difference. The bloods had all been drawn between 9 and 10 A.M. to minimize variations resulting from what was then considered the diurnal rhythm (Mattingly, 1962).

F. Urinary Excretion of Corticosteroids in Normal Persons

Although we shall discuss later in greater detail the urinary excretion of individual C_{21} corticosteroids in connection with metabolic alterations in adrenocortical tumors, it may be convenient to summarize briefly their excretion in normal individuals. Using paper chromatographic methods, Cost and Vegter (1962) obtained the values in a group of 9 males and 7 females (Table 12-4).

TABLE 12-4

Urinary Excretion of Adrenocortical Steroids and Their α-Ketolic Metabolites in Normal Individuals[a]

Steroid and designation	Mean (mg/24 hours)	Range (mg/24 hours)
Cortisol	0.07	0.03–0.09
Cortisone	0.09	0.06–0.14
Corticosterone	0.02	0.0–0.04
11-Dehydrocorticosterone	0.01	0.0–0.03
Tetrahydrocortisol	1.0	0.6–1.6
Allotetrahydrocortisol	0.4	0.1–0.7
Tetrahydrocortisone	2.7	1.0–3.8
Tetrahydro-11-deoxycortisol	0.06	0.02–0.10
Tetrahydrocorticosterone	0.20	0.10–0.36
Allotetrahydrocorticosterone	0.20	0.08–0.36
Tetrahydro-11-dehydrocorticosterone	0.16	00.6–0.24

[a] Based on data of Cost and Vegter (1962). All values are means from 16 individuals; value for allotetrahydrocortisol is based on values of 11 individuals.

The urinary adrenocortical steroids or their metabolites may be grouped into several classes that have proved useful in diagnosis of adrenocortical dysfunction. These are (a) the 17-hydroxycorticoids (17-OHCS), (b) the 17-ketogenic steroids, and (c) the 11-hydroxycorticoids (11-OHCS).

The 17-OHCS include those steroids that are characterized by a 17,21-diol-20-one side chain such as in cortisol and cortisone. This group can be analyzed for by the Porter-Silber reaction which is based on the interaction of phenylhydrazine–sulfuric acid with an extract of urine that has been subjected to enzyme hydrolysis in order to liberate the free 17-OHCS (Silber and Porter, 1954; Margraf and Weichselbaum, 1967). The values depend to some degree on the particular modification of the method used. The normal values, expressed in terms of cortisol, range between 4 and 12 mg/day and are somewhat higher in males than in females (Forsham, 1968).

The 17-ketogenic steroids, known in Britain as "17-oxogenic" steroids, include all those corticosteroids which, on oxidative fission of the 17,20,21 side chain, yield 17-ketosteroids. Specifically therefore, this class would include, in addition to the 17-hydroxycorticoids, those adrenocortical steroids with (a) the 17,20,21-triol group such as cortolone or cortol, (b) the 17,20-diol group to be found in pregnanetriol, and the 17-21-ketol grouping present in 17α-hydroxyprogesterone. The 17-ketogenic steroids may be analyzed for either directly or indirectly (Norymberski *et al.*,

1953; Appleby *et al.*, 1955). The direct method involves, as the first step, the reduction of preformed 17-ketosteroids by sodium hydroboride and so eliminates these from the final color reactions. Sodium bismuthate is then employed to split off the side chain of the C_{21} corticosteroid and simultaneously oxidizes the hydroxyl group at C-17 in order to form 17-ketosteroid. The indirect method consists in determining concurrently on two different urine specimens the 17-ketosteroid content in the usual manner and the "total 17-ketosteroid" content after bismuthate oxidation. The difference between the two measurements represents the 17-ketosteroids formed from 17-ketogenic steroids on oxidation. The 17-ketosteroids are estimated by interaction with *m*-dinitrobenzene according to the Zimmerman reaction. Employing the indirect method for the 17-ketogenic steroids, Norymberski *et al.* (1953) obtained a mean value of 13.2 and a range of 9.6–19.2 mg per 24 hours for eight determinations on 7 adult males. The mean value on 9 females was somewhat lower, namely, 8.9 mg with a range of 4.6–13.4 mg per 24 hours. Also employing the indirect method, Albert *et al.* (1968) reported the normal range to be 4–14 mg per 24 hours in 54 males and 2–12 mg per 24 hours in 48 females.

The method for 11-OHCS was described in connection with their determination in plasma. The range of urinary excretion in normal men is 108–386 μg per 24 hours and somewhat lower in normal women, namely, 78–311 μg per 24 hours (Mattingly and Tyler, 1972).

G. Biochemical and Physiological Effects of Adrenal Corticosteroids

Our knowledge of the normal actions of the adrenal corticosteroids has been accumulated from a host of observations on *in vitro* systems, adrenalectomized animals, human adrenocortical insufficiency, and attempts at various replacement therapies. Limitation of space prevents us from indicating the full scope of these actions, either on a phenomenological or molecular basis. A wide variety of biochemical and physiological systems is affected. These include (a) carbohydrate, protein, and lipid metabolism; (b) water and electrolyte metabolism; (c) hematological alterations; (d) secretory systems; (e) inflammatory and allergic phenomena; and (f) resistance to noxious stimuli. We shall briefly refer to some of these effects in connection with our discussion of the adrenocortical neoplasms but, for a fuller description of the normal actions, the reader is referred to various standard texts and monographs (White *et al.*, 1973; Cope, 1972; Symington, 1969; Ashmore and Morgan, 1967).

III. Cushing's Syndrome

A. Introduction

As we have pointed out in Chapter 11 (Section IV), apart from the use of hormonal medications, Cushing's syndrome may be produced in any of three ways: (a) adrenocortical hyperplasia or adrenocortical neoplasms, benign or malignant; (b) the excessive production of ACTH by the anterior pituitary; and (c) the secretion of ACTH by neoplasms in tissues other than the adrenal or pituitary. This secretion is known as ectopic, and we shall discuss it more fully in Chapter 16. The term, "Cushing's disease," is applied very often to the form of Cushing's syndrome which is the result of excessive production of ACTH by the anterior pituitary (Liddle, 1967). In the preceding chapter, we discussed chiefly the biochemical aspects of Cushing's disease. In this chapter, we shall discuss more broadly these aspects of Cushing's syndrome.

For purposes of orientation, a few anatomic details about the normal and neoplastic adrenal gland and cortex are appropriate. The average weight of the two normal adrenals, as determined at autopsy of ill individuals, ranges between 4 and 14 gm and averages about 5–6 gm (Forsham, 1968). Study of adrenals from 50 adults who died from car accidents or other sudden causes gave an average value of 4.16 gm with 99% of the values ranging from 2 to 6 gm (Symington, 1969). Each gland normally measures 40–60 mm in length, 20–30 mm in width, and 3–6 mm in thickness and is surrounded by areolar tissue containing fat. In turn, this is covered by a thin fibrous capsule attached to the gland by many fibrous bands. The adrenal cortex forms the bulk of the adrenal gland and consists of three zones: the outermost zona glomerulosa just under the capsule; the middle, very wide zona fasciculata; and the innermost zona reticularis. These zones are normally related to the type of steroids formed by the adrenal cortex, under the stimulus of ACTH. The peripheral glomerulosa produces mostly mineral corticoids, chiefly aldosterone, and is affected little by ACTH, whereas glucocorticoids, 17-ketosteroids, progestins, and estrogens are produced predominantly by the two inner zones of the cortex under the influence of ACTH (Forsham, 1968).

In a review of 308 cases of Cushing's syndrome in the literature, Neville and Symington (1967) observed the incidence of "normal" and hyperplastic adrenal glands to be 74.4%; of adenomas, 12.3%; and of carcinoma, 13.3%. In their own study of the adrenal cortex from 81 patients with Cushing's syndrome, Neville and Symington (1967) found

69 cases of bilateral adrenocortical hyperplasia, 7 cases of carcinoma, and 5 of adenoma. In the cases of simple bilateral hyperplasia, the cortex was wider than normal and had a broad irregular brown inner layer and an outer yellow layer.

Adrenocortical tumors, both benign and malignant, have been reported to account for Cushing's syndrome in about 15–25% of patients with this condition. About 76% of these occur after the age of 12 and, irrespective of age, about 80% are present in females (Symington, 1969). When the clinical symptomatology does not involve virilism, the tumor weighs about 10–70 gm. When there is evidence of hirsutism and virilism, the tumor is more likely to be large and malignant. Adenomas are usually encapsulated spherical yellow growths which have compressed the adrenal cortex. Histologically, the yellow areas are composed of normal-looking clear zona fasciculata-type cells arranged in regular cords and are full of lipid (Symington, 1969).

The malignant tumors are larger, usually more than 100 gm in weight, and are associated with signs of virilism or feminism. The tumor may weigh as much as 1000–4000 gm, is encapsulated, and may be a soft, gray-white or pink, lobulated, friable mass. Miscroscopically, clear cells full of lipid are rare. The viable cells are almost always compact in type and are arranged in large trabeculae separated by fibrovascular bands (Symington, 1969).

B. Clinical Relation of Symptomatology in Cushing's Syndrome to Corticosteroid Production

The signs and symptoms of Cushing's syndrome in adrenocortical hyperplasia and neoplasia may be related quite closely to the type of corticosteroids elaborated (Forsham, 1968). Thus, in the "pure" type of Cushing's syndrome, cortisol is secreted in excessive amounts and the following symptoms are prominant: (a) obesity with centripetal fat distribution, sparing the extremities and having supraclavicular fat pads and a buffalo hump; (b) red cheeks and purple striae over abdomen, thighs, and upper arms; (c) easy bruisability with ecchymoses; (d) muscle wasting; (e) osteoporosis with frequent compression fractures of the vertebrae and dorsal curvature of the spine; (f) diabetes, overt or latent; and (g) alkalosis with low serum potassium and chloride concentrations.

In the "mixed" type of Cushing's syndrome, which occurs particularly in adrenocortical carcinoma, excessive amounts of 17-ketosteroids or androgens are secreted and the following additional symptoms and signs become manifest in the female: (a) hirsutism and baldness, (b) deepen-

ing of the voice, (c) acne, (d) enlargement of the clitoris, and (f) increase of musculature with changes toward a male figure.

C. Plasma Levels of Corticosteroids in Cushing's Syndrome

1. Plasma 17-Hydroxycorticosteroids

It will be recalled (Section II,D) that this group includes those steroids that are characterized by a 17,21-diol-20-one side chain, such as cortisol and cortisone. Bliss *et al.* (1953) found that the mean value of 267 determinations of plasma 17-OHCS on 120 normals was 13 ± 6 (SD) μg per 100 ml. The range was 2–34 μg per 100 ml. The samples were drawn between 8 and 8:30 A.M., with the subject in the fasting state. The levels were substantially elevated in patients with Cushing's syndrome before therapy as, for example, a range of 36–103 μg and an average of 64 μg per 100 ml in eleven determinations on 2 patients with adrenal carcinoma and a range of 27–44 μg and an average of 33 μg per 100 ml in four determinations on 2 patients with the syndrome, but without any adrenal tumor (Perkoff *et al.*, 1954).

The procedures used in obtaining these values involve extraction of both the protein- and nonprotein-bound corticosteroids. In investigating these two fractions in a variety of conditions, Doe *et al.* (1960) observed that the mean value of plasma 17-OHCS in 11 normal subjects was 17.2 μg per 100 ml, of which 16.3 μg per 100 ml was protein-bound. In 4 patients with Cushing's syndrome resulting from bilateral adrenal hyperplasia, the mean plasma total 17-OHCS was 32.7 μg per 100 ml, of which 16.1 μg per 100 ml was protein-bound. Hence, it may be seen that the elevated plasma total 17-OHCS in Cushing's syndrome is the result of unbound or free corticosteroids.

The question arises whether plasma 17-OHCS and cortisol, which constitute a major portion of this group, are subject to episodic secretion in Cushing's syndrome. Normal subjects had a circadian rhythm in the plasma 17-OHCS values with peak values of about 13–27 μg per 100 ml at about 6 A.M. and low levels of approximately 2–12 μg per 100 ml at 6 P.M. In 7 patients with Cushing's syndrome, the plasma levels of 17-OHCS ranged from 18 to 30 μg per 100 ml at 6 P.M. According to Liddle (1967), the circadian pattern is frequently lost in Cushing's syndrome with late afternoon plasma 17-OHCS levels often as high as those in the morning. In contrast, Hellman *et al.* (1970b), using samples at 20-minute intervals, found that the secretion of cortisol in a 46-year-old woman with Cushing's syndrome was episodic and that

the general pattern was merely an exaggeration of that established for normal individuals (Hellman *et al.*, 1970a; Weitzman *et al.*, 1971). The peaks of plasma cortisol concentration in the patient with Cushing's syndrome ranged from about 26 to 46 μg per 100 ml, as compared with peak concentrations ranging from about 4 to 20 μg per 100 ml obtained in a control patient (Hellman *et al.*, 1970b). The amount produced per episode in the patient was 3.9–12.0 mg, and the total daily production was 80 mg. The amounts produced in the control patient were 0.8–3.0 mg per episode. The total production per 24 hours in 7 normal subjects averaged 16 mg with a range of 13–21 mg (Weitzman *et al.*, 1971).

2. Plasma 11-Hydroxycorticosteroids in Cushing's Syndrome

As we have previously noted (Section II,E,3), this group of compounds includes the C_{21} corticosteroids with a hydroxyl group at C-11, chiefly corticosterone and cortisol. Since the plasma concentration of corticosterone is very low (Peterson, 1957; see also Table 12-3), the procedure for this group essentially measures cortisol. Mattingly and Tyler (1972) found that at midnight the mean normal level of free plasma 11-OHCS, which presumably measures cortisol but not cortisone, was 3.3 μg per 100 ml in a group of 28 unstressed sleeping subjects. The upper limit was 8 μg per 100 ml. This range is lower than that reported for a control series of hospitalized patients. Nineteen of 21 patients with Cushing's syndrome had midnight levels above the upper limit of normal. The levels in these 19 patients ranged from 9 to 65 μg per 100 ml. Of the 2 patients with normal levels on the first determination, one had a level of 23 μg per 100 ml on a subsequent occasion. In contrast, elevated midnight levels of about 10–15 μg per 100 ml were observed in only 3 of 21 obese patients and at concentration of 9–10 μg per 100 ml in only 3 of 12 hirsute females. The normal range for plasma 11-OHCS measured in 100 individuals between 9 and 10 A.M. was 6–24 μg per 100 ml with a mean level of 14.7 μg per 100 ml. These morning levels were elevated in 15 of the 21 patients with Cushing's syndrome. Only one of 27 obese patients and none of 18 hirsute patients had levels outside the normal range (Mattingly and Tyler, 1972).

3. Plasma Cortisol Concentrations

As we have just seen, plasma concentrations of 17-OHCS or 11-OHCS represent to a large degree the plasma concentration of cortisol, and

the elevation of the plasma concentration of these corticosteroids in Cushing's syndrome reflects very well the elevation of plasma cortisol itself. The data of Peterson (1957) on the plasma concentrations of corticosterone and cortisol are shown in Table 12-5. The time of sampling was not stated, but the substantial elevations of plasma cortisol in Cushing's syndrome are clearly apparent. The study of Hellman *et al.* (1970b) on the episodic secretion of cortisol in Cushing's syndrome has already been noted and also attests to the high levels of cortisol characteristic of this condition.

D. Urinary Excretion of Corticosteroids in Cushing's Syndrome

As has been pointed out (Section II,F), the normal values for daily urinary excretion of 17-OHCS vary somewhat with the particular method employed but usually lie between 4 and 12 mg, expressed in terms of cortisol, and are somewhat higher for males than for females. In a series of 7 female and 2 male patients with Cushing's syndrome resulting from adenoma of the adrenal cortex, Liddle (1967) obtained values ranging from 15 to 31 mg and averaging 22 mg per 24 hours. In 4 females and 1 male patient with Cushing's syndrome resulting from adrenocortical carcinoma, the 17-OHCS excretions ranged from 12 to 60 mg and averaged 27 mg/day.

As we have previously noted (Section II,F), the range of urinary

TABLE 12-5

Plasma Corticosterone and Cortisol Levels in Normal Subjects and in Patients with Cushing's Syndrome[a]

Group	Corticosterone (μg/100 ml)	Cortisol (μg/100 ml)	Type of Cushing's syndrome
Normal subjects[b]	1.1 ± 0.4	14.0 ± 1.2	—
Cushing's syndrome	1.4	33.0	Unilateral tumor
	1.1	23.0	Unilateral tumor
	1.0	60.0	Bilateral hyperplasia
	0.7	38.0	Bilateral hyperplasia
	1.5	70.0	Adrenocortical carcinoma
	2.0	156.0	Adrenocortical carcinoma
	1.7	67.0	Adrenocortical carcinoma
	0.7	23.0	Adrenocortical carcinoma

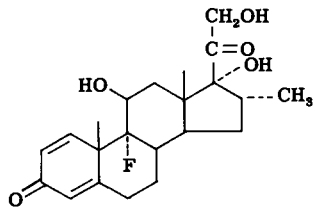
[a] From data of Peterson (1957).
[b] Thirty subjects consisting of 18 males and 12 females.

11-OCHS in normal subjects is 78–311 μg per 24 hours for women and 108–396 μg per 24 hours for men. This determination is essentially a measure of cortisol. The 21 patients with Cushing's syndrome studied by Mattingly and Tyler (1972) had urinary excretions ranging from 390 to 6150 μg per 24 hours, all well above the upper limit of normal.

The class of 17-ketogenic steroids (17-KGS) includes cortisone, cortisol, cortol, and cortolone. Various estimates of the 24-hour urinary excretion of 17-KGS run from about 9 to 19 mg (Norymberski *et al.*, 1953) or, according to Albert *et al.* (1968) from 4 to 14 mg for normal men and somewhat lower for normal women. In their study of Cushing's syndrome, Mattingly and Tyler (1972) employed a normal range of 5–18 mg per 24 hours and found that 13 patients had levels ranging from about 20 to 113 mg that were above the upper limit of normal. Seven patients had levels within the normal range. None of 27 female obese patients and only 2 of 18 hirsute patients had elevated excretions of 17-KGS.

E. Effect of Dexamethasone on Plasma Levels and Urinary Excretion of Corticosteroids

Dexamethasone is a synthetic analog of cortisol in which a double bond links C-1 and C-2 and in which a fluoride group is attached to C-9 and a methyl group to C-16, both in α position.



Dexamethasone

Earlier in this chapter (Section II,B), we described the pathways and regulatory mechanisms involved in the secretion of ACTH and action on the adrenal cortex. Dexamethasone has a marked potency for inhibiting ACTH release, and thus may serve as an aid in the diagnosis of Cushing's syndrome. Available evidence indicates that ordinarily corticotropin-releasing factor (CRF) may be liberated from the hypothalamus following stimuli arriving over either one of two functionally distinct pathways, a corticoid-insensitive or a corticoid-sensitive pathway. It is only the latter that appears to be blocked by dexamethasone (Yates, 1967).

The dexamethasone test involves the oral administration of 1 mg of dexamethasone between 11 and 12 P.M. The concentration of plasma 17-OHCS is determined on a blood specimen taken immediately before dexamethasone and again at 8 A.M. the following morning. In general, this procedure causes marked decreases in the plasma levels of 17-OHCS in normal persons or in those with miscellaneous conditions, but it has little effect on patients with Cushing's syndrome (Nugent *et al.*, 1965; Pavlatos *et al.*, 1965). This is illustrated by Table 12-6. In normal persons, in patients with exogenous obesity, and in miscellaneous diseases, the plasma 17-OHCS levels after dexamethasone were all below 5 μg per 100 ml. Indeed, 26 of the 46 individuals in these 3 groups had zero concentrations of plasma 17-OHCS and only 2 attained levels as high as 4.1 μg per 100 ml. In contrast, in the group of 17 patients with Cushing's syndrome, all the values for plasma 17-OHCS after dexamethasone were higher than 13.0 μg per 100 ml. Two obese, hirsute women had values of 10.5 and 10 μg per 100 ml after dexamethasone, and it is possible that mild abnormalities of adrenocortical secretion were present in these cases. From a diagnostic point of view, these observations support the conclusion that a single morning plasma 17-OHCS level less than 5μg per 100 ml excludes Cushing's syndrome.

TABLE 12-6

Effect of Dexamethasone on Plasma Values of 17-OHCS in Normal Individuals, in Other Conditions, and in Cushing's Syndrome[a]

Group	Number	Before dexamethasone Mean ± SE (μg/100 ml)	After dexamethasone Mean ± SE (μg/100 ml)	Extent of suppression (%)
Normal subjects	16	15.1 ± 1.05	1.1 ± 0.38	93
Exogenous obesity	20	14.8 ± 1.1	0.6 ± 0.2	96
Diseases other than obesity[b]	10	15.6 ± 1.5	1.6 ± 1.2	90
Hirsutism and obesity	2	20.3 ± 1.6	10.3 ± 1.6	43
Cushing's syndrome[c]	17	29.7 ± 3.9	27.5 ± 3.8	7

[a] Based on data of Pavlatos *et al.* (1965). Blood was drawn before the oral administration of 1 mg of dexamethasone and again at 8 A.M. the next morning from the fasting subject.

[b] This group included patients with diabetic retinopathy, Stein-Leventhal syndrome, virilization syndrome, iodiopathic hirsutism, nonfunctioning carcinoma of the adrenal gland, etc.

[c] The 17 patients included 10 with bilateral adrenal cortical hyperplasia, 5 with adrenocortical adenoma, and 2 with adrenocortical carcinoma.

However, some overlap of values has been reported to occur between patients with Cushing's syndrome and those without demonstrable pituitary-adrenal dysfunction. Accordingly, it has been proposed to determine, in addition to measurement of plasma 17-OHCS at 8 A.M., the amount of 17-OHCS excreted in the urine during a 5-hour period from 7 A.M. to noon following the midnight dose of dexamethasone (Tucci *et al.*, 1967). Figure 12-5 shows the increasing discrimination obtained by using the ratio of the urinary 17-OHCS to urinary creatinine excreted during the 5-hour period.

A variant of the dexamethasone suppression procedure has been recently submitted by Mattingly and Tyler (1972). This consists in the administration by mouth at 6-hour intervals of 2 mg of dexamethasone daily starting at noon for 48 hours, followed by 9 mg daily for 48 hours. The joint determinations of the basal urinary 11-OHCS excretions, the midnight and morning plasma 11-OHCS levels, and the results of the dexamethasone suppression test made it possible to form a firm preoperative diagnosis of pituitary-dependent Cushing's syndrome in 90% of the patients studied by Mattingly and Tyler (1972).

F. Effect of Metyrapone in Cushing's Syndrome

The structural formula of metyrapone is indicated by its systematic name, 2-methyl-1,2-bis(3-pyridyl)-propan-1-one. Its essential action is to inhibit β-hydroxylation of the steroid ring in the 11 position and thus prevent the formation of cortisol from 11-deoxycortisol. As a result of the fall in circulating cortisol, the normal hypothalamic pituitary system is stimulated to increase the output of ACTH, which then leads to an increase in 17-deoxycortisol (Forsham, 1968). Since 11-deoxycortisol has the same 17,21-diol-20-one side chain as cortisol, it and its metabolites are analyzable in the urine as 17-OHCS and 17-KGS. The rise in urinary 17-OHCS or 17-KGS is dependent on three factors. First, the decrease in cortisol must be large enough to be sensed by the ACTH-regulatory mechanism. Second, the pituitary must have the capacity to respond to decreased cortisol levels with an increased ACTH secretion. Third, the adrenals must have reserve capacity to respond to increased secretion of ACTH with increased secretion of 17-OHCS.

These considerations were made the basis of a diagnostic procedure (Liddle, 1967). A 24-hour urine is collected for determination of 17-OHCS or 17-KGS. The following day, 500 mg of metyrapone is given by mouth at hourly intervals from 7 A.M. to noon for a total of 3 gm and the collection of a second 24-hour urine is begun at 7 A.M. (Forsham, 1968). In Cushing's syndrome as a result of primary adrenocortical neoplasms, whether adenoma or carcinoma, there is a failure to respond to metyra-

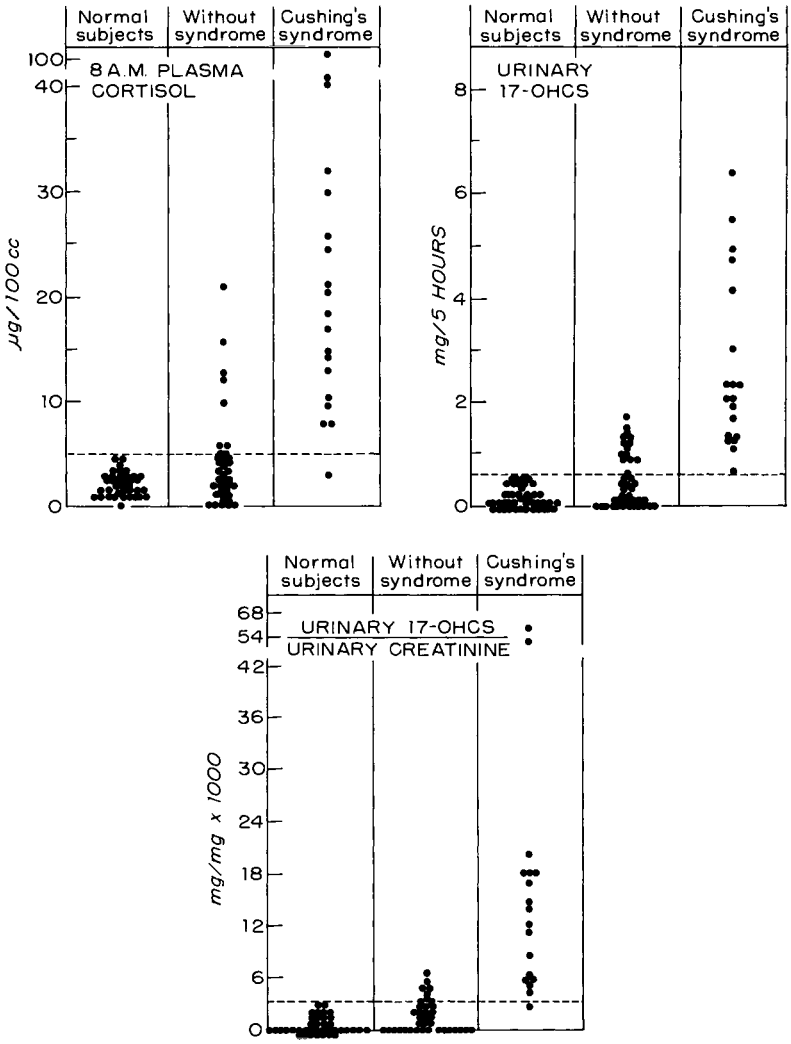


Fig. 12.5 Comparison of 8 a.m. plasma cortisol values, urinary 17-OHCS excretion and urinary 17-OHCS creatinine ratio during a 7 a.m. to noon collection in 3 groups of subjects following 1 mg of dexamethasone at midnight. From Tucci *et al.* (1967). Reproduced by permission of the American Medical Association.

pone with an increase in total 17-OHCS or 17-KGS. This failure may be explained by one or more of the mechanisms indicated above. In Cushing's disease which, as the reader will recall, is that variety of Cushing's syndrome resulting from pituitary-dependent hypercortisolism and is characterized by adrenocortical hyperplasia, there is a vigorous

pituitary-adrenal response to metyrapone with a large increase in urinary excretion of 17-OHCS (Forsham, 1968; Liddle, 1967).

These features are illustrated in the recent report by Burke and Beardwell (1973). After metyrapone, the mean daily excretion of 17-KGS was increased two- to threefold in the group with pituitary-dependent Cushing's syndrome and nodular adrenal hyperplasia, but no increase occurred in the patients with primary adrenal adenoma, carcinoma, or ectopic ACTH-producing tumors (Table 12-7). On testing with metyrapone, 96% of pituitary-dependent patients had a definite rise in 17-KGS excretion, but none of the patients with nonpituitary-dependent Cushing's syndrome exhibited such rises.

Burke and Beardwell (1973) also determined the urinary excretion of free (unconjugated) cortisol and deoxycortisol. The normal excretion of free cortisol was less than 95 μg per 24 hours, whereas the values observed in patients with Cushing's syndrome ranged from a low value of 110 μg per 24 hours to values of 4013–9497 μg per 24 hours in cases of ectopic neoplasms. However, the measurement of urinary excretion of free cortisol and deoxycortisol was not as satisfactory as the excretion of 17-KGS in following the response to metyrapone.

G. Cushing's Syndrome in Infants and Children

In 1969, Loridan and Senior reviewed the occurrence of Cushing's syndrome in childhood, found reports of 30 patients in whom this dis-

TABLE 12-7

Effect of Metyrapone on Excretion of 17-KGS in Various Groups of Patients with Cushing's Syndrome[a]

Group	Number	17-Ketogenic steroids		
		Basal (mg/day)	After metyrapone (mg/day)	After metyrapone (% of basal)[b]
Pituitary-dependent (with or without pituitary tumor)	27	24.2	72.0	316
Nodular adrenal hyperplasia	4	30.4	65.8	243
Primary adrenal adenoma	7	27.3	21.2	80
Adrenal carcinoma	3	114	117	95
Ectopic ACTH	5	103	73	79

[a] Based on data of Burke and Beardwell (1973).

[b] These values represent averages of the percentages calculated for each patient.

order was present before 1 year of age, added 3 cases of their own, and also reported another patient, 3 years of age. Each of the 4 patients presented a clear picture of Cushing's syndrome with ballooned cheeks, a buffalo hump, and excessive weight gain. In addition, there was a failure of normal growth. A review of the 3 cases of Cushing's syndrome in infancy and the 30 reported in the literature showed the following incidences of various clinical and biochemical findings: generalized obesity, 31/33; masculinization, 20/31; hirsutism, 18/21; hypertension, 21/26; increased hematocrit (>40%), hemoglobin (>14 mg per 100 ml or >90% or red blood cell count >5,000,000), 14/24; abnormal glucose tolerance test, 9/18; retarded bone age, 8/17; osteoporosis, 12/19; increased 17-ketosteroid excretion (>1 mg per 24 hours), 18/24; and increased 17-OHCS excretion (>4.2 mg/m^2 of body surface per 24 hours), 10/13. The excretion of 17-ketosteroids ranged from normal levels up to 120 mg per 24 hours, but the highest values were found in carcinoma of the adrenal cortex. In the infants, hyperplasia and adenoma together accounted for over half of the cases. Several cases have been described in which symptoms were present before 1 month of age, but there seems to be little evidence that these result from a genetic disorder.

McArthur *et al.* (1972) have recently reported a series of Cushing's disease in 7 male and 6 female children, ranging in age from approximately 4–15 years. The two outstanding and most common clinical features were truncal obesity and short stature, and the other features were similar to those found in adult Cushing's syndrome.

The biochemical findings were not particularly outstanding. Plasma cortisol (8 A.M.) was determined in 6 cases and was well within the 8 A.M. normal range for normal adults, 9–32 μg per 100 ml, employed by McArthur *et al.* (1972). The 4 P.M. values ranged from 19.7 to 32.7 μg per 100 ml and were all above the upper limit, 2–18 μg per 100 ml, for normal adults, and were stated to reflect loss of the normal diurnal rhythm. The values for 17-ketosteroid excretion were found to be increased in 7 of 11 patients, but markedly so, namely, to 77 mg per 24 hours, in only 1 patient. The determination of urinary 17-KGS, performed in 3 cases, ranged from 10.5 to 16.3 mg per 24 hours and was higher than the normal values for children (0.1–4 mg per 24 hours for birth to 10 years and 2–9 mg per 24 hours for 11–14 years). In 3 patients in whom the procedure was performed, administration of 8 mg of dexamethasone per day suppressed the urinary excretion of 17-KGS by over 90% on the third day. In one of these cases, 1 mg/day for 2 days produced a suppression of 40% and in a second case, 1 mg/day for 3 days, a suppression of 88%. These results are not easily compared to those in adults with Cushing's syndrome in whom a single dose of

1 mg at midnight failed to suppress the plasma cortisol and urinary steroid excretion.

In a general survey of the literature, McArthur *et al.* (1972) noted that, in children less than 7 years of age, Cushing's syndrome may either result from hyperplasia or adrenocortical tumor. In children more than 7 years old, hyperplasia may occur with equal or greater frequency than adrenal tumor.

IV. The Neoplastic Adrenogenital Syndrome

A. Introduction

The adrenogenital syndrome may be divided into congenital and noncongenital forms. The former is now well recognized as an inherited inborn error of metabolism, and is related to specific enzyme defects in the biosynthesis of steroids by the adrenal cortex. These enzymic defects lead to adrenocortical hyperplasia, and this condition is therefore termed "congenital adrenal hyperplasia." Compelling as its biochemical aspects are, they are outside the scope of our present discussion. We shall concern ourselves here with the noncongenital form of the adrenogenital syndrome which is almost always the result of neoplasms of the adrenal cortex. This syndrome includes the virilizing syndrome, usually in the female, pseudosexual precocity in the prepuberal male, and the feminizing syndrome in the male. As was previously noted (Section I), of 91 cases of adrenocortical carcinoma reported by Hutter and Kayhoe (1966), 19% were instances of virilization, 12% of feminization, and 4% had both Cushing's syndrome and virilization.

B. Virilizing Adrenocortical Neoplasms in the Adult Female

A previously normal female may develop such symptoms and signs as hirsutism, an increase in heterosexual sex drive, an increasing tendency to amenorrhea, and cessation of ovulation. Angular baldness, deepening of the voice, atrophy of the external genitalia with enlargement of the clitoris, disappearance of fat deposits, and increasing muscular mass are some of the other manifestations (Baulieu *et al.*, 1967).

In general, the total neutral 17-ketosteroids of urine are increased in patients with virilizing adrenogenital tumors. The levels may range from about 20 to several hundred mg/day (Baulieu *et al.*, 1967). About 75% of normal urinary 17-ketosteroids consist of three compounds which are of adrenal origin. These, as shown in Fig. 12-6, are dehydroepiandrosterone (DHEA), androstenedione, and, according to Forsham (1968),

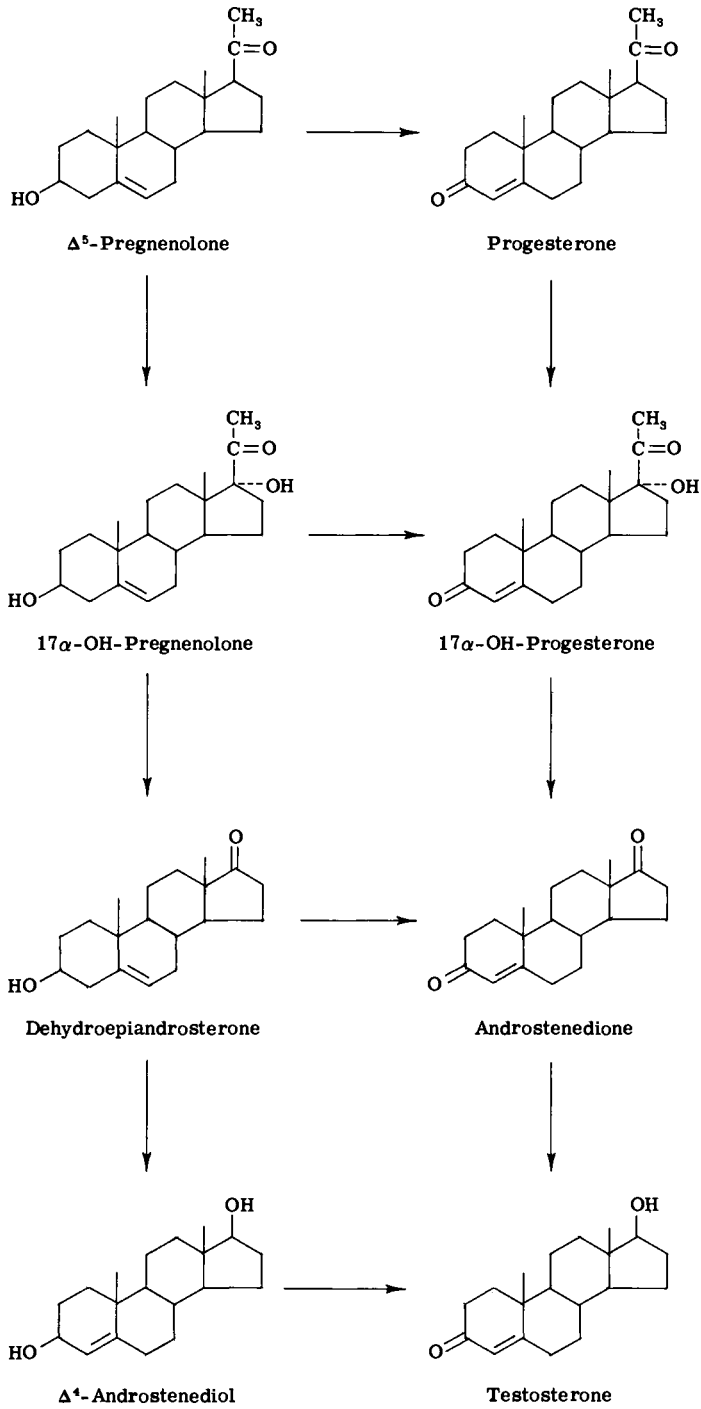


Fig. 12-6 Pathways of androgen metabolism.

androstenediol. Margraf and Weichselbaum (1967) listed dehydroepiandrosterone, etiocholanolone, and androsterone as the principal 17-ketosteroids excreted in human urine.

The increase of urinary 17-ketosteroids and their nature in virilizing adrenal tumor may be illustrated by the case of a 28-year-old woman reported by Baulieu (1962). The excretions, in milligrams per 24 hours, were DHEA and its sulfate, 49.6; 7-ketodehydroepiandrosterone and its sulfate, 3.0; androsterone and sulfate, 3.1; androsterone and glucuronide, 12.4; and etiocholanolone and glucuronide, 18.6. Etiocholanolone and androsterone are immediate metabolites of androstenedione and testosterone. Dehydroepiandrosterone was increased chiefly as the ester sulfate and, to a smaller extent, as the glucuronide. In most cases, conjugated DHEA constituted more than 50% of the total urinary 17-ketosteroids (Baulieu *et al.*, (1967). Dehydroepiandrosterone sulfate, but not free DHEA, was present in the tumor and in adrenal venous plasma. These considerations, as well as analysis for other steroids in the tumor and plasma, indicated the possibility that DHEA sulfate was produced by the tumor.

Although the urinary excretion of 11-hydroxy-17-ketosteroids is usually normal, rare cases may show an increase in 11β-hydroxyandrosterone (Baulieu *et al.*, 1967). Testosterone excretion is increased substantially as, for example, in the female patient reported by Dluhy *et al.* (1971), where the values ranged from 564 to 860 μg/day, as compared with values less than 20 μg/day in normal females (Dluhy *et al.*, 1971; Forsham, 1968). The urinary excretion of cortisol and its metabolites is usually well within the normal range, particularly prior to the development of metastases. A few cases show a slight increase in urinary excretion of 17-OHCS, with the increase mainly resulting from the excretion of tetrahydro-11-deoxycortisol. Aldosterone secretion is almost always normal but may be elevated in a few cases that are associated with arterial hypertension. Alterations in estrogen excretion are irregular. Cases of virilizing adrenal tumor have been reported with greatly elevated production of androgens without any increase of urinary estrogens, whereas other cases were characterized by increased excretion of estrone, estradiol, and estriol.

The usual concentration of testosterone in the plasma of normal adult women has been variously estimated as 34 ± 18 (SD) ng per 100 ml (Lobotsky *et al.*, 1964) and 40 ± 13 (SD) ng per 100 ml (Bardin *et al.*, 1968). Values for the plasma concentration of testosterone in males is about 600–700 ng per 100 ml (Lobotsky *et al.*, 1964; Bardin *et al.*, 1968). In virilizing adrenal tumors, the vein coming from the adrenal tumor shows high concentrations of testosterone and androstene-

dione, attesting to the secretion of these androgens by the tumor. The secretion of cortisol by the adrenal may also be affected. In contrast, the effluents from the unaffected adrenal or the ovaries show no such gradient. These features are well illustrated in Table 12-8.

Understanding of steroid metabolism in patients with adrenocortical neoplasms or other types of clinical syndromes producing virilization in females is aided by determining the metabolic clearance rates (MCR) and the production rates (PR) of various adrenocortical steroids. In general, these are determined by a constant infusion technique in which both [7α-^{3}H]androstenedione and [4-^{14}C]testosterone are administered together in 5% ethanol in saline. The MCR is calculated from the rate of infusion of the isotope divided by the concentration of isotope in the specific steroid per volume of plasma (Bardin and Lipsett, 1967; Bardin *et al.*, 1968). The production rate, PR, is equal to MCR multiplied by the plasma steroid concentration. Figure 12-7 shows that the hepatic clearance is essentially the same for normal women, virilized women, and normal men; that the value for clearance at extrahepatic sites for virilized women lies between that of normal women and normal men; and that the latter clearance accounts for the difference in the values for MCR among these 3 groups. The production rate of testosterone in virilized women is substantially greater than in normal women (Bardin *et al.*, 1968).

Several diagnostic procedures have been proposed for detection of virilizing adrenocortical tumors which help to differentiate them from

TABLE 12-8

Adrenal, Ovarian, and Peripheral Plasma Concentrations of Androgens and Cortisol in a Patient with a Right-Sided Adrenogenital Adenoma and Virilism[a]

Source of plasma sample	Testosterone (ng/100 ml)	Androstenedione (ng/100 ml)	Cortisol (μg/100 ml)
Peripheral blood	96	278[b]	18.0[c]
Left adrenal	70	50	14.0
Right adrenal	1,070	16,600	140.0
Left ovary	68	800	14.0
Right ovary	100	810	11.0

[a] From Kirschner and Bardin (1972). No information is available concerning time of day at which samples were taken. Reproduced by permission of Grune and Stratton, Inc.

[b] The mean normal value is 130 $\pm$ 30 (SD) for men and 150 $\pm$ 50 (SD) for women.

[c] The early morning values for cortisol are 11.7–16.0 μg per 100 ml (see Table 12-3).

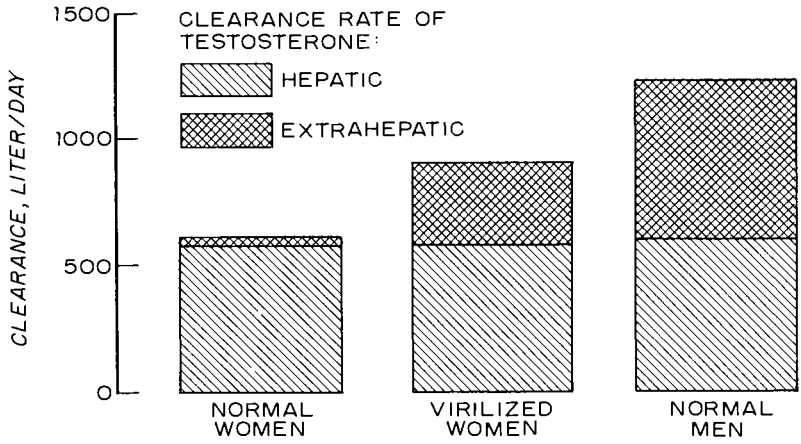


Fig. 12-7 Metabolic clearance rates of testosterone in normal and virilized women. Hatched area: hepatic clearance rate of testosterone. Cross-hatched area: clearance of testosterone at extrahepatic sites. From Kirschner and Bardin (1972). Reproduced by permission of Grune and Stratton, Inc.

other types of adrenocortical tumors. Simple tomograms usually reveal the presence of a large tumor. In contrast to cortisol-producing tumors, as in Cushing's syndrome, the contralateral adrenal is not atrophic (Forsham, 1968). In patients with pure virilizing adrenal tumors, administration of ACTH produces no increase in the urinary excretion of 17-ketosteroids. Dexamethasone in such instances fails to affect excretion of 17-ketosteroids or 17-OHCS (Baulieu *et al.*, 1967). Without these evocative procedures, the extent of excretion of 17-ketosteroids (17-KS) is not a differential measure between the various types of adrenocortical cancer. Lipsett *et al.* (1963) observed that, when an adrenal cancer produced steroids, the patient excretes large amounts of 17-KS, no matter whether the tumor causes Cushing's syndrome, virilization, or feminization, or is unaccompanied by significant clinical features of endocrinopathy. The range of 17-KS excretion in the various stages of Cushing's syndrome, expressed as mg per 24 hours, is as follows: hyperplasia, 10–40; adenoma, 3–15; and carcinoma, 25–800 (Lipsett *et al.*, 1963). The 17-KS excretion in adult females with virilizing adrenocortical tumors is within the same range (Bardin *et al.*, 1968; Dluhy *et al.*, 1971).

C. The Neoplastic Adrenogenital Syndrome in Infancy and Childhood

When the adrenogenital neoplasm develops during infancy or childhood, it results in sexual precocity. As Goldstein *et al.* (1946) have

expressed it: "Little girls become little boys and little boys little men." Boys show signs of early masculinization with enlargement of the penis, appearance of pubic and sometimes facial hair, acne, deepening of the voice, and excessive muscular development. In girls, sexual precocity is combined with masculinization. Auxiliary and pubic hair develop early, the clitoris is enlarged, and growth of hair on the face, trunk, and extremities may appear. Excessive muscular development may also be present. Goldstein *et al.* (1946) summarized 54 cases from the literature and reported an additional case of their own. Of these 55 cases, 43 were female. The majority of the adrenal carcinomas were on the left side. The liver and lung were the most common sites of metastases. The weights of the tumors, obtained either by operation or at autopsy, were available in 19 cases, ranged from 10 to 3900 gm and were greater than 1000 gm in 6 of these 19 cases.

Although this review showed that, in those instances in which the determination was done, the urinary excretion of 17-KS was increased, later studies have provided more detailed information on this and other urinary corticosteroids. Sobel *et al.* (1958) reported the case of a 7½-year-old girl who had developed signs of virilism about 6 months previously. Her urinary excretion of 17-KS rose under observation from 70 to 203 mg (normal 1–5 mg for that age) per 24 hours, 17-OHCS from 20 to 90 mg (normal 1–10) per 24 hours, and aldosterone up to 205 μg (normal 1–8 μg) per 24 hours. Within the week following hypophysectomy, the urinary 17-KS decreased to about half the preoperative level and acne improved. Thereafter, the disease advanced steadily and urinary steroid excretion rose. Administration of amphenone [3,3-di(-*p*-aminophenyl)butane-2-dihydrochloride] at various times induced a distinct reduction in the excretion of 17-OHCS, 17-KS, and aldosterone, and cessation of this therapy counteracted these reductions. Kenny *et al.* (1968) studied 5 female and 2 male children, from less than 1 month to 8½ years in age. The 17-KS excretions were moderately elevated up to about 26.9 mg per 24 hours in 5 of these cases, substantially elevated to 110 mg per 24 hours in an 8½-year-old girl and to 73 mg per 24 hours in a 6-year-3-month-old boy. The case reported by Helson *et al.* (1971) illustrates well the association of active growth of adrenal carcinoma with greatly increased excretion of 17-KS and the development of virilization. Temporary improvement following removal of a left adrenal tumor was characterized by greatly decreased urinary excretion of 17-KS. No metastases were observed. Approximately 2½ years later, x-ray revealed bilateral lung infiltrates, and urinary 17-KS excretion had increased to 77 mg per 24 hours. More lasting clinical improvement following administration of prednisone and *o,p'*-dichlorophenyl dichloroethane (*o,p'*-DDD) was marked by return of this excretion to normal levels.

D. Feminizing Adrenal Tumors

This rare tumor is characterized by isosexual precocity in the female and by feminization in the male. The literature on this type of tumor in the male was exhaustively reviewed by Gabrilove *et al.* in 1965. Of 52 such cases, 5 were under the age of 15. The incidence of the chief signs and symptoms were gynecomastia, 98%; palpable tumor, 58%; atrophy of testis, 52%; and diminished libido and/or potency, 48%. In the series of Gabrilove *et al.* (1965), 41 tumors were carcinomas, 7 adenomas, and 4 of questionable nature.

The excretion of 17-KS tends to remain normal in patients with adenomas and to be elevated, sometimes quite substantially, in those with carcinomas. Thus, in the series by Gabrilove *et al.* (1965), of 6 patients with adenoma, the urinary excretion of 17-KS was less than 10 mg/day in 4 patients and 24 and 61 mg/day in the other 2 patients. Of 24 cases with carcinoma in whom the determination was performed, 5 patients had excretions ranging from about 200 to 600 mg per 24 hours. If the 17-KS excretion is higher than 100 mg/day, the diagnosis of carcinoma is warranted (Gabrilove *et al.*, 1965). The fraction of dehydroepiandrosterone (DHEA) may be increased in patients, either with adenoma or carcinoma.

Chemical estimations of the urinary estrogens were reported for 19 patients in the series reviewed by Gabrilove *et al.* (1965), and significant elevations, sometimes up to 100 times normal, were found in each case. In general, the excretions appear to be moderately increased in patients with benign tumor or a benign course and substantially increased in adrenocortical carcinoma. For example, Dempsey and Hill (1963) reported the case of a 19-year-old male with an adrenocortical adenoma. The initial daily excretion was 222 μg estrogens as compared with a normal range in males of 5–22 μg and a mean of 11 μg. The level of plasma estrogens may also be elevated in some cases (Rappaport *et al.*, 1963).

Fragmentary and incomplete data with respect to other corticosteroids are available in only a few cases in the literature and do not permit any substantial generalizations. Thus, in the urine of 3 patients with carcinoma analyzed for the excretion of 17-KGS, the values were high in 2 of these cases and normal in the third (Gabrilove *et al.*, 1965). The case reported by Dempsey and Hill (1963) had a normal excretion of 17-OHCS, but a substantially elevated excretion of 17-KS, namely, about 50–60 mg, and consisted chiefly of DHEA. The administration of ACTH had no effect on the excretion of total estrogens, of 17-KS, or of 17-OHCS. The administration of human chorionic gonadotropin

(HCG) and of pregnant mare's serum gonadotropin (PMS) increased the excretion of estrogen 2½- and 5-fold, respectively. Human chorionic gonadotropin had little effect on 17-KS and 17-OHCS excretion, but PMS appeared to cause a 25% increase in 17-KS excretion. A right adrenal tumor weighing 202 gm was removed and this resulted in decreases of total and fractional estrogens, pregnanetriol, 17-KS, and 17-OHCS to normal levels. This detailed analysis of the preoperative urinary excretion of various metabolites suggested that the pathway of biosynthesis in the tumor tissue was the conversion of Δ^5-pregnenolone, through DHEA, into Δ^4-androstenedione, and then into estrone and its metabolites, a pathway similar to that which occurs in ovarian tissue (Dempsey and Hill, 1963).

V. Tumors of Aldosterone Excess

A. Introduction

In 1955, Conn demonstrated that a syndrome characterized predominantly by arterial hypertension, antidiuretic hormone-resistant polyuria, and muscle weakness accompanied by hypokalemic alkalosis resulted from the excessive secretion of aldosterone by tumors of the adrenal cortex. In less than 5% of subjects is the syndrome associated with hyperplasia rather than with discrete tumors. The vast majority of cases of primary aldosteronism are caused by benign adrenocortical adenomas and approximately 60% of these weigh less than 3 gm. In a few cases, this syndrome results from large and extensive malignant adrenocortical neoplasms (Alterman *et al.*, 1969; Brooks *et al.*, 1972; Brown *et al.*, 1972; Filipecki *et al.*, 1972).

It will be recalled that aldosterone stimulates reabsorption of sodium, chloride, and bicarbonate ions in the distal tubules of the kidney and in the sweat glands, salivary glands, and gastrointestinal mucusa. The excretion of potassium, hydrogen, ammonium, and magnesium ions is increased. Consequently, when an aldosterone-secreting adrenocortical tumor develops and excessive aldosterone is secreted, the following sequence of events tends to occur, the extent depending on the length of the disease: loss of potassium from the body with hypokalemia and episodes of muscle weakness, retention of sodium, and development of hypertension. In general, the frequencies of various symptoms and signs in primary aldosteronism, expressed in percentages, were as follows: hypertension, 100; hypokalemia, 100; polyuria, 92; polydypsia, 84; alkalosis, 76; albuminuria, 68; paresthesias, 58; and periodic paralysis, 38 (Conn, 1960; Forsham, 1968).

B. Steroid Content of Adrenocortical Adenomas and Carcinomas in Patients with Primary Hyperaldosteronism

The contents of aldosterone and corticosterone are elevated in the cortical adrenal adenomas of patients with primary hyperaldosteronism (Biglieri *et al.*, 1963; Kaplan, 1967). For example, Kaplan's study (1967) showed the mean content of aldosterone ranged from 2.7 to 30.4 and averaged 10.3 μg/gm of tissue in the adrenal tissue of patients with primary hyperaldosteronism and was markedly elevated above the content, 0.11–0.62 μg/gm with an average of 0.27 μg/gm, in normal glands or the content, 0.37 μg/gm (0.11–0.75 μg), in the adenomas from patients with essential hypertension. A similarly marked elevation of corticosterone, namely, a mean of 22.0 μg/gm tissue, was observed in the adrenal adnomas of patients with primary aldosteronism as compared with the content, a mean of 3.14 μg/gm, in normal adrenal glands, or 4.44 μg/gm in the glands of patients with essential hypertension. No such differences were observed for cortisol. The blood pressure readings in the patients with primary aldosteronism averaged about 180/120 mm Hg and were of the same order of magnitude as those in the group of patients with essential hypertension.

C. Plasma Electrolytes, Renin, and Aldosterone in Primary Hyperaldosteronism

An estimate of the frequencies with which abnormal values for these biochemical parameters appear in this condition may be gathered from the study by Brown *et al.* (1968) of a group of 17 male and 33 female patients ranging in age from 21 to 60 years. All had raised arterial blood pressure (160/110 to 240/130, mean 207/122 mm Hg) and adrenal cortical adenomas were found in 37 of 38 cases in which examination was made. Although plasma potassium concentrations varied in most cases, these were consistently lower than 3.7 mEq/liter in 27 patients (range 1.5–3.6, mean 2.6 mEq/liter), and intermittently so in all but one of the remainder. The plasma renin concentration was subnormal at least once in 42 patients and consistently so in 24 of these. It was often below normal or in the lower range of normal in 18 patients and normal in the remaining 8 patients.

Fifty-four determinations of the plasma aldosterone concentration were performed in 39 patients, having a daily dietary intake of approximately 130 mEq sodium and 50 mEq potassium. These conditions were not the same as those for which normal values for plasma aldosterone were

obtained (see Table 12-2). Blood samples were drawn in the morning before breakfast when they would probably give peak values. Brown *et al.* (1968) stated that the plasma aldosterone concentration was elevated in 38 of the 39 patients and ranged from 18.5 to 193 ng per 100 ml. The secretion rate of aldosterone was measured in 7 patients, and raised values were found in each (383–2500 μg per 24 hours). Plasma corticosterone concentrations were measured in 12 patients and were found to be within the normal range of 0.1–2.3 μg per 100 ml in 11 patients. In 1 patient, the concentration was 3.3 μg per 100 ml. The plasma concentration of sodium was determined 489 times in this series and most of the results fell within the normal range, although there were a number of slightly elevated values. The total plasma CO_2, measured on 421 occasions, was mostly within the normal range. We shall presently note that there are several types of primary aldosteronism, and that the findings we have described above may vary with the particular type (Biglieri *et al.*, 1972).

D. Urinary Excretion of Steroids in Primary Hyperaldosteronism

As has been pointed out earlier in this chapter (Section II,E,2), the plasma level of aldosterone is dependent on the relative dietary intake of sodium and potassium. The urinary excretion of aldosterone is also dependent on this factor. Biglieri *et al.* (1967) found that the normal excretion on an intake of 120 mEq sodium ranged from 4 to 17 μg per 24 hours. In a series of 134 measurements on 28 patients with primary hyperaldosteronism, 122 determinations were at or above the upper levels of the normal range. Sixteen determinations ranged from 50 to 98 μg per 24 hours. We shall consider the urinary excretion of other steroids in connection with the various types of hyperaldosteronism.

In order to accentuate the differences in aldosterone excretion between patients with renovascular or essential hypertension and those with hypertension presumably associated with aldosteronism, Biglieri *et al.* (1967) devised the deoxycorticosterone acetate (DOCA) test. This compound was administered intramuscularly every 12 hours for 3 days following a 4–6-day metabolic control period with 120 mEq of sodium intake per day. The urinary aldosterone excretion was measured on the third day of DOCA administration and compared with that of the control period. The results showed clearly that, whereas normal subjects and patients with essential or renovascular hypertension had marked decreases of approximately 50–90% in urinary aldosterone excretion, 13 of 15 of those with primary hyperaldosteronism showed slight decreases

or marked increases up to 80%. An adrenal adenoma was found in all but 1 of these 13 patients. In the remaining 2 of the 15 patients, the DOCA test resulted in decreases of 40% in urinary aldosterone excretion and no adrenal adenoma was observed on bilateral adrenal exploration. Thus, this DOCA suppression test constitutes a valuable aid in the diagnosis of aldosterone-producing adrenal adenomas (Forsham, 1968).

E. Types of Primary Hyperaldosteronism

Although our chief interest here has been to discuss that type of hyperaldosteronism which is associated with adenoma or carcinoma of the adrenal cortex, for purposes of comprehensive understanding several other types may be described briefly (Biglieri *et al.*, 1972).

The coexistence of hypertension, hypokalemia, increased aldosterone excretion, and subnormal plasma renin activity characterize primary aldosteronism resulting from adenoma (APA). Removal of the adenoma usually corrects these abnormalities. However, a certain number of cases with these clinical and biochemical findings and either micro- or macronodular adrenal hyperplasia or even normal-appearing glands do not respond to bilateral adrenalectomy, and the designation of idiopathic hyperaldosteronism (IHA) has been suggested for this group. The third group, indeterminate hyperaldosteronism (IndHA), includes and characterizes those patients in whom the administration of DOCA is followed by a decrease of aldosterone excretion to normal levels, a finding not obtained in other groups. Finally, the fourth group, glucocorticoid remediable hyperaldosteronism (GRHA), although rare, is present in children. Treatment with replacement doses of glucocorticoid hormones restores blood pressure to normal levels and corrects the hypokalemia and hyperaldosteronism.

The mean values for the blood pressure in the first two groups were as follows: APA, 198/120 mm Hg; IHA, 209/130 mm Hg; and were higher than the value 160/110 mm Hg for IndHA, but not significantly so. The electrolyte abnormalities were more pronounced and statistically different in the APA group, with a mean potassium value of 2.7 mEq/liter, as compared with 3.3 mEq/liter in the IHA group and 4.0 mEq/liter in the group with IndHA. The concentrations of serum sodium and bicarbonate were significantly higher in the patients with APA than in the other two groups. The mean values for aldosterone excretion, expressed as micrograms per 24 hours, were APA, 36; IHA, 24; and IndHA, 22. The urinary excretions of tetrahydrodeoxycorticosterone (THDOC) and of tetrahydrocorticosterone (THB) were within normal limits in patients with IHA (Biglieri *et al.*, 1972).

It was not possible to determine any single clinical feature or biochemical finding that clearly differentiated patients with classic APA from those with IHA. However, there were some general distinctions. Patients with IHA exhibited a less striking hypokalemic alkalosis, lower levels of aldosterone excretion, and somewhat higher plasma renin activity than patients with APA. This suggested that a greater degree of physiological control persists in IHA than in APA. Preoperative distinction between these 2 types of primary hyperaldosteronism was best achieved when the basal recumbent plasma renin concentration on 120 mEq sodium intake per day was plotted against the urinary aldosterone excretion on the third day after administration of DOCA. The combination of these two parameters has predictive value when analyzed by linear discriminant analysis. Eleven patients with APA and 2 patients with IHA were correctly predicted prospectively by this method of analysis (Biglieri *et al.*, 1972).

F. Adrenocortical Carcinoma and Hyperaldosteronism

The vast majority of cases of primary aldosteronism result from adenomas secreting aldosterone and the symptoms are related to mineralcorticism. The question may arise whether there are adrenocortical carcinomas which exhibit hyperaldosteronism without evidence of the secretion of steroids other than aldosterone, particularly glucocorticoids such as cortisol. In general, the several cases that have been reported show findings similar to the adenomas (Crane *et al.*, 1965; Alterman *et al.*, 1969; Brooks *et al.*, 1972; Filipecki *et al.*, 1972). For example, the 64-year-old female described by Crane *et al.* (1965) had, in addition to the ussual clinical features of hyperaldosteronism, the characteristic metabolic alkalosis with hypokalemia. The aldosterone excretion was elevated to 32 μg per 24 hours, and the excretions per 24 hours of 17-KS, 17-KGS, cortisol, cortisone, and tetrahydrocortisone were normal on two occasions. The excretion rate of tetrahydrodeoxycortisol (THS) was increased.

In a case reported by Brooks *et al.* (1972), the aldosterone excretion ranged between 25 and 48 μg/day, as compared with a normal range in their laboratory of 0–16 μg/day. The excretion of 17-KS was normal, but that of 17-KGS was slightly elevated to 26–47 mg/day, as compared with a normal range of 5–20 mg/day. A large adrenal carcinoma, weighing 1000 gm, was removed at operation.

The preceding data indicate that adrenocortical carcinomas with hyperaldosteronism show occasional, moderately elevated excretions of corticosteroids other than aldosterone. In reviewing 10 cases in the litera-

ture prior to their own report, Alterman *et al.* (1969) reported some values for 17-OHCS and 17-KS that were substantially elevated. Yet, there was very little clinical evidence in these 10 cases of the effects of hypercortisolism or hyperandrogenia.

G. Secondary Aldosteronism

The term, "secondary aldosteronism," applies to a group of disorders in which larger than normal quantities of aldosterone are secreted in response to some extra-adrenal disorder. This group of disorders includes conditions which cause a decrease in effective blood volume such as simple dehydration, sodium depletion, exsanguination, hypoalbuminemia, or venous sequestration of blood in hepatic cirrhosis and conjestive heart failure. These conditions lead to increased production of renin and angiotensin and, secondarily, to increased secretion of aldosterone. Luetscher (1967) has outlined the dynamic processes which involve changes in other biochemical and physiological parameters such as extracellular fluid volume, urinary and serum levels of sodium and potassium, and blood pressure.

H. Diagnostic Procedures

In the preceding sections, we have indicated some biochemical characteristics which form the basis for diagnosis of primary hyperaldosteronism. These may be briefly summarized. The triad of resting hypertension, serum potassium levels lower than 3 mEq/liter on a normal diet and in at least three separate blood samples, and failure in urine concentration after intramuscular administration of 20 units of antidiuretic hormone (Pitressin) is strongly suggestive of the disease (Forsham, 1968). Elevated excretion of aldosterone and low plasma levels of renin constitute additional diagnostic aids (Conn *et al.*, 1965; Brown *et al.*, 1968; Biglieri *et al.*, 1972). In the "spironolactone test," this aldosterone antagonist is given at a dose of 100 mg 4 times a day for 3 days on a constant dietary intake of at least 6 gm of sodium chloride (Forsham, 1968). Serum potassium levels are determined immediately before and at the end of the 3 days. If the initial low potassium level is the result of hyperaldosteronism, the use of the antagonist causes a rise of at least 1 mEq/liter (Forsham, 1968). We have already described in detail the procedure proposed by Biglieri *et al.* (1967) in which the administration of DOCA to patients with essential renovascular hypertension causes marked decreases in aldosterone excretion but

only slight decreases or even substantial increases in patients with adrenocortical adenomas and hypertension.

Adrenal venography, particularly in combination with adrenal vein assay for aldosterone, has also been called upon as a diagnostic aid. In 1963, Emerson achieved the catheterization of the left adrenal vein by percutaneous femoral vein puncture and the radiographic visualization of adrenocortical adenomas after injection of a contrast medium. Kahn and his associates (1971) found that percutaneous adrenal venography was superior to adrenal arteriography and, together with adrenal vein assay for aldosterone, achieved an 85% accuracy. The use of percutaneous adrenal venography has been further explored in patients with various suspected adrenocortical disorders (Nicolis *et al.*, 1972; Melby, 1972). Table 12-9 shows the results of adrenal venography coupled with measurement of aldosterone in the venous effluent obtained by percutaneous bilateral adrenal vein catheterization in 38 patients with primary aldosteronism. The anatomic nature of the adrenocortical lesion was determined at operation. In a series of 9 patients with aldosterone-producing solitary adenomas, the adrenal venous plasma aldosterone from the adrenal gland bearing the tumor ranged from 230 to 8010 ng per 100 ml and averaged 4500 ng per 100 ml. The aldosterone content from the contralateral vein ranged from 50 to 680 ng per 100 ml and averaged 195 ng per 100 ml.

Conn and his associates (1972) have studied the photoscanning of tumors in primary aldosteronism after intravenous administration of

TABLE 12-9

Use of Venography in Visualization of Adrenal Gland and Venous Aldosterone Assay in Patients with Primary Aldosteronism[a]

Group	Radiographic vs. anatomical diagnosis (No.)	(%)	Adrenal-venous aldosterone vs. anatomical diagnosis (No.)	(%)
Solitary adenoma				
<8 mm	0/3	0	3/3	100
>8 mm	19/22	86	20/22	90
All tumors	19/25	76	23/25	92
Idiopathic aldosteronism (bilateral hyperplasia)	5/13	38	10/13	77

[a] From Melby (1972). Reproduced by permission of the American College of Physicians.

[^{131}I]19-iodocholesterol. In 4 patients whose aldosterone-producing tumors had been visualized by adrenal venography, scintillation scanning gave clear visual evidence of concentration of radioactivity at the site of the tumor. In a case of bilateral adrenal hyperplasia, only diffuse adrenal uptake of radioactivity was demonstable without any areas of concentration.

VI. Nonfunctioning Adrenocortical Neoplasms

We have noted that the various types of adrenocortical neoplasms are usually characterized by the formation of one or another type of physiologically active steroids such as cortisol in Cushing's disease, androgens or estrogens in neoplasms causing the adrenogenital syndrome, and neoplasms that lead to excessive formation of aldosterone. However, there appears to be a certain number of adrenocortical neoplasms which do not produce any recognizable clinical evidence of excessive secretion of steroids and which have been termed "nonfunctional." In presenting their own series of 38 cases of adrenocortical carcinomas, Lipsett *et al.* (1963) reviewed the literature and, including their own 9 adult cases, found a total of 81 cases of adrenocortical carcinomas without any endocrine syndromes. In considering these cases, Lipsett *et al.* (1963) abjured the use of the term, "nonfunctional," noting that ". . . we shall consider a cancer functional if steroid production by the tumor can be demonstrated by chemical means." Of the 8 male patients who had no clinical evidence of excessive steroid secretion, 4 patients had high urinary levels of 17-KS, and 3 of the 4 also excreted large amounts of 17-OHCS.

The absence of recognizable clinical evidence of excessive secretion of steroids does not necessarily mean that physiologically active steroids are not being formed and excreted in more than normal amounts. Fukushima and Gallagher (1963) studied the urinary steroids in 3 patients with metastatic "nonfunctioning" adrenocortical carcinoma. No excessive production of cortisol or adrenal androgen metabolites occurred. The isolation of relatively large amounts, namely, 5.6 and 2 mg per 24 hours, of Δ^5-pregnene-3β,20α-diol and of 5 and 4.8 mg per 24 hours of pregnane-3α,20α-diol from the urine of 2 patients indicated that the metastases were at least capable of the first step in corticosteroid biosynthesis, namely, the conversion of cholesterol to pregnenalone. Δ^5-Pregnene-3α,16α,20α-triol was isolated from the urine of one of these patients. No abnormal steroids were found in the urine of the third patient.

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13

Neoplasms of the Thyroid

I. Introduction*

A. General Structure and Functions of the Normal Thyroid Gland

Although the incidence and morbidity of thyroid neoplasms are lower than those of other types of cancer, the subject has many aspects of interest to clinical investigators (Ingbar and Woeber, 1968; Studer, 1969). The first question concerns the significance of thyroid nodules which are very prevalent in the general population—4% by palpation and even higher on examination of the thyroid at necroscopy (Ingbar and Woeber, 1968). But there are several other reasons for the unusual interest: the extreme variability of the natural history of thyroid neoplasms, the degree of histological and biochemical differentiation, the reproducibility of some neoplasms in laboratory animals, and, most importantly, the applicability to neoplastic processes of our considerable knowledge of the biochemical features and functions of the thyroid cell.

* The following abbreviations are used most commonly in the present chapter: BEI = butanol-extractable iodine; BMR = basal metabolic rate; bTSH = bovine thyroid-stimulating hormone; DIT = diiodotyrosine; hTSH = human thyroid-stimulating hormone; ITDase = iodotyrosine deiodinase; LATS = long-acting thyroid stimulator (stimulating substance); MIT = monoiodotyrosine; PBI = protein-bound iodine; T_3 = triiodothyronine; T_4 = thyroxine; TBG = thyroid hormone-binding globulin; TBPA = thyroid hormone-binding prealbumin; TRF = thyrotropin-releasing factor; TSH = thyroid-stimulating hormone.

The structure and functions of the normal thyroid gland are responsive to a variety of stimuli which the organism encounters during life. Such, for example, are puberty, pregnancy, the amount of iodine and organic iodine compounds in the food and water of a particular locality, extremes of environmental temperature, and nervous strain. Many of these stimuli are made effective through other endocrine glands and neurological structures.

The normal thyroid gland in the North American adult weighs about 20 gm. It consists of two lobes joined by a thin band of tissue. Each lobe is approximately 2.0–2.5 cm in both thickness and width at the largest diameters and is about 4.0 cm in length. The anatomic unit is the acinus or follicle which varies in diameter from about 0.05 to 0.5 mm and averages about 0.2 mm or 200 μm. The follicle is normally lined by a single layer of epithelium, consisting of closely packed cuboidal cells. These are usually 15 μm high, but this height varies with the degree of glandular stimulation, becoming columnar when active and flat when inactive. From 20 to 40 follicles are separated by connective tissue septa to form lobules, each supplied by a single artery (Wollman, 1965). The epithelial cells secrete a clear, amber-colored, sticky fluid into the lumen of the follicle. Electron microscopy has revealed the presence of numerous microvilli extending from the apical aspects of the acinar cells into this fluid or colloid.

The biochemical and physiological functions of the normal thyroid gland are vast in scope. These include the synthesis, secretion, and metabolism of the thyroid hormones; the regulation of thyroid hormone activity; the metabolic effects of the thyroid hormones; and the diagnostic tests of thyroid gland function and thyroid hormone economy. It is beyond the range of this volume to describe the normal aspects in any detail, but we shall note briefly some of the essential features in connection with our discussion of the various thyroid neoplasms.

B. Classification of Thyroid Neoplasms

Several schemes of classification of thyroid neoplasms have been proposed (Woolner *et al.*, 1961; Hazard, 1968; Ingbar and Woeber, 1968; Ackerman and del Regato, 1970; Meissner, 1971). For purposes of convenience, we shall refer chiefly to that of Ackerman and del Regato (1970). The benign neoplasms are designated as adenomas and, in general, arise from the epithelium of preexisting follicles, are well encapsulated, have few mitoses, range in weight from 25 to 200 gm and do not invade adjacent tissues of metastasize to noncontiguous areas. In about 5% of these adenomas, the follicular cells are larger and have

acidophilic granular cytoplasm. These benign tumors are known as Hürthle cell adenomas. In contrast to the benign adenoma, the thyroid nodule does not have a complete connective tissue capsule (Ackerman and del Regato, 1970).

The malignant neoplasms of the thyroid may be classified into (a) papillary adenocarcinomas characterized microscopically by well-defined papillary fronds and frequently by psammobodies (small calcific spherules), (b) follicular (alveolar) adenocarcinomas distinguished by well-defined follicles, and (c) medullary carcinomas characterized microscopically by undifferentiated neoplasm with masses of amyloid. In addition to these types of malignant neoplasms, so-called small-cell, giant cell, and Hürthle cell carcinomas, as well as plasmacytomas, lymphosarcomas, fibrosarcomas, and teratomas are present much less frequently (Ackerman and del Regato, 1970). Of the 885 cases of thyroid carcinoma observed by Woolner *et al.* (1961) at the Mayo Clinic over a 30-year period from 1926 through 1955, 541, or 61.6%, were papillary; 157, or 17.7%, were follicular; 57, or 6.5%, were solid medullary with amyloid stroma; and 130, or 14.7%, were anaplastic. Meissner (1971) has given the following approximate values: papillary, 50%; follicular, 25%; medullary, 10% and anaplastic, 15%. As is true of other malignant neoplasms, those of the thyroid tend to metastasize to neighboring or distant organs. However, regional metastases of the well-differentiated neoplasms may be slow to appear and may remains in cervical and mediastinal nodes for a number of years. The organs most frequently affected are lymph nodes, lungs, bone, liver, kidneys, and brain (Ackerman and del Regato, 1970). According to R. D. Leeper (personal communication, 1974), the experience at Memorial Hospital shows the liver and kidneys to be relatively uncommon sites.

C. Incidence of Malignant Thyroid Neoplasms

Estimates of the incidence of malignant neoplasms of the thyroid in the United States are rather crude (Silverberg and Vidone, 1966). A recent survey noted that cancer of the thyroid is responsible for ½–1% of all clinical cancer in the United States, to have an incidence of 25 patients per million population per year, and to account for approximately a thousand deaths annually (Gordon *et al.*, 1961). Segi and Kurihara (1972) listed 331 male and 576 female deaths for the year 1966 and 274 male and 598 female deaths for 1967 in the United States. The mortality rates are about 30–50% higher in England and Wales and approximately 4 times as much in Switzerland (Segi and Kurihara, 1972). Most recently, Silverberg and Holleb (1973) have estimated an incidence of 2900 and a mortality of 1150 for the United States in 1973.

Silverberg and Vidone (1966) pointed out that incidences based upon general autopsy records may be unreliable since examination of the thyroid may not be done or, if done, may be inadequate. In a study of all autopsies on adults during a 10-month period at Yale-New Haven Hospital in which the neck organs were available for examination, the incidences in a study of 300 thyroids were : normal thyroid, 28.0%; non-neoplastic conditions, 65.6%; benign adenoma or other benign tumor of the thyroid, 3.0%; primary carcinoma, 2.7%; and metastatic malignant tumors from other sites, 5.0%. The total of 104.3% indicates that one diagnosis was applicable to several cases.

D. Thyroid Nodules and Neoplasms

The prevalence of thyroid nodules in the general population has been estimated to be about 4% on the basis of palpation and even higher at autopsy. As we have seen, the incidence of cancer of the thyroid is much lower, but any particular case of nodular goiter raises the very immediate questions of whether the nodular thyroid gland is likely to harbor a carcinoma, what the course of such a carcinoma would be, and what the relative benefits and hazards of treatment would be (Ingbar and Woeber, 1968). Shimaoka *et al.* (1962) found that 7% of a series of 235 patients with nodular goiter had thyroid carcinoma on histological examination. Ingbar and Woeber (1968) observed a similar incidence in their own smaller series of about 100 cases. These investigators have considered the various clinical and laboratory critera that would indicate the presence of carcinoma in a nodular goiter: greater incidence of carcinoma in patients less than 40 years of age and in men rather than women; presence of areas in thyroid that do not take up radioiodine and, hence, are "cold" on scintillation scanning (scintiscan); history of radiation to the head, neck, or chest during childhood; and recent painless growth in neck. Obviously, a judicious appraisal of these various criteria is necessary to indicate the likelihood of carcinoma.

II. Biochemistry and Physiology of the Thyroid Hormones

A. Synthesis, Secretion, and Metabolism of the Thyroid Hormones

1. Extrathyroidal Metabolism of Iodine

Iodine enters the human body both in its inorganic form, as iodine, and organically. Normally, these substances are in the diet and ingested

water and this intake may vary widely with the country and, within a particular country, with the geographical area.

The normal pathways of iodine metabolism in a state of iodine balance on an average daily intake of 160 μg inorganic iodine have been conveniently summarized by Ingbar and Woeber (1968), with numerical values representing the normal means on the Eastern seaboard of the United States (Fig. 13-1). Approximately 90% of the total body iodine stores is present in the thyroid, predominantly in the inorganic form. At any time in equilibrium, the concentration of inorganic iodide in the extracellular fluid is approximately 0.2–0.3 μg per 100 ml and the total content is about 60 μg I^-. Iodide is removed from the extracellular fluid in very small amounts via the skin, the expired air, lactation, and fecal excretion, but most of it is removed through the kidney and by the thyroid. The urinary excretion of iodine per day is 150 μg, chiefly in the inorganic form.

Iodide is almost completely filterable at the glomerulus. However, a substantial amount is reabsorbed through the tubules, so that the renal iodide clearance in adults normally approximates 35–40 ml/minute

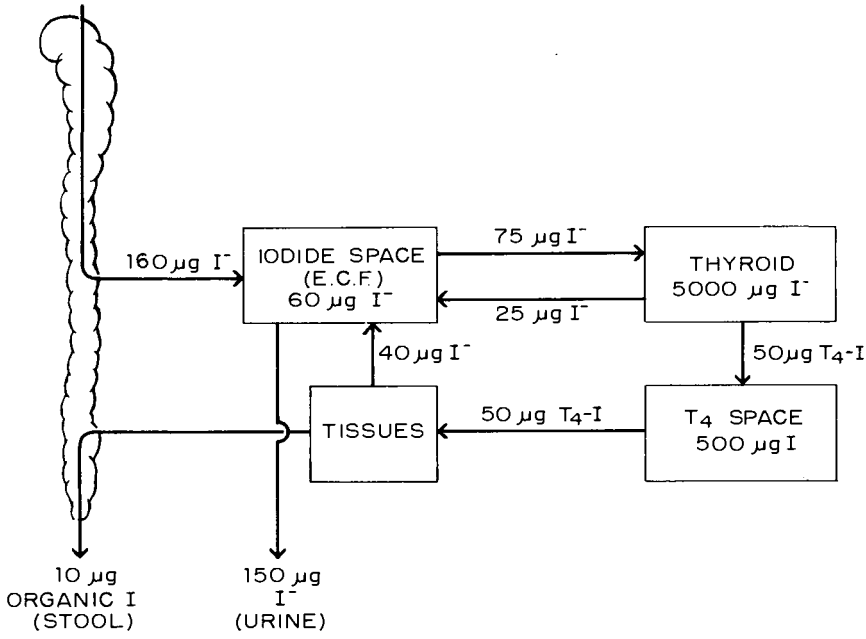


Fig. 13-1 Schema depicting normal pathways of iodine metabolism in a state of iodine balance. Reproduced from Ingbar and Woeber (1968) in "Textbook of Endocrinology" (R. H. Williams, ed.) by permission of W. B. Saunders Company.

(Ingbar and Woeber, 1968). The urinary excretion of iodine is subject to considerable variation, chiefly because of the level of daily intake (Reith, 1933; Curtis *et al.*, 1937). Rall (1950) studied the distribution of urinary iodine in three euthyroid subjects following the administration of radioiodine. Expressed as percent of the total urinary iodine, the values were thyroxin, 0.3–2.0; diiodotyrosine, 3.0–10.6; and inorganic iodide, 88.8–95.0.

The fecal excretion of iodine is about 10 μg/day in normal persons (Curtis *et al.*, 1937; Ingbar and Woeber, 1968) but may be increased when gastrointestinal absorption is impaired as in chronic diarrheal states (Hiss and Dowling, 1962) or under the influence of certain dietary constituents such as soybean products (Pinchera *et al.*, 1965).

Iodine is present in the thyroid gland and other tissues as free iodine, inorganic iodide, and in various organic forms; the total iodine content of the adult human body ranges between 20 and 50 mg (Sturm and Buchholz, 1928). The iodine content of the human thyroid is about 1–2 mg/gm of dry tissue (Gutman *et al.*, 1932), a total of 5–10 mg in the whole thyroid, or about one-fifth of the total body store. The concentrations in such major tissues as muscle, skin, and skeleton are very low but, because of their mass, these tissues contain about one-half, one-tenth, and one-seventeenth, respectively, of the total body store of iodine (Sturm and Buchholz, 1928). The iodine concentrations in the other glands of internal secretion, namely, the anterior pituitary, ovary, adrenal cortex, and the parathyroid have been reported to be about fourfold that of other body tissues (Sturm and Buchholz, 1928).

2. Iodide Transport into the Thyroid

Synthesis of the thyroid hormones involves four major steps: (a) the entrance of inorganic iodide (I^-) into the thyroid gland; (b) oxidation of iodide and organic iodination; (c) formation of the iodothyronines; and (d) the incorporation of the iodothyronines into the specific thyroprotein, thyroglobulin. We shall subsequently describe the reactions involved in the metabolism of thyroglobulin, that is, in the release of the thyroid hormones, their passage into the circulation, and their further fate.

The thyroid contains a mechanism which enables it to accumulate iodine in concentrations ten- to several hundredfold greater than the concentration in the serum or surrounding medium (Wolff, 1964). This mechanism has been variously referred to as the iodide-concentrating, iodide-transport, or trapping mechanism. The ability to concentrate I^- is also shared by several other organs such as the stomach, salivary

glands, choroid plexus, ciliary body, and skin, but these organs do not possess the thyroid's ability to mediate the conversion of iodide into other organic compounds.

The transport of iodide into the thyroid is highly dependent upon continuous generation of phosphate bond energy (Ingbar and Woeber, 1968). Transport is inhibited by anoxia, by inhibitors of oxidative metabolism such as fluoride and cyanide, by agents that uncouple oxidative phosphorylation such as 2,4-dinitrophenol, and by exposure to oubain and related cardiac glycosides which inhibit the ATPase system and the cellular sodium-potassium pump. Since the iodide transport system is also effective in transporting other monovalent ions such as perchlorate, monofluorosulfonate, difluorophosphate, or fluoroborate, these ions can act as competitive inhibitors of iodide transport. Among the physiological factors affecting the thyroid iodide transport, the most important appears to be thyroid-stimulating hormone (TSH) which enhances the transport under most circumstances. Hypophysectomy diminishes it.

3. Oxidation of Iodide and Incorporation into Organic Compounds (Organification)

Although the sequence of reactions involved in the conversion of iodide into organic compounds, including the thyroid hormones, is not elucidated completely, evidence for the following reactions has been obtained. An iodide-peroxidase has been demonstrated to be present in thyroid tissue from many species. This appears to mediate the interaction of iodide, which has been transported into the gland, and hydrogen peroxide, which is generated through the autoxidation of flavin enzymes, to form either hypoiodide ion (IO^-) or iodinium ion (I^+). These are evanescent but are capable of actively iodinating the tyrosine found in peptide linkage rather than free tyrosine that is later incorporated into protein. The reaction is mediated by tyrosine iodinase, appears to be dependent on the extent of thyroid stimulation by TSH, and is susceptible to inhibition by a great variety of pharmacological substances (De Groot, 1965; Ingbar and Woeber, 1968).

Figure 13-2 depicts the structures of the iodotyrosines which are formed by the reactions we have just discussed and of the hormonally active thyronines or coupled iodotyrosines. Although various model systems have been set up *in vitro* to demonstrate possible mechanisms for such coupling between the iodotyrosine molecules, it would appear that *in vivo* coupling occurs between molecules of monoiodotyrosine (MIT) or diiodotyrosine (DIT) contained within thyroglobulin. The

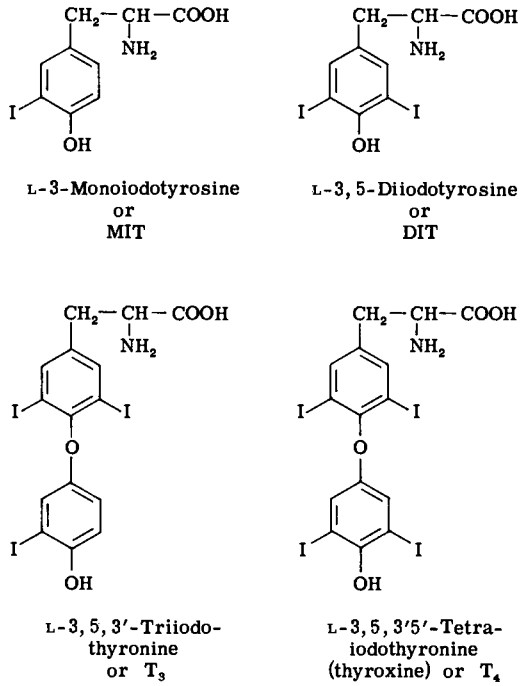


Fig. 13.2 Structural formulas of the thyroid hormones and their percursors.

coupling between two DIT residues to yield a thyroxine (T_4) structure would involve the loss of an alanyl side chain from one ring and the formation of an ether bridge.

As we have noted, thyroglobulin serves as the matrix for tyrosine iodination and coupling of iodotyrosines occurs and also acts as the storage site of thyroid hormones. Thyroglobulin is a glycoprotein of approximately 660,000 molecular weight and is composed of four chains. In thyroglobulin obtained from rats with adequate iodide in the diet, about 7 tyrosyl groups are iodinated to the level of MIT, 6 to the level of DIT, and 1 or 2 to the level of iodothyronine (De Groot, 1965). Direct analyses of normal human thyroids show that the organic iodine is partitioned as follows: MIT, 17–28%; DIT, 24–42%; triiodothyronine (T_3). 5–8%; and T_4, 35% (Ingbar and Woeber, 1968). The carbohydrate component constitutes approximately 10% by weight of the thyroglobulin molecule and consists of glucosamine, mannose, fucose, galactose, and sialic acid molecules.

Although thyroglobulin is the major iodoprotein in the normal thyroid

gland, several others are present in small amounts. For example, a soluble iodoprotein variously designated as S-1 iodoprotein or thyralbumin is present to the extent of only a few percent of the total iodoprotein, but may increase in disease. The iodinated amino acids are chiefly MIT, some DIT, and a very small amount of T_4 (Ingbar and Woeber, 1968).

4. Release and Transport of Thyroid Hormones

Most T_4 and T_3 appears to be released from thyroglobulin by the action of proteases and peptidases present in the thyroid gland. The activity of these proteases is enhanced by the administration of TSH (Pastan, 1966), and the mechanism of this release has been illumined by the histochemical and electron microscope studies of Wollman (1965). When the thyroid is stimulated by TSH, a phagocytosislike ingestion of colloid occurs at the apical end of the epithelial cell. The engulfed colloid forms colloid droplets which contain a material that is highly similar to thyroglobulin or is thyroglobulin itself. At the same time, electron dense granules, which contain esterases and acid phosphatase and are probably lysosomes, begin to migrate from the basal region of the cell to the apical end. These electron dense particles then fuse with the colloid droplets to form vesicles which migrate back to the basal end of the cell and reassume the character of electron dense particles. Iodothyronines liberated from the thyroglobulin are now apparently free to enter the circulation. Iodotyrosines are also liberated, but these are deiodinated by the action of a microsomal iodotyrosine dehalogenase and thus do not enter the circulation. The dehalogenase does not act upon peptide-bound iodotyrosines or on the free iodothyronines.

Upon entering the circulation, the free iodothyronines become bound chiefly to two specific plasma proteins in firm but dissociable bonds. One of these proteins migrates electrophoretically at alkaline pH between the α_1- and α_2-globulins, was found to bind T_4, and was designated the thyroid hormone-binding globulin or TBG (Gordon *et al.*, 1952; Larson *et al.*, 1952; Robbins and Rall, 1952). The second protein also binds T_4 but migrates anodally to albumin and is known, therefore, as T_4-binding prealbumin or TBPA (Ingbar, 1958). A small fraction, approximately 10%, of T_4 is also bound to albumin (Oppenheimer, 1968). Triiodothyronine is bound primarily to TBG and secondarily to serum albumin (Deiss *et al.*, 1953; Robbins and Rall, 1955, 1960), but is not bound to TBPA (Ingbar and Woeber, 1968).

The relative concentrations of free and bound T_4 or T_3 in serum are determined by the concentration of the protein in serum, the number

of sites on the protein molecule, and the affinity of the T_4- or T_3-protein complex.

The combination between the free thyroid hormones and their binding proteins may be expressed mathematically by the formulas for a reversible binding equilibrium or for the conventional mass action relationship (Robbins and Rall, 1957, 1960; Ingbar and Braverman, 1966; Ingbar and Woeber, 1968). The protein-bound thyroid hormones are in equilibrium with the free forms, but it is the latter which are available to the tissues, exert or induce various metabolic actions, and undergo metabolic degradation (Ingbar *et al.*, 1965).

The concentrations of TBG and TBPA in normal human serum average about 1.5 and 27.7 mg per 100 ml, respectively (Oppenheimer *et al.*, 1966; Oppenheimer, 1968). The corresponding maximal T_4-binding capacity of TBG is about 20 μg per 100 serum and that of TBPA ranges from 216 to 342 and averages about 274 μg per 100 ml serum.

Various estimates of the normal concentration of T_4, both bound and free, in human serum range from 4 to 11 μg per 100 ml. By multiplying these values by 0.653, the proportion of iodine in T_4, the normal range of T_4 iodide, may be expressed as 2.6–7.2 μg per 100 ml (Rosenberg, 1972). The proportion of free T_4 is very low (Oppenheimer, 1968; Rosenberg, 1972). For example, Ingbar *et al.* (1965) submitted a value for a group of 87 normal persons of 4.03 ± 1.08 ng per 100 ml, or $0.050 \pm 0.009\%$, of the total T_4.

The concentration of total T_3 in normal human serum is much less than that of T_4. Rosenberg (1972) has summarized values in the literature as ranging from 100 to 200 ng per 100 ml. Sterling (1970) obtained 220 ± 27 ng per 100 ml, or a normal range of approximately 170–270 ng per 100 ml. Expressed as thyronine iodine, these values would be 115–187 ng per 100 ml. The mean value for the fraction of free T_3 was about tenfold that for the fraction of free T_4 in a series of 12 normal persons in whom these parameters were determined concomitantly (Ingbar *et al.*, 1965).

5. Degradation of Thyroid Hormones

The overall metabolism of thyroid hormones has usually been studied by observing the fate of a single injection of the hormone containing radioiodine only in the 3′ or 3′,5′ positions of the beta ring. In a review of such studies, Oddie *et al.* (1966) judged that in the young or middle-aged adult the extrathyroid pool of T_4-iodine is approximately 500 μg. When labeled T_4 is injected intravenously into a normal subject, it mixes rapidly within its distribution space, then decreases in concentra-

tion at an exponential rate. In about 15 days, the cumulative radioactivity in the feces, presently almost completely in an organic form, amounts to about 10–20% of the radioactivity. The remainder of the hormone is deiodinated and apportioned between the urine and the thyroid gland. The amount cumulatively excreted in the urine is almost entirely in inorganic form and amounts to about 55% of the administered dose. The remainder, about 30%, accumulates in the thyroid and presumably distributes itself therefore, in the usual proportions that unlabeled iodine does, namely, between the thyroid hormones, the iodotyrosine and inorganic iodide (Ingbar and Freinkel, 1955).

Metabolic degradation of the thyroid hormones may involve either the alanyl side chain or an attack on the phenolic groups, particularly with regard to deiodination. Conversion of the hormones to their pyruvic acid analogs may occur either through oxidative deamination or direct transamination. Reduction of the pyruvic acid derivatives yields lactic acid analogs and the acetic acid derivatives may be formed by decarboxylation. The production of tetraiodothyroacetic acid (Tetrac) and triiodohydroacetic acid (Triac) has been demonstrated by a number of workers (Stanbury, 1960). In 1952, Taurog *et al.* showed that T_4 is conjugated by the formation of glucuronide or sulfate esters of the phenolic hydroxyl group and that these esters are excreted in the bile. This aspect of the metabolism of the thyroid hormones has been studied further by Flock and her associates (1960) and by Barker and Shimada (1964). Deiodination is also an important step in the degradation of the thyroid hormones (Tata, 1960; Lissitzky *et al.*, 1961).

B. Regulation by Thyroid Hormone Activity

1. Introduction

Leeper (1969) has reviewed in some detail recent concepts of the factors involved in thyroid hormone regulation. Figure 13-3 indicates that thyrotropin-releasing factor (TRF) is formed in the hypothalamus and arrives at the pituitary via the hypophysial portal blood system. This in turn mediates the release of thyroid-stimulating hormone (TSH) from the anterior pituitary. In the thyroid gland, the TSH stimulates the synthesis and secretion of the thyroid hormones. We have already noted that T_4 and T_3 are bound to specific thyroid-binding proteins and that this binding is a factor in determining the amounts of free thyroid hormones in the circulation. Figure 13-3 also shows the feedback effect of free T_4 on thyrotropin release and the effect of other compounds on the equilibrium between free and protein-bound thyroid hormones.

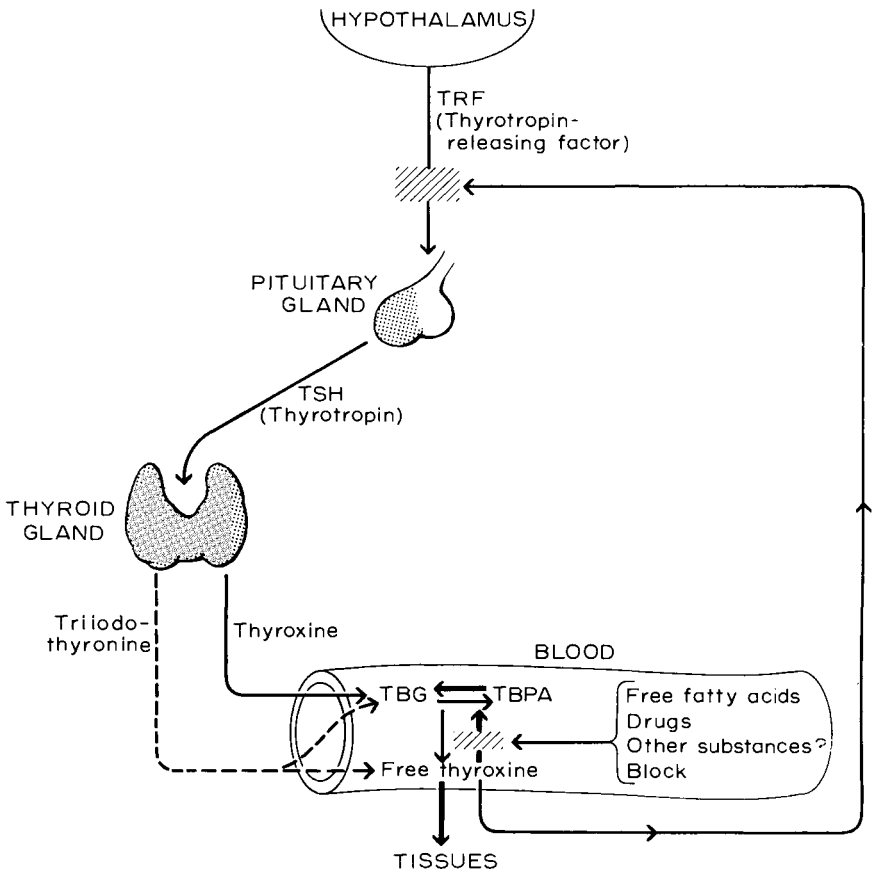


Fig. 13-3 Schematic outline of factors controlling thyroid hormone levels in blood and tissue, according to Leeper (1969). Reproduced by permission of Academic Press.

2. Thyrotropin-Releasing Factor

In 1955, Harris reported that the destruction of the ventromedian nuclei and the paraventricular nuclei in the hypothalamus led to depression of thyroid function. A highly purified preparation of TRF was obtained from porcine hypothalmi by Schally *et al.* (1969), and its complete structure was determined as L-pyroglutamyl-L-histidyl-L-proline amide (Nair *et al.*, 1970). The synthesis of this compound was soon accomplished, and its activity was essentially the same as that of the isolated product (Baugh *et al.*, 1970). The structure is given in Fig. 13-4.

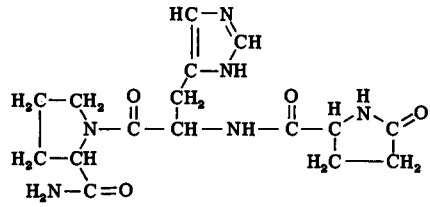


Fig. 13.4 Structure of TRF, L-pyroglutamyl-L-histidyl-L-proline amide.

Assays can be carried out in either an *in vitro* system or *in vivo* and depend upon the ability of TRF to release TSH from the pituitary. For example, in the method of Redding *et al.* (1966), the test hypothalamic material, which is free of TSH, vasopressin, and MSH, is injected into mice treated with ^{131}I, 1 μg T_4 and codeine. Blood ^{131}I levels obtained 2 hours after injection are proportional to the amount of TRF injected.

3. Thyroid-Stimulating Hormone (Thyrotropin)

In 1922, it was observed that bovine pituitary glands activate the thyroid of hypophysectomized tadpoles (Smith and Smith, 1922). Since then, increasingly successful efforts have been made to purify TSH as well as to understand the mechanisms by means of which it enhances the processes that lead to the synthesis and secretion of thyroid hormone. Bovine TSH has a molecular weight of 28,300 and consists of two polypeptide chains, one of 13,000 and the other of 14,700. The complete amino acid sequence of each has been established, and the nature of its carbohydrate moiety has been studied (White *et al.*, 1973). In 1963, Condliffe obtained a 100-fold purification of human thyrotropin. Like bovine thyrotropin, it is a glycoprotein with a molecular weight of approximately 28,000, probably consisting of two subunits. The carbohydrate component constitutes about 15% of the weight of the molecule.

Several methods are available for the assay of TSH (Leeper, 1969; Hershman and Pittman, 1971b). Utiger (1965) and Odell *et al.* (1965) have developed a radioimmunoassay for the determination of very small quantities of TSH in human plasma or serum. In general, radioimmunomethods, as developed in the fundamental studies of Berson and Yalow (1967) depend on the capacity of unlabeled antigen to displace ^{131}I-labeled antigen from specific antibody.

The volume of distribution of circulating TSH has been reported to be 5.8% of the body weight (Odell *et al.*, 1967). This indicates that the hormone is confined to the blood circulation and is not present in extracellular fluid. The following values have been obtained for the

concentration in normal or euthyroid persons of TSH in plasma, expressed as microunits (μU) per milliliter: 2.7 (Odell *et al.*, 1967) and 3.9 ± 2.0 (SD) (Hershman and Pittman, 1971b).

The action of TSH on the thyroid and the mechanisms by which this action is accomplished have been summarized in several reviews (Ingbar and Woeber, 1968; Field, 1968; Leeper, 1969; Utiger, 1971; Pastan, 1971). Thyroid-stimulating hormone enhances practically all processes leading to the synthesis and secretion of hormone. Elimination of TSH secretion by hypophysectomy or suppression in the intact animal is followed by decreased activity of the thyroid iodide transport mechanism and by inhibition of organic iodine binding. The fraction of organified iodine present in the iodothyronines is decreased, evidence of a decrease in the rate of coupling of iodotyrosines. The fractional release of glandular ^{131}I is decreased, indicating a decrease in proteolysis of thyroglobulin. All of these effects are reversed by the administration of TSH. In man, the effects of TSH on iodine metabolism are evident in an increase in the rate of release of glandular ^{131}I, increased thyroid ^{131}I uptake, and increased thyroid iodide clearance. The concentration of labeled and unlabeled protein-bound iodine (PBI) in the plasma is also increased. Pastan *et al.* (1966) have demonstrated that the first step in this action, as with other polypeptide hormones, is the binding of the hormone to the target tissue.

The metabolic effects of TSH on the thyroid have been studied at great length (Ingbar and Woeber, 1968; Field, 1968; Leeper, 1969), and may be noted here only briefly and illustratively. Thyroid-stimulating hormone has several effects on carbohydrate metabolism. For example, it increases the rate of oxidation of glucose via the hexose monophosphate, glycolytic and tricarboxylic acid pathways. The synthesis of phospholipids, usually measured by the incorporation of ^{32}P into total phospholipids, is also accelerated. When administered *in vivo*, TSH increases the content of RNA. *In vitro*, it increases the rate of incorporation of various purine precursors. The increased incorporation of amino acids into protein may be demonstrated *in vitro* in preparations of isolated thyroid cells Thyroid-stimulating hormone rapidly elevates tissue levels of cyclic 3′,5′-AMP in thyroid slices and, in thyroid homogenates, TSH activates the enzyme, adenyl cyclase, which forms cyclic AMP from ATP.

4. Long-Acting Thyroid Stimulator

In one of the bioassays for TSH which depended on the discharge of organic iodine from the thyroid gland and the rise in serum protein-

bound ^{131}I, it was found that the blood of some hyperthyroid patients gave a delayed maximal response (Adams and Purves, 1956; Adams 1958; McKenzie, 1967). It appeared, therefore, that in certain conditions blood contains a long acting thyroid-stimulating substance (LATS), most easily defined by McKenzie (1967) as giving in mice a maximal ^{131}I response at 9 hours instead of 2 hours after injection.

Since its original discovery in 1956 (Adams and Purves, 1956), the properties of LATS have been investigated in some detail (Leeper, 1969; Werner, 1971). There is much evidence to indicate that it is a 7S γ-globulin and that it possesses antibody activity. Long acting thyroid-stimulating substance resembles TSH in its action on glucose oxidation, ^{32}P uptake, and iodine release, but the time course is delayed (Leeper, 1969); LATS has also been considered as associated with the ophthalmopathy of Grave's disease and, more generally, with autoimmune thyroid disease (Werner, 1971).

C. Metabolic Effects of Thyroid Hormones

1. Introduction

The biochemical and physiological effects of thyroid hormones on organs other than the thyroid constitute a vast area of compelling interest. However, it is beyond the scope of this volume to discuss these in any but the barest outline. Most of our information in these connections has been obtained by using the thyroidectomized animal or the patient with hypothyroidism or hyperthyroidism. Hamolsky and Freedberg (1960) reviewed and listed some of the biochemical systems affected by thyroid hormone *in vivo* or *in vitro*. For example, among the parameters that are inhibited or depressed are oxidative phosphorylation, muscle creatine and phosphocreatine, various dehydrogenases, alkaline phosphatase, D-amino acid oxidase, transhydrogenase, and ascorbic acid oxidase. Illustrative of those systems that are enhanced or increased are succinoxidase, TPNH-cytochrome c-oxidase, fatty acid component oxidase, cytochrome c, carotene to vitamin A conversion, mitochondrial swelling, and oxygen consumption of red cells.

2. Increased Calorigenesis and Oxygen Consumption

That the oxygen uptake of the body as a whole is increased in hyperthyroidism has been known since the classic demonstration by Magnus-

Levy in 1895, and the determination of the oxygen uptake was used for many years as a diagnostic method. After the injection of a single large dose of thyroxine into the whole organism, excised tissues, with the exception of spleen, brain, and testes, exhibit an increased oxygen uptake after varying lag periods. Myocardial tissue and gastric mucosa are most sensitive in this respect. Triiodothyronine (T_3) causes a more prompt but a less lasting effect than thyroxine (T_4) (Barker and Klitgaard, 1952). Thyroxine given to normal animals leads to a swelling of the mitochondria and to increases in both the numbers of mitochondria and cristae per cell, and it has been postulated that the thyroid hormones increase the number of miteochondrial respiratory units (Buchanan and Tapley, 1971).

3. Protein Metabolism

The extent and direction of the effect which thyroid hormone has on protein metabolism depends upon the metabolic state of the organism and the amount of thyroid hormone administered or present in the body (Ingbar and Woeber, 1968). Thus, in thyroidectomized rats, administration of moderate amounts of T_4 increases protein synthesis and decreases nitrogen excretion. With larger doses, protein synthesis is decreased and the concentration of free amino acids is increased in plasma, liver, and muscle. The effects on protein synthesis are most generally reflected in the overall growth rates. Growth is retarded in the hypothyroid immature animal or young patient, may be restored toward normal by administration of moderate amounts of thyroid hormone, and is inhibited by excessive doses (Ingbar and Woeber, 1968).

4. Carbohydrate Metabolism

The effects of thyroid hormone here, as in other metabolic actions, depend upon the amount which is administered or produced in the organism. Practically all aspects of carbohydrate metabolism are affected. These have been demonstrated in the animal organism and include the regulation of the magnitude of the glycogenolytic and hyperglycemic actions of epinephrine, potentiation of the effects of insulin on glycogen synthesis and utilization, enhancement of rate of intestinal absorption of glucose and galactose, and rate of uptake of glucose by adipose tissue and muscle (Hoch, 1962; Ingbar and Woeber, 1968).

In a series of patients subjected to total thyroidectomy as a possible therapeutic measure for cardiac disease, the oral glucose tolerance curves

were, on the average, somewhat flatter than before operation, that is, the glucose tolerance was increased (Gilligan *et al.*, 1934). In the presence of infused glucagon, the average intravenous glucose tolerance curve in a group of hypothyroid patients was significantly higher, and that in a group of hyperthyroid patients was signficantly lower, than the average curve in a group of control euthyroid patients also receiving glucagon (Lamberg, 1965). Since glucagon acts chiefly by accelerating glycogenolysis in the liver, it would appear likely that the shape of the curves reflects the extent of the glycogen stores in these 3 groups of patients (Lamberg, 1965).

The decrease in blood glucose following intravenous tolbutamide was significantly faster and more marked in hyperthyroid patients than in euthyroid controls and significantly less in hypothyroid patients. Lamberg (1965) has suggested that excessive thyroid hormone production, as illustrated by clinical hyperthyroidism, results in an increase in glucose utilization. This, in turn, would induce depletion of the glycogen stores and possible "hyperinsulinization" through a constant elevation of the fasting blood glucose level in the blood.

5. Lipid Metabolism

The effects of thyroid hormones on lipid metabolism have been reviewed by Hoch (1962) and by Ingbar and Woeber (1968) and may be briefly noted here. There is considerable conflicting evidence concerning the effect of the hormones on the rate of synthesis of lipids. Much of this conflict may result from the use of the whole organism in some instances and from tissue preparations in others. As in the effects of carbohydrate metabolism, dose may play a role. For example, Fletcher and Myant (1960) found that injection of rats with 20 μg of T_4 increased the synthesis of cholesterol from acetate in cell-free liver fractions, whereas 30–50 μg decreased the rate.

One of the most interesting and visible effects of the action of thyroid hormone in man is the level of cholesterol in serum or plasma. There are many observations in the literature showing that the concentration of serum or plasma cholesterol is decreased in patients with hyperthyroidism and increased in those with hypothyroidism (Epstein and Lande, 1922; Mason *et al.*, 1930; McGee, 1935; Skanse, 1949). For example, in a group of patients with severe exophthalmic goiter in crisis or on the verge of crisis, with a mean basal metabolic rate of +66%, Hurxthal (1933) found the blood chloresterol to average 82 mg per 100 ml; the lowest value was 48 mg per 100 ml. The close relationship between serum levels of cholesterol and the effect of thyroid hormone is also

evident in the treatment of patients with hypothyroidism. For example, Hurxthal (1933) reported the case of a woman, 25 years of age, who had myxedema of 12 years' duration. The concentration of blood cholesterol was 356 mg per 100 ml and the basal metabolic rate (BMR) was —40%. After treatment with thyroid for a period of 1½ years, the BMR had increased to —1% and the level of cholesterol had fallen to 185 mg per 100 ml.

Cholesterol is not the only lipid whose concentration in blood and possibly metabolism are affected by the state of thyroid hormone activity. The concentrations of total lipid, total fatty acids, and phospholipid in blood are increased significantly in hypothyroidism and, conversely, are decreased in hyperthyroidism (Boyd and Connell, 1936; Hoch, 1962). The mechanisms of these alterations in man have received some study. Myxedema and surgical or medical thyroidectomy decrease the rate of cholesterol synthesis (Gould *et al.*, 1955), and this lowered rate is restored to normal by thyroid hormones (Lipsky *et al.*, 1955).

6. Other Metabolic Effects

Limitation of space prevents any substantial discussion of the other metabolic effects which thyroid hormones exert (Bodansky and Bodansky, 1952; Hoch, 1962; Ingbar and Woeber, 1968). However, some illustrations may be given. Thus, the requirements for water-soluble vitamins are increased in hyperthyroidism and the conversion of some of them to the coenzyme form may be impaired. Thyroid hormones are also required for the synthesis of vitamin A from carotene and for the conversion of vitamin A to retinene, the pigment involved in dark adaptation (Ingbar and Woeber, 1968). The metabolism of creatine is under the influence of thyroid hormones and, in hyperthyroidism, creatine excretion is increased and creatinine excretion is decreased, whereas the opposite occurs in hypothyroidism. Calcium and phosphorus metabolism, particularly as related to bone, are frequently affected in altered clinical thyroid states (Bodansky and Bodansky, 1952).

7. Interdependence with Other Hormones

Although this area is of considerable interest, only a few aspects may be noted briefly (Hoch, 1962). Increased amounts of T_4 potentiate the actions of epinephrine, whereas decreased amounts of T_4 diminish or abolish some of them. The potentiating effect of thyroid hormone on insulin action has been clearly demonstrated in animals and, in human hyperthyroidism, the hypoglycemic response to insulin is increased

(Elrick *et al.*, 1961). Hellman *et al.* (1959) explored the interrelationship between thyroid hormone and androgens. The daily endogenous production of androsterone and etiocholanolone averaged approximately 1.0 mg for a group of hypothyroid myxedematous patients as compared with 4.5 mg for a group of control patients. Androsterone constituted 44% of the sum of these two steroids in the control subjects, but only 15% in the hypothyroid group. The administration of 200 μg of T_3 per day for a period of at least 10 days did not essentially alter the amounts of steroid excretion, but the fraction of androsterone increased in both the control and the hypothyroid group. Administration of androsterone caused significant decreases of the serum chloresterol level in myxedematous patients, in subjects with hyperchloresterolemia of other origin, and in normocholesteremic subjects, thus supporting the hypothesis that some of the peripheral manifestations of excess or deficit of thyroid hormone might be mediated by the metabolites of steroid androgens.

D. Diagnostic Tests of Thyroid Hormone Economy

1. Introduction

A great variety of biochemical procedures have been used to assess the function and regulation of the thyroid gland (Ingbar and Woeber, 1968; Werner, 1971; Selenkow, 1973). These may perhaps be most conveniently classed according to Ingbar and Woeber (1968) as (a) tests of thyroid gland function, (b) tests related to the concentration and physical state of thyroid hormones in the blood, (c) tests that assess the integrity of the homeostatic mechanism, and (e) special tests. Limitation of space prevents us from discussing these in any detail. We shall limit ourselves to a few procedural notes and to listing illustrative results that have been obtained in euthyroid individuals (Table 13-1).

2. Tests of Thyroid Gland Function

Tests of thyroid gland function include measurements of thyroid radioiodine accumulation and tests that assess the turnover and release of thyroid iodine. As we have already noted, the oral or intravenous administration of ^{131}I-labeled iodide to an individual is followed by a uniform mixing of this iodide with endogenous, stable iodide within the iodide distribution space, including the blood compartment. Immediately upon

its entrance into the plasma, the ^{131}I begins to be removed by the thyroid and excreted through the kidney. Since the thyroid and kidney are the main pathways for the removal of iodide from the plasma, the maximum urinary excretion and maximum thyroid accumulation should approach 100% of the administered dose; measurement of the urinary ^{131}I excretion should, after subtraction, indicate thyroid uptake. Some typical values for the 24-hour thyroid uptake, the 24-hour urinary excretion, and the thyroid iodide clearance are shown in Table 13-1.

To test the turnover or release of thyroid iodine, the protein-bound radioiodine ($PB^{131}I$) of the serum is measured 48 or 72 hours after the administration of a moderately large dose of ^{131}I. Normal values range from 0.2 to 0.4% of the administered dose. Another useful measure is the "conversion ratio" which expresses the ratio of $PB^{131}I$ to the sum of the inorganic ^{131}I and $PB^{131}I$ present in the serum at a definite time interval, usually 24 hours, after administration of the ^{131}I. The normal value of this ratio is between 0.2 and 0.5.

3. Tests Related to the Concentration and Physical State of the Thyroid Hormone in the Blood

This group of procedures include the measurement of protein-bound iodine (PBI) and total iodine, butanol-extractable iodine (BEI), total and free thyroxine and *in vitro* uptake tests. As we indicated earlier in this chapter, the serum ordinarily contains iodine chiefly in the form of inorganic iodide, free and protein-bound thyroxine (T_4), and triiodothyronine (T_3). Thyroglobulin and nonspecific iodoproteins are present in smaller amounts and iodinated dyes may be present as the result of prior roentgenological diagnostic procedures. These compounds are separable to some extent by virtue of their solubility characteristics (Ingbar and Woeber, 1968). Treatment with trichloroacetic acid results in the precipitation of the following iodine compounds: protein-bound T_3 and T_4, iodinated dyes, thyroglobulin, and iodoprotein. The supernatant contains inorganic iodide. Washing of the precipitate with butanol results in the extraction of T_3, T_4, some inorganic iodide, and the iodinated dyes. The precipitate is composed of thyroglobulin and iodinated proteins. Alkaline washing of the butanol extract results in the elimination of inorganic iodide in the aqueous phase.

Measurement of the PBI concentration in plasma or serum is probably the most widely used procedure for the diagnosis of abnormal thyroid function. The mean values obtained for groups of normal individuals by various investigators have been essentially similar. Several such

values, expressed as micrograms per 100 ml serum, may be cited in illustration: 5.7 (Riggs *et al.*, 1941), 4.6 (Connor *et al.*, 1949), 5.4 ± 0.94 (Kydd *et al.*, 1950), and 6.2 ± 1.3 (Bodansky *et al.*, 1958). Table 13-1 also lists the results of some other studies. In general, a range of 4.0–8.0 μg per 100 ml would represent the results of most studies on normal mean values. The determination of serum PBI involves the precipitation of serum proteins, usually with trichloroacetic acid. The digestion of serum without protein precipitation yields values about 1.0 μg per 100 ml higher than those for the PBI, and this increment represents chiefly inorganic iodide.

As we have already seen, extraction of serum with butanol breaks up the association between T_4 and T_3 with their binding proteins and T_4, T_3, iodide, and any iodotyrosines present in the plasma enter the butanol phase. Subsequent washing with a sodium carbonate–sodium hydroxide mixture removes the iodides and iodotyrosines and yields a better measure of T_4. Normally, the BEI obtained in this way is about 0.6 μg per 100 ml less than the PBI. If a greater difference is obtained, the likelihood exists that abnormal products, usually iodoproteins, are being released into the circulation from the thyroid gland.

In the absence of abnormal iodinated products, the concentration of PBI or BEI closely reflects the concentration of T_4, but the specific measurement of this compound may itself be accomplished by several methods. In 1961, Pileggi *et al.* utilized column chromatography with anion-exchange resin Dowex-1 and several years later, West *et al.* (1965) proposed methods based on thin layer chromatography and silica gel chromatography. Both methods eliminated spuriously high increments in the values obtained by PBI and BEI methods used in the analysis of the serum of patients who had received various organic iodine x-ray contrast media or who had been treated with potassium iodide. West *et al.* (1965) obtained a mean value for T_4 iodine of 5.24 ± 1.06 (SD) μg per 100 ml in 117 euthyroid patients (Table 13-1).

The measurement of serum T_4 may also be accomplished by a displacement method. This procedure depends on the capacity of increments of stable T_4 to displace progressively radioactive iodine-labeled T_4 from thyroid hormone-binding globulin (TBG) in a sample containing this protein and albumin (Murphy and Pattee, 1964). The mean value obtained in 40 euthyroid subjects and expressed as T_4 iodine was 6.6 ± 1.3 (SD) μg per 100 ml (Table 13-1), corresponding to a mean T_4 level of 10.1 μg per 100 ml (range 6.1–13.8).

Most methods for the determination of free T_4 depend upon the ability of this compound to pass through a semipermeable membrane during dialysis or utrafiltration. Actually, the passage of labeled free T_4 is

measured since the serum is enriched with a very low concentration of ^{131}I-labeled T_4 which is dissociable into the free and bound T_4 in the same proportion as the unlabeled compound. Oppenheimer *et al.* (1963) obtained a normal value for free T_4 of 3.02 ± 0.37 (SD) $\times 10^{-11}$. This is equal to 2.34 ± 0.029 (SD) ng per 100 ml or $0.0277 \pm 0.0029\%$ of the total T_4. Increased values for this percentage are found in hyperthyroidism or in conditions characterized by a decrease in the activity of binding protein. Conversely, decreased values for the proportion of free T_4 occur in hypothyroidism and in conditions in which the concentration of thyroid hormone-binding prealbumin (TBPA) in the serum is decreased or in which the binding activity of TBG is increased (Ingbar and Woeber, 1968).

A measure of the reverse binding capacity of the T_4-binding proteins may be obtained by any one of several *in vitro* uptake tests (Ingbar and Woeber, 1968; Rosenberg, 1972). Trace quantities of labeled T_4 are added to the serum and this presumably dissocates in the same proportion as the unlabeled hormone. The serum is then incubated with an insoluble particulate material such as an anion-exchange resin, dextran-coated charcoal, or even the patients' red cells, any of which compete with the serum proteins in binding T_4. At the end of a stated time, the proportion of labeled hormone bound to the insoluble phase is determined. Since T_3 is bound more weakly to serum proteins, this compound is most often employed in uptake tests. The "resin uptake," or "T_3 uptake," is influenced by several factors but, in general, tends to be high in hyperthyroidism and low in hypothyroidism.

4. Tests Based on Metabolic Responses

Several of the many metabolic responses of the human organism to defective or excessive secretion of the thyroid hormones have found an ancillary use in the diagnosis and management of patients with these conditions. We have considered the relationship of serum cholesterol levels to the thyroid state earlier in this chapter. The determination of the basal metabolic rate (BMR) was a classic procedure for assessing the thyroid function for many years. Although its use has steadily decreased, it still claims some attention (Becker, 1971). The BMR reflects the calorigenic effects of the thyroid hormones which we have described earlier in this chapter. Practically, the oxygen consumption of individuals is measured in a basal state, with due regard for technical factors that may affect the apparatus and psychological factors that may influence the patient. The utility of this procedure is illustrated by Fig. 13-5,

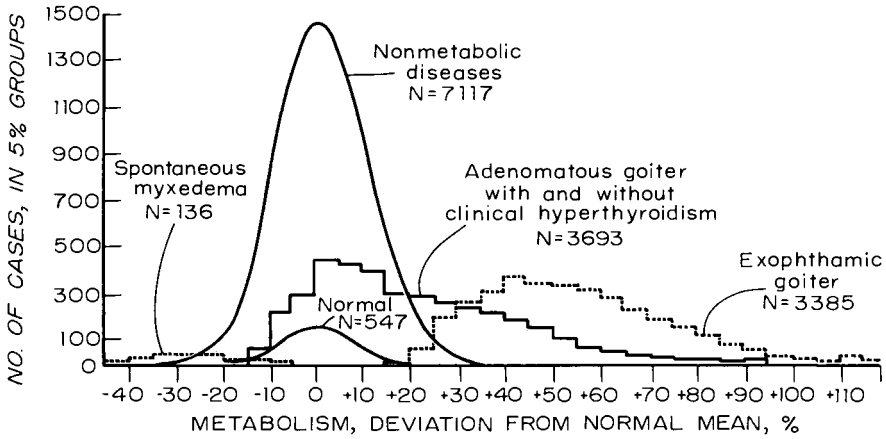


Fig. 13-5 Comparison of normal distribution of basal metabolism with that for 3 groups of thyroid disease and a nonmetabolic disease group. The frequencies are the number of cases corresponding to each group seen at the Mayo Clinic during a 10-year period. The total area under each curve is therefore proportional to the number of individuals in the corresponding group sent to the metabolism laboratory during this period. After Boothby *et al.* (1937). Reproduced by permission of the American College of Physicians.

based on the distribution of the BMR's obtained in large series of cases studied by Boothby *et al.* (1937) over a 10-year period at the Mayo Clinic. It may be noted that there is a degree of overlap between the different groups which reflects the limited utility of BMR's as a diagnostic aid and the necessity for the use of additional procedures such as we have been describing in this section.

Creatine metabolism is deranged in hypothyroidism and hyperthyroidism, and a creatine tolerance test based upon this feature was proposed by Shorr in 1934 and by Thorn in 1936. However, this test has rarely been employed in recent years. An oral tyrosine tolerance test was proposed more recently. In normal fasting individuals, the plasma tyrosine concentration averaged 12.5 μg/ml, as contrasted with 20.0 μg/ml in 14 patients with hyperthyroidism (Soos *et al.*, 1961). The levels of other amino acids, except glutamic acid, are normal in hyperthyroidism. In a typical study on the tyrosine tolerance test (Melmon *et al.*, 1964; Rivlin *et al.*, 1965), the fasting level of plasma tyrosine was 18.5 ± 0.8 μg/ml in a group of 9 hyperthyroid patients. After an oral dose of 50 mg tyrosine per kg body weight, peak values of approximately 80–100 μg/ml were reached in $\frac{1}{2}$–$1\frac{1}{2}$ hours after ingestion, whereas levels of only 35–40 μg/ml were attained in euthyroid subjects. Although fasting plasma levels

and tolerance curves of tyrosine were lower in hypothyroid than in euthyroid patients, the differences were slight and not significant. In view of various conflicting reports, Becker (1971) did not believe that this procedure has proved adequate for routine clinical use, but Ingbar and Woeber (1968) held that further studies may prove its usefulness.

5. Tests of Integrity of Homeostatic Mechanisms

The thyroid-suppression test or the T_3 suppression test is based on the principle that administration of thyroid hormone in doses adequate to meet peripheral requirements suppresses the radioiodine uptake of the normal thyroid gland by suppressing TSH release from the pituitary. There are several methods of carrying this procedure out. In most of these, T_3 is used as the suppressive agent because of its more rapid action. Immediately after the control determination of thyroid radioiodine uptake, a dose of T_3 is given daily for several days and a second determination of uptake is done. The size of the duration of the dose has varied with the particular clinic (Werner, 1956; Wayne *et al.*, 1964; Ingbar and Woeber, 1968). The reduction of uptake to less than 50% of the initial value is considered a normal response. Failure of the 2-hour or the 24-hour uptake to decrease to less than 50% of the initial value indicates autonomous function by the thyroid such as is seen in thyrotoxicosis.

The TSH test is of aid in detecting 2 types of thyroid hypofunction. In the first, the thyroid is intrinsically normal but fails to be stimulated by TSH. This situation occurs in patients with pituitary or hypothalamic disease or in normal individuals who have been receiving suppressive doses of exogenous thyroid hormone. In the second type of hypofunction, diseases of the thyroid gland itself render the gland unresponsive, either partially or completely, to the administration of exogenous TSH.

There are several procedures for performing such a TSH test, and the work of Taunton *et al.* (1965) may be used in illustration. Following control measurements of PBI and 24-hour thyroidal ^{131}I uptake, several dosage schedules were explored. The optimal schedule consisted in the injection at three successive 24-hour intervals of 5 USP units of TSH, followed 24 hours later by a determination of the residual count and the administration of a second dose of ^{131}I. The increase or accumulation of ^{131}I in the thyroid is then determined after another 24 hours. The results, expressed as a fraction of this second intake of ^{131}I, were $41 \pm 16\%$ (SD) in 10 clinically euthyroid patients, $32 \pm 10\%$ (SD) in 5 patients with panhypopituitarism, and $2 \pm 1\%$ in 3 patients with extreme panhypopituitarism (Sheehan's syndrome).

6. Miscellaneous and Special Tests for Thyroid Economy

There are a number of other tests which may be mentioned briefly and will be discussed more fully in connection with our consideration of thyroid neoplasms. Though not a biochemical procedure, external scanning is of considerable diagnostic importance and should be described briefly.

The accumulation of ^{131}I, ^{125}I, or of other isotopes such as technicium (^{99m}Tc) or gallium (^{67}Ga) in the thyroid after suitable administration can be visualized by scintillation scanning. It is thus possible to determine the anatomic configuration of the thyroid, its alteration in disease and the presence of "hot" and "cold" areas, namely, areas of increased and decreased function, respectively.

The capacity of the thyroid to incorporate inorganic iodide into the organic compounds which constitute the thyroid hormones may be ascertained through the use of the percholorate discharge test (Harden, 1971). Perchlorate ion inhibits the uptake of iodide by the thyroid and discharges thyroid iodine which has been taken up by the thyroid but not bound organically. Radioiodine is administered and uptakes are measured before, and at intervals after the oral ingestion of perchlorate. In normal individuals, increases in the uptake cease. When organic binding is impaired, loss of thyroid radioiodine occurs. The test is considered positive if 10% of the radioactivity present in the gland is discharged (Wayne *et al.*, 1964).

We have already noted earlier in this chapter that Utiger and his associates (Utiger, 1965; Odell *et al.*, 1965) developed methods for the radioimmunoassay of TSH in human plasma or serum. Antibodies to human TSH were employed. The values in normal euthyroid adults and children range from undetectable amounts to 3 ng/ml plasma. Patients with hyperthyroidism are also in this range, whereas those with primary hypothyroidism have elevated values, ranging up to 212 ng/ml (Utiger, 1971). Using the International Human Thyrotropin Standard, Hershman and Pittman (1971a,b) found the normal serum TSH in 173 euthyroid individuals to be 3.9 ± 2.0 (SD) μU/ml. In 61 patients with primary hypothyroidism, the serum TSH ranged from 24 to 800 and averaged 107 μU/ml. In 23 patients with hyperthyroidism and in 10 patients with hypothyroidism secondary to hypopituitarism, serum TSH was undetectable. Procedures for chromatography of iodinated products in the serum and the determination of thyroid antibodies in the serum will be described, when necessary, in connection with our discussion of neoplasms of the thyroid.

TABLE 13-1

Biochemical Tests for Thyroid Hormone Economy: Results in Euthyroid Individuals

Type of test	No. of individuals	Result	Reference
24-Hour thyroid uptake of ^{131}I as % of intake	11	28.3 ± 4.6[a]	Keating *et al.* (1947)
	181	25.3 ± 8.7[b]	McConahey *et al.* (1956)
	75	17.6 ± 6.6[c,d]	Maisey *et al.* (1973)
Thyroid iodide clearance rate (ml/minute)	181	8.2 ± 6.5[b]	McConahey *et al.* (1956)
24-Hour urinary excretion of radioiodine as % of intake	110	60.6 ± 0.79[a]	Skanse (1949)
	73	53.7	Berson *et al.* (1952)
	181	58.8 ± 9.6[b]	McConahey *et al.* (1956)
PBI (μg/100 ml serum)	80	5.4 ± 0.94[c]	Kydd *et al.* (1950)
	100	6.2 ± 1.3[c]	Bodansky *et al.* (1958)
	2182	5.21 ± 1.17[a]	Lowrey and Starr (1959)
	130	4.9 ± 0.10[a]	Wayne *et al.* (1964)
T_4 iodine (μg/100 ml serum)	40	6.6 ± 1.3[c]	Murphy and Pattee (1964)
	117	5.24 ± 1.06[c]	West *et al.* (1965)
Free T_4 (10^{-11} *M* ng/100 ml serum)	18	3.02 ± 0.35[c]	Oppenheimer *et al.* (1963)
	8	1.4 ± 0.1[a]	Nicoloff *et al.* (1972)
T_3 (ng/100 ml)	31	220 ± 27[c]	Sterling *et al.* (1969)
	8	106 ± 17[a]	Nicoloff *et al.* (1972)
Thyroid-suppression test as % suppression of initial thyroid ^{131}I uptake	14	70 ± 14[e]	Starr and Liebhold-Schueck (1953)
TSH-stimulation test thyroid uptake as % of uptake after TSH administration	10	41 ± 16[c]	Taunton *et al.* (1965)
Circulating TSH (μU/ml serum)	173	3.9 ± 2.0[c]	Hershman and Pittman (1971b)

[a] Standard error of mean.

[b] Standard deviation calculated from 95% range. Skew distribution.

[c] Standard deviation of mean.

[d] Iodine uptake values have fallen progressively over the years because of introduction of iodide into foods.

[e] Calculated from data of oral intakes of 35–140 μg once a day for 7 days.

The results of various biochemical tests for thyroid function and economy in euthyroid individuals are summarized in Table 13-1.

III. Biochemistry of Thyroid Neoplasms

A. Thyroid Nodules and Adenomas

As we indicated in Section I, the incidence of thyroid nodules in the general population is approximately 4%. Many of them are "cold" nodules, that is, they fail to concentrate radioiodine either before or after TSH administration, as revealed by scintiscan. These nodules are of clinical concern because the question arises whether they are precursors of cancer. In 1948, Dobyns and Lennon prepared radioautographs from patients who had previously received tracer doses of radioiodine and observed in general, but not without marked exceptions, a parallelism between the degree of function and the degree of cellular differentiation of adenomas. According to Malamos *et al.* (1969), patients with toxic adenoma constitute a very small proportion, about 10%, of all patients with hyperthyroidism. They also have a lower incidence of abnormal uptake curves and elevated $PB^{131}I$ values than those with hyperthyroidism.

In an attempt to elucidate the inability of cold thyroid nodules to concentrate ^{131}I before or after TSH administration, De Rubertis *et al.* (1972) studied various metabolic parameters in 11 cold thyroid adenomas, 2 medullary carcinomas, and surrounding normal thyroid tissue. These investigators observed that basal adenyl cyclase activity, [1-^{14}C]glucose oxidation and ^{32}P incorporation into phospholipids were significantly greater in the adenomas than in the contiguous normal thyroid tissue. The concentration of basal cyclic AMP and incorporation of [^{3}H]adenine into ^{3}H-labeled cyclic AMP were not different. Upon incubation with TSH, the adenyl cyclase activity and ^{32}P incorporation were increased significantly in the adenomas in comparison with that of adjacent normal thyroid. In other words, it would appear that the adenyl cyclase–cyclic AMP system is intact in the adenomas. Receptor sites for TSH are present on the cells of the adenoma, and the failure of the cold nodules to concentrate ^{131}I is the result of a subsequent impairment of the iodine metabolism. Burke and Szabo (1972) have found more recently that "hot" nodules may also be more responsive to TSH *in vitro* than normal thyroid tissue. They have suggested that both acquisition of autonomy and loss of function in thyroid nodules may, occasionally, be restricted to iodine metabolism.

B. Papillary and Follicular Carcinomas of the Thyroid

1. Hyperthyroidism

Much of the older work on the biochemistry of thyroid carcinoma was reported without regard to the histological character of the tumor. However, since follicular and papillary carcinomas constitute about 75% of all thyroid carcinomas, the data applied chiefly to these 2 types. This work indicated that these cancers rarely led to an increase in the production of thyroid hormone and, indeed, that in the vast majority of patients there was reduced function in the tumors when compared to normal thyroid tissue in the same gland. For example, Keating *et al.* (1947) obtained the following results in a series of 75 patients: BMR, -7.0 ± 1.3(SD) %; 24-hour radioiodine thyroid uptake, 24.4 ± 1.3 (SD) %; and 24-hour radioiodine urinary excretion, 63.7 ± 1.1 (SD) %. These values were within the normal range. Similarly low incidences of hyperthyroidism in large series of patients with thyroid cancer have been obtained by other investigators (Crile, 1936; Skanse, 1949; Pemberton and Black, 1948). Skanse (1949) also observed that the serum PBI value was normal in 7 of 8 cases of carcinoma of the thyroid in whom the determination was done and low in 1 case.

Leiter and his associates (1946) described a patient who had had clinical symptoms of hyperthyroidism for 6 years prior to admission. A large encapsulated mass, later diagnosed as "adenocarcinoma of the thyroid," had been removed 4 years before admission. Metastatic lesions developed subsequently and, after admission, the BMR values ranged between +32 and +39%. The urinary radioiodine excretion during 4 days was low, about 12% of the administered dose.

The case of Leiter *et al.* (1946) is representative of those cases in which hyperthyroidism resulted from hyperfunction developing in metastatic lesions after the original tumor and the normal thyroid gland had been removed. It is much more rare to encounter cases in which thyrotoxicosis is the result of hyperfunctioning of the primary carcinoma itself. Thus, Sussman *et al.* (1968) studied a 6-year-old female child with mixed follicular and papillary carcinoma, with the latter type predominating. Initially, the child showed elevated PBI values ranging from 12.0 to 19.0 μg per 100 ml serum and T_3 resin uptakes of 51 and 53%, substantially higher than the normal range of 27–36%. The ^{131}I uptake was normal, namely, 24% at 24 hours. These results were obtained after 3 months of potassium iodide administration and while the patient was receiving corticosteroid. Later and after discontinuation of the iodide

therapy, the PBI value was 25 μg per 100 ml, T_4 was 19.4 μg per 100 ml, but the ^{131}I uptake was 34% at 24 hours and, hence, still within the normal range. Ghose *et al.* (1971) have also reported an unusual instance of hyperthyroidism associated with primary and metastatic thyroid carcinoma in a 68-year-old female. Radioactive iodine scanning revealed predominant intake in the tumor and suppression of function in the remaining normal thyroid tissue. Propylthiouracil treatment was instituted, and the thyrotoxic state abated while the function of the previously suppressed normal tissue increased. These findings indicated that the tumor tissue was the cause of the patient's hyperthyroidism.

2. Serum Thyrotropin in Thyroid Cancer

Serum levels of TSH are substantially elevated in patients with primary hypothyroidism and are very low and usually undetectable in hyperthyroidism and in hypothyroidism secondary to hypopituitarism (Odell *et al.*, 1965; Hershman and Pittman, 1971a,b). Accordingly, the TSH test differentiates between thyroid hypofunction resulting from intrinsic disease of the thyroid gland and that resulting from hypothalamic or pituitary disease.

The serum levels of TSH in papillary or follicular carcinoma of the thyroid have been studied by several groups of investigators. Employing methods based on a cross-reaction between human TSH (hTSH) and antibodies to bovine TSH (bTSH) and an immunofluorescent assay, Hargadine *et al.* (1970) found that almost all patients with thyroid cancer had significantly higher serum levels than individuals with adenoma, thyroiditis, or hyperthyroidism. Nineteen of the 23 cases studied had papillary thyroid carcinoma. One case of medullary carcinoma, 1 of 2 cases of mixed tumor, and 1 case of adenocarcinoma also showed substantially elevated serum TSH levels. The normal ranges were 2–4 μU/ml for 17 men and 4–10 μU/ml for 15 females. When L-thyroxine was administered to the patients, the serum TSH levels tended to decrease as might be anticipated but, at each dosage, these levels were higher in the patients with thyroid cancer than in those with other thyroid diseases or nonthyroid cancers.

Also employing bovine TSH as a standard, Valenta and his associates (1970a,b) studied a group of 90 patients with histologically confirmed thyroid cancer, some of whom exhibited hyperthyroidism. The results were similar to those of Hargadine *et al.* (1970). As we have seen (Section III,B,2), the serum TSH level is low and usually undetectable in patients with hyperthyroidism. The occurrence of high serum TSH levels in hyperthyroidism associated with thyroid carcinoma led Valenta

et al. (1970b) to formulate the presence of an impairment of the feedback link of the thyroid-pituitary regulating mechanism. Indeed, this impairment was considered to hold more generally in patients with thyroid carcinoma regardless of the presence of hyperthyroidism, for the mean level in such a group was significantly higher than in euthyroid patients. Valenta *et al.* (1970a) suggested that hyperplasia of the pituitary cells secreting TSH, which is found in some thyroid carcinoma patients (Russfield, 1955), could be primary or secondary to chronic suppression of the function of normal thyroid gland containing carcinoma. Another possible mechanism, namely, that elevation of serum TSH arises as a result of destruction of normal thyroid tissue by the thyroid carcinoma, with the resulting tendency to hypothyroidism and the elicitation of the feedback mechanism, was considered unlikely by Hargadine *et al.* (1970).

These interesting speculations may, however, be rendered irrelevant by more recent findings. In contrast to the results of Lemarchand-Béraud *et al.* (1969) and of Hargadine *et al.* (1970), Mayberry *et al.* (1971) failed to obtain elevated plasma TSH levels in individuals with thyroid cancer when antibodies to human instead of to bovine TSH were employed in the assay. This discordance was more persuasively demonstrated by Greenspan *et al.* (1972) who performed concurrent radioimmunoassays with antibodies to the two species of TSH. Table 13-2 shows that a group of 32 patients with thyroid cancer had a substantially

TABLE 13-2

Plasma Thyrotropin Levels in Normal Individuals and Patients with Various Diseases, as Determined by Radioimmunoassay against Antibodies to Bovine TSH and Human TSH[a]

Group	No. of subjects	Assay with bTSH Mean ± SE (μU/ml)	Assay with hTSH Mean ± SE (μU/ml)
Normal	30	2.9 ± 0.18	2.9 ± 0.2
Other thyroid diseases	30	7.4 ± 1.45[b,c]	10.7 ± 2.8[b]
Other cancer	23	4.5 ± 0.74	4.7 ± 0.9
Thyroid cancer	32	22.4 ± 5.6[b,c]	5.3 ± 1.3

[a] From Greenspan *et al.* (1972). Reproduced by permission of Dr. M. B. Lipsett, Editor-in-Chief, *Journal of Clinical Endocrinology and Metabolism* and The Endocrine Society.

[b] Significantly elevated ($p < 0.01$) with respect to normal levels.

[c] That these results might have been spuriously high has been indicated by a recent report (Greenspan *et al.*, 1974).

and significantly elevated mean value for levels of plasma TSH when these were determined with antibodies to bTSH, but had normal levels when assayed with antibodies to hTSH. Of interest was the finding that a significantly elevated mean value was obtained for patients with other thyroid diseases by either type of assay, although there was considerable overlap of individual values between the ranges of this group and the group of normal persons. Recently, Greenspan and his associates (1974) have reported that even the elevated levels of TSH which his group obtained in thyroid cancer with bTSH might have been spurious, partly as a result of previous immunization of some patients with injections of bovine TSH and partly because of nonspecific protein effects in the radioimmunnoassay system.

3. Long-Acting Thyroid Stimulator in Patients with Thyroid Cancer

We have previously noted (Section II,C,4) that in certain conditions human blood may contain a long-acting thyroid stimulator (LATS). This is defined as positive when, 9 hours after injection in mice, the value for serum ^{131}I is higher than the 2-hour value and is at least 150% of the initial, preinjection value (Leeper, 1969; Valenta *et al.*, 1970b). Long-acting thyroid stimulator was found to be present in the plasma of 10 out of 22 patients with thyroid carcinoma.

4. Biochemical Characteristics of Human Thyroid Carcinomatous Tissue

Several studies have been carried out on normal and carcinomatous thyroid tissue to determine whether there are any characteristic biochemical differences. The intracellular as well as the tissue distribution of iodotyrosine deiodinase (ITDase) has been the subject of several studies. Stanbury and Morris (1958) noted that ITDase is a microsomal enzyme which requires TPNH and that it is similar in the thyroid, liver, and kidney. Kusakabe and Miyake (1966) observed that, in the normal thyroid gland, the enzyme is distributed chiefly between the mitochondrial and microsomal fractions and that its activity is relatively low in the soluble fraction. The hyperthyroid gland shows an increased amount of activity in the soluble fraction, approximately equal to that in the other two fractions and to the activities of these fractions in the normal thyroid gland. Analysis of a thyroid cancer of the papillary type showed similar ranges of activity in the mitochondria and microsomes but virtual absence of the enzyme from the soluble fraction. The ITDase of the extract of the particulate fraction, presumably the com-

bined mitochondria and microsomes of normal thyroid, liver, or kidney, migrated electrophoretically toward the cathode, while the deiodinase of the soluble fraction from normal thyroid and liver migrated toward the anode. This difference indicates the possibility that the enzyme exists in isoenzymic forms.

The activities of the nucleases and adenosine deaminase in normal and various types of diseased thyroid tissue have been explored by Goldberg and Goudie (1968). Several of the enzyme activities were altered, but such changes were not necessarily specific for the particular disease. Thus, the activity of acid RNAase in the supernatant or soluble fraction, expressed as percent of the whole cytoplasmic activity, was decreased significantly both in the thyroid tissue from patients with cancer and with thyroiditis. The activity in the microsomal fraction was elevated significantly in both these types of disease tissue. The absolute activities of DNAase I and II activity tissue, expressed as units per milligram protein, were elevated significantly in the supernatant and mitochondrial fractions of cancer thyroid tissue and as units per gram wet weight in the microsomal fraction, but significant elevations of these enzyme activities also occurred in one or more of the fractions of thyroid tissue from patients with thyrotoxicosis or thyroiditis. Similarly, significant elevations of adenosine deaminase activity occurred in the supernatant fractions of thyroid tissue from patients with thyroid cancer as well as in patients with thyrotoxicosis and thyroiditis.

Valenta (1966) has pointed out that functioning thyroid carcinoma differs from the normal thyroid gland in lower accumulation and faster turnover of radioiodine. Several types of abnormal synthesis of thyroid hormone have been described. These include cases of thyroid carcinoma: (a) producing iodotyrosines predominantly (Hales *et al.*, 1964), (b) trapping radioiodine without organifying it (Valenta, 1966), and (c) secreting an abnormal iodoprotein with many of the properties of serum albumin (Robbins and Rall, 1960).

It has been held since the observations of Seidlin *et al.* (1948) and of Rawson and his associates (1948) that metastasizing thyroid carcinoma in patients with normal thyroid glands only occasionally, and then to a small extent, accumulates radioactive iodine. It is after total surgical or radiothyroidectomy that iodine uptake in metastases becomes appreciable. Valenta *et al.* (1968a) showed that, in extracts of thyroidal carcinomatous tissue obtained from patients not subjected to radioiodine uptake, the thyroglobulin-iodine was 1/10 to 1/1000 of that in extracts obtained from normal thyroids. Moreover, they determined the extent to which organification, as determined by ultracentrifugal analysis and paper electrophoresis of the proteins extracted from the primary tumor or metastases, was present in various functional types of tumor (Valenta *et al.*, 1968b).

Thyroglobulin-type proteins, namely, thyroglobulins 19S and 4S, were present in substantial amounts in tumors capable of accumulation and organification of iodine either at the time of examination or after thyroidectomy (radiothyroidectomy). In those specimens which had been labeled, the majority of the radioactivity was bound to thyroglobulin, but about 5% of the total radioactivity was present in an albuminlike protein. In 7 specimens from 5 patients with nonfunctioning papillary or follicular thyroid carcinoma, as determined by lack of ^{131}I uptake, the 19S thyroglobulinlike proteins were virtually absent. This was also true of all nondifferentiated thyroid tumors such as small or giant cell carcinomas. Seven specimens from 7 patients with "cold" adenomas as well as 2 specimens of normal thyroid gland had substantial fractions of 19S thyroglobulin.

C. Medullary (Solid) Carcinoma of the Thyroid

1. Introduction

The medullary or solid carcinoma of the thyroid constitutes about 10% of all thyroid carcinomas but, from a biochemical point of view, is the most interesting of the various types. Its existence as a distinct clinicopathological type of thyroid carcinoma was first described by Hazard *et al.* in 1959. It occurs most often in patients over 40 years of age, but occasionally in younger adults. Grossly, the tumor is usually a hard gray-white, well-demarcated mass. Microscopically, the tumor consists of cells growing in solid, sheet-like masses which are separated by a hyaline amyloid-containing stroma. Many variants of the cells and cellular arrangements can be observed, but there is no transition either to papillary or follicular carcinoma. Although the tumor is a slow-growing one, it possesses considerable capacity for metastasizing (Hazard *et al.*, 1959). Evidence has been accumulating that the occurrence of the tumor may be familial and may be associated with pheochromocytoma or with cutaneous or mucous membrane neuromas (Meissner, 1971). As will be noted presently, medullary carcinoma has multiple biochemical effects, namely, the production of thyrocalcitonin, prostaglandins, serotonin, histaminase, and ACTH.

2. Thyrocalcitonin (Calcitonin) in Medullary Thyroid Carcinoma

In Chapter 10, we mentioned but did not consider further the role of thyrocalcitonin in calcium metabolism. This hormone, which lowers

the serum calcium in lower animals (Foster, 1968) and has also been reported in man (Aliapoulis *et al.*, 1966), was first believed to arise in the parathyroid gland. However, the thyroid origin of the hormone was later suggested by Hirsch *et al.* (1963) who found that parathyroidectomy in rats, when performed by hot-wire cautery, produced a more rapid fall in serum calcium than surgical removal of the parathyroid glands. Cautery apparently caused trauma to the thyroids, and cautery of the thyroid gland alone produced profound hypocalcemia even when the parathyroid glands were not disturbed. Subsequently, calcitonin was extracted in large amounts from the thyroid glands of a large variety of animal species and of man (Foster, 1968). Although, for some time there was disagreement on whether there were 2 types of calcitonin, one arising in the parathyroid and the other in the thyroid, most investigators hold that, with rare exceptions, calcitonin is secreted only by the thyroid gland and, in species where these exist, by the ultimobranchial bodies (Moseley *et al.*, 1968; Foster, 1968).

Within a few years after calcitonin was recognized to be present in high concentrations in the thyroid gland, it was purified 50,000- to 100,000-fold from the glands of various species and found to be a polypeptide consisting of about 32 amino acids and having a molecular weight of 3,600 (Foster, 1968). As many as four active fractions may exist and the calcitonin of one species differs from another with regard to the position of the various amino acids and, indeed, the number of a particular amino acid in the sequence (Neher *et al.*, 1968).

It has long been appreciated that the normal thyroid gland contains cells that differ from the T_4- and T_3-secreting cells. By means of immunofluorescent studies on pig and dog thyroid, it was shown that these "parafollicular" cells contain calcitonin (Foster *et al.*, 1964; Bussalati and Pearse, 1967). They were designated as "C" cells to associate them with the secretion of calcitonin. In their study of medullary carcinoma, Williams *et al.* (1966) described the typical cell as having an ill-defined cytoplasmic membrane and plentiful finely granular eosinophilic cytoplasm. They suggested that these cells resembled the parafollicular (light, clear, C) cells of the normal thyroid gland. Meyer and Abdel-Bari (1968) confirmed the histological findings in a medullary carcinoma from a 50-year-old patient and obtained an extract of the tumor which had a thyrocalcitoninlike activity at least 100 times that found in the normal human thyroid gland. Using a biological assay method dependent on the hypocalcemic effect produced by injection of tissue extracts in rats, Tashjian and Melvin (1968) found that extracts of thyroid medullary carcinoma from 2 patients contained 1000–2000 times more hypocalcemic activity than normal human thyroid tissue.

3. Serum Calcium and Phosphorus in Medullary Thyroid Carcinoma

The serum calcium level is not usually lowered in patients with medullary carcinoma. Thus, the case reported by Meyer and Abdel-Bari (1968) and the 3 cases described by Stephens and Braunstein (1969) had concentrations of serum calcium within the normal range. On the other hand, of 2 cases reported by Tashjian and Melvin (1968), 1 case with extensive metastases to the liver had markedly low serum calcium levels of 6.0–6.5 mg per 100 ml, low serum phosphate levels of 2.2–3.0 mg per 100 ml, and frequent episodes of tetany; the second patient had serum calcium levels ranging from 8.1 to 8.9 mg per 100 ml, slightly below or at the lower region of the normal range. Perhaps the serum calcium level is dependent on the progress of the disease and the occurrence of metastatic spread. For example, in the case reported by Verdy *et al.* (1971), the serum calcium level was 10.6 mg per 100 ml and within the normal range when the patient was first seen but, 6 years later, when metastatic spread was more pronounced and the plasma calcitonin was higher, the plasma calcium had decreased and now oscillated between 7.0 and 8.2 mg per 100 ml. The serum phosphorus was normal, ranging from 3.8 to 4.4 mg per 100 ml. In general, calcitonin tends to produce hypocalcemia and hypophosphatemia, whereas parathyroid deficiency is associated with hypocalcemia and hyperphosphatemia.

4. Serum Calcitonin in Medullary Thyroid Carcinoma

Using a bioassay method, Tashjian and Melvin (1968) found that the plasmas of 2 patients with medullary thyroid carcinoma contained considerable hypocalcemic activity as compared with normal human plasma or plasmas from hypocalcemic patients with hypoparathyroidism. In the first of their 2 cases of medullary carcinoma, the level of thyrocalcitonin was 9 Medical Research Council milliunits (MRC mU) per milliliter when the plasma calcium was 6.0 mg per 100 ml. In the second case, the plasma thyrocalcitonin was 8 mU/ml when the plasma calcium ranged between 8.7 and 8.9 mg per 100 ml.

The introduction of radioimmunoassay methods has facilitated the further study of serum calcitonin. Employing a radioimmunoassay method based on antiserum prepared to pure synthetic human calcitonin M, Tashjian and his associates (1970) were able to express the concentration of calcitonin in terms of nanograms per milliliter of serum. Figure 13-6 shows that the levels of calcitonin in 81 samples of 58 control subjects ranged between 0.02 and 0.40 ng/ml, whereas all samples from

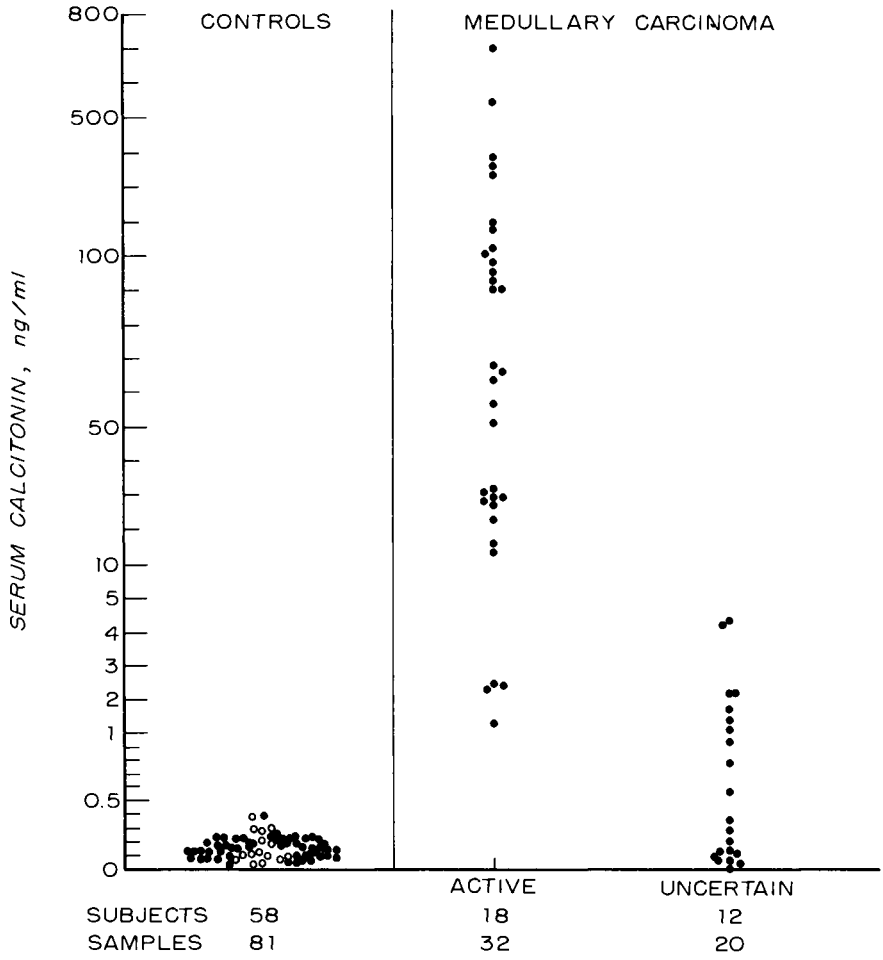


Fig. 13-6 Serum calcitonin in control subjects and patients with medullary carcinoma. Controls were normal laboratory personnel (○) or patients without evidence of thyroid disease or disorders of mineral metabolism (●). Cases of medullary carcinoma were designated "active" when there was clinical evidence of tumor and "uncertain" after treatment of the tumor and when there was no clinical evidence of disease. After Tashjian *et al.* (1970). Reproduced by permission of the *New England Journal of Medicine*.

18 patients with clinical evidence of medullary carcinoma showed serum calcitonin concentrations ranging between 1.0 and 700 ng/ml. Twelve patients with previously diagnosed medullary carcinoma who had no clinical evidence of disease after treatment showed low serum calcitonin levels, ranging from 0.02 to 4.3 ng/ml. Infusion of calcium led to increases of serum calcitonin in 13 of 21 control subjects and led to much

greater rises in 10 of 13 patients with medullary carcinoma. In a series studied by Hill *et al.* (1973), 12 of 14 patients had basal serum calcitonin values that were higher than those of control subjects. Maximal values obtained during a 4-hour calcium stimulation test were all higher than those obtained in a stimulation test with controls.

As we have already indicated and as may be stressed again, the concentration of calcitonin in the serum does not always correlate directly with the level of serum calcium. For example, of 39 serum samples on 29 patients with chronic hypercalcemia (>11 mg Ca per 100 ml) resulting from hyperparathyroidism, cancer, or idiopathic in nature, 5 showed slightly elevated levels, 0.5–1.3 ng/ml, of calcitonin. Of 9 samples from 6 subjects with chronic hypocalcemia (<8 mg Ca per 100 ml) either idiopathic or resulting from hypoparathyroidism, all serum calcitonin values were below 0.2 ng/ml. The upper limit of the normal range is 0.4 ng/ml (Tashjian *et al.*, 1970).

5. Serum Calcitonin in Familial Medullary Thyroid Carcinoma

During the past few years, abundant evidence has arisen that medullary thyroid carcinoma may have a familial occurrence and several kindreds have been described (Verdy *et al.*, 1971; Block *et al.*, 1972; Ljungberg, 1972; Hill *et al.*, 1973). As is to be expected, the incidence of familial thyroid medullary thyroid carcinoma in these kindreds is much higher, 2.6–27%, than in the general population which can be calculated to be 0.05–0.1%. In the study by Verdy *et al.* (1971), the patient developed metastases late in her course, diffuse increase in skeletal density, and an elevation in plasma calcitonin concentration. There were fairly good indications that 4 of her 9 living children had osteopetrosis. This diagnosis was confirmed roentgenologically in the 2 children available for study.

Block *et al.* (1972) have pointed out that medullary thyroid carcinoma is characterized biologically by a greater virulence and tendency to metastasize than the more common follicular and papillary types of thyroid carcinoma. They have suggested that these aspects make it highly desirable to determine serum calcitonin levels before the clinical effects become evident, particularly in the "familial" type of medullary thyroid carcinoma. Employing a radioimmunoassay, Block *et al.* (1972) found the upper limit of normal for the level of serum calcitonin to be 0.25 ng/ml at basal conditions and 0.55 ng/ml during or after a stimulation test involving the intravenous infusion of 15 mg calcium gluconate per kg of body weight given over a 4-hour period. Of 48 members of 2 unrelated families with medullary thyroid carcinoma, 12 were found

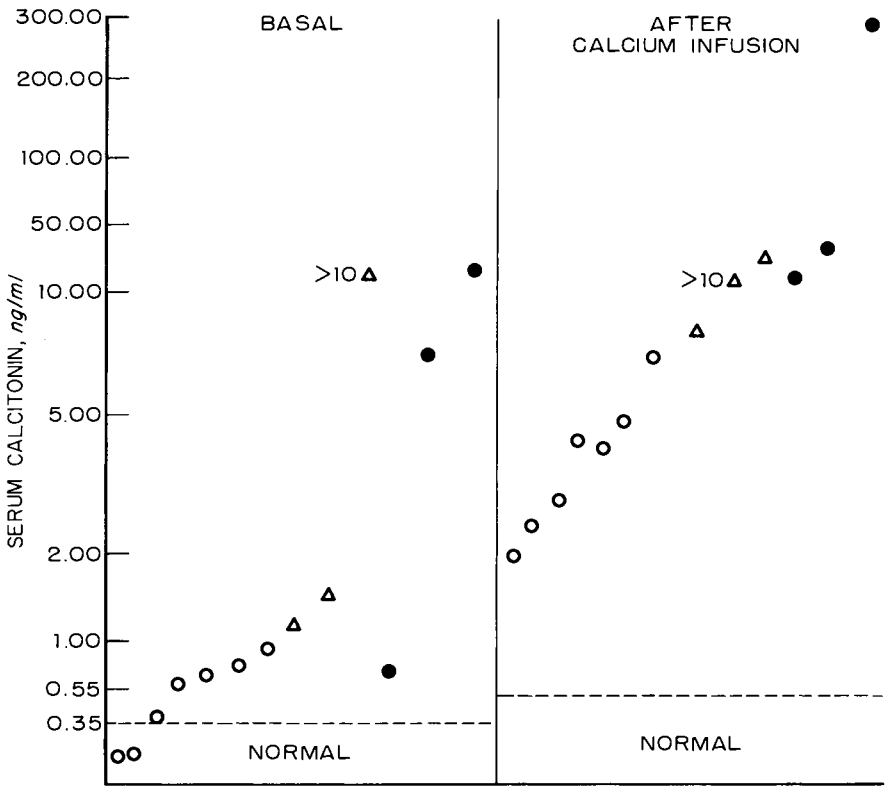


Fig. 13-7 Degree of elevation of serum calcitonin correlates well with extent of carcinoma in familial medullary carcinoma of thyroid. (○) Thyroid and neck examination negative preoperatively; (△) thyroid nodules palpable preoperatively; no lymph node metastases; and (●) bilateral palpable thyroid carcinoma; lateral cervical lymph node metastases. After Block *et al.* (1972). Reproduced by permission of the American Medical Association.

to have elevated basal levels of serum calcitonin and all of these as well as 2 additional patients with normal basal levels had elevated levels as a result of the calcium infusion test. The rises produced by the evocative test were substantial and, in some cases, impressive. For example, 2 patients with basal levels of 7 and 10 ng calcitonin per ml had rises to approximately 150 and 300 ng/ml, respectively.

In 8 of the 14 members with elevation of serum calcitonin, no clinical abnormality in the neck or evidence indicative of a thyroid carcinoma could be found. Although nodules were palpable in the thyroid of the remaining 6 patients, the patients were not aware of the abnormality.

Thirteen of the 14 patients were operated on and, in each, the presence of medullary thyroid carcinoma was confirmed histologically. Figure 13-7 shows that there is a correlation between the extent of carcinoma in familial medullary carcinoma of the thyroid and the degree of elevation of serum calcitonin. Diagrams of the pedigrees of the 2 families indicated that the carcinoma appeared as an autosomal dominant. Serum calcitonin assay has been advocated as an excellent tool for genetic analysis in families of patients with medullary thyroid carcinoma (Jackson *et al.*, 1971).

6. Association of Medullary Carcinoma of the Thyroid with Other Tumors

The coexistence of medullary thyroid carcinoma and pheochromocytoma, particularly as a familial occurrence, has already been mentioned (Section III,C,1; Block *et at.*, 1972). It has been studied by several investigators (Ljungberg *et al.*, 1967; Ljungberg, 1972) and, indeed, has been extended to include cases in which there is further association with multiple mucosal neuromas (Schimke *et al.*, 1968; Gorlin *et al.*, 1968; Baum, 1971). Medullary carcinoma of the thyroid may also be associated with parathyroid adenoma and hypercalcemia (MacGillivray and Anderson, 1971) and with a syndrome consisting of mucosal neuromas, marfanoid features, myopathy, and pigmentation (Cunliffe *et al.*, 1970). Recently, Sizemore *et al.* (1973) have also reported a relationship between the Zollinger-Ellison syndrome and medullary carcinoma of the thyroid. It has been suggested that these different associations all have a common histogenetic basis, namely, that neuroendocrine cells, originating in the neural crest, migrate into the primitive alimentary tract mucosa, find a resting place in the various endocrine glands, and develop into tumors that arise in these sites (Schimke *et al.*, 1968; Gorlin *et al.*, 1968; Weichert, 1970; Hill *et al.*, 1973).

It is not surprising that the association of medullary carcinoma of the thyroid with other conditions will be reflected in varying combinations of biochemical parameters characteristic of each. For example, MacGillivray and Anderson (1971) reported a case of association of medullary carcinoma of the thyroid and a parathyroid adenoma. The biochemical findings were primarily those of the latter, namely, hypercalcemia, hypophosphatemia, and nephrocalcinosis, whereas the serum calcitonin was normal. MacGillivray and Anderson (1971) also reviewed 17 other examples from the literature of the association between medullary carcinoma and parathyroid enlargement. Eleven of these did not have hypercalcemia; this finding was more characteristic of medullary

TABLE 13-3

Biochemical Parameters in the Zollinger-Ellison Syndrome and Medullary Carcinoma of the Thyroid[a]

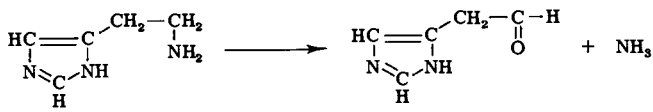
Component	Normal range	Patients with Z-E syndrome	Patients with medullary carcinoma of thyroid
Serum calcium (mg/100 ml)	8.9–10.1	9.2–10.0	8.7–10.0
Basal gastric acid secretion (mEq/hour)	<6	16–107[b]	—
Serum immunoreactive human gastrin (pg/ml)[d]	0–193	450–85,000[b,e]	16–60[c]
Plasma immunoreactive calcitonin (pg/ml)[b]	65–393	50–3,700[e]	8,000–285,000[e]

[a] Based on data of Sizemore *et al.* (1973). Sixteen normal individuals, 8 patients with the Z-E syndrome, and 7 patients with medullary carcinoma were studied.
[b] Chicken antiserum to human calcitonin M (Ciba) was employed.
[c] Mean value was significantly lower.
[d] Guinea pig antiserum to porcine gastrin was used.
[e] Mean value was significantly higher than normal.

carcinoma and the action of calcitonin than of hyperparathyroidism. Patients with medullary thyroid carcinoma have significantly lower concentrations of gastrin than normal individuals, and patients with the Zollinger-Ellison (Z-E) syndrome have significantly higher concentrations of calcitonin (Table 13-3).

7. Serum Histaminase Activity in Medullary Thyroid Carcinoma

The enzyme, histaminase, catalyzes the oxidative deamination of histamine with the formation of imidazole acetaldehyde and ammonia:



Histamine Imidazole acetaldehyde

It has long been known that this enzyme is elevated in the serum of pregnant women (Ahlmark, 1944). Recently, Sjoerdsma's laboratory developed a new and sensitive radioassay for histaminase and undertook

to explore alterations in the serum activity of this enzyme in a variety of clinical conditions (Baylin *et al.*, 1970).

The mean value in a group of 10 normal males and 12 normal females, 20–62 years of age, was 1.4 ± 1.0 (SD) pmoles of histamine deaminated per ml of serum per hour. The upper limit of normal was, accordingly, 3.4 pmoles/ml/hour. A group of 16 patients with various miscellaneous diseases such as pulmonary infections, chronic renal disease, Paget's disease, and hyperthyroidism showed no elevations. Of a group of 20 patients with various types of neoplastic disease (such as lymphoma; metastatic carcinomas of the lung, kidney, and breast; metastatic carcinoids; or hepatoma) only one, a women with metastatic carcinoma of the breast, had an elevated serum histaminase level of 5.0 pmoles/ml. In contrast to these findings, 4 of 7 patients with medullary thyroid carcinoma had elevated levels, the highest being 60 pmoles/ml/hour. Two of the three normal serum histaminase values were present in post-operative patients who were without evidence of recurrent disease.

Apparently, the high activity of histaminase in the serum resulted from the high content of this enzyme in the tumor tissue. Analysis of 8 specimens from 7 patients with medullary thyroid carcinoma yielded values ranging from 260 to 1960 and averaging 1395 pmoles histamine deaminated per gm of tissue per hour. In contrast, analysis of adjacent normal thyroid tissue in 5 patients yielded an average of 65 and a range of 15–170 pmoles histamine deaminated per gm per hour. Other neoplastic tissues, including 3 carcinomas of the breast, 2 sarcomas, 1 carcinoid tumor, and 1 pheochromocytoma, had low histaminase activity.

The basis for the elevated histaminase activity in the neoplastic tissue of patients with medullary thyroid carcinoma is largely unknown at present. The available evidence indicates that the deaminating action on histamine is specific, for monoamine oxidase activity is not elevated in either the serum or neoplastic tissue of patients with medullary thyroid carcinoma (Baylin *et al.*, 1970). It may be postulated that histaminase is produced and secreted in excessive amounts by the parafollicular or C cells of the carcinoma, but cytological study has not yet confirmed this (Baylin, 1971).

The excessive production of histaminase by medullary thyroid carcinoma and its entrance into the circulation and possibly other tissues of the body raise the possibility that this phenomenon might be utilized diagnostically. Baylin *et al.* (1972) found that the mean value for serum histaminase activity in 62 normal individuals was 1.6 ± 0.95 (SD) units/ml of serum where the unit was expressed as 1 pmole of histamine deaminated per hour of incubation. The serum histaminase activity in 42 patients with medullary thyroid carcinoma ranged from 1.2 to 90

units/ml and 21 patients had activities greater than 3.5 units, the upper limit of normal. Fourteen of these 21 patients came from families with inherited medullary thyroid carcinoma and 18 of the 21 had metastatic disease. Twenty-six unaffected members of the families with inherited disease had serum histaminase activity ranging from 0.5 to 4.1 units/ml, with only 2 of these subjects having values higher than 3.5 units, the upper limit of normal. In a series of 31 patients who had concurrent determinations of serum calcitonin levels and serum histaminase activity, 15 had high serum calcitonin levels but normal histaminase activity. Two had elevated serum histaminase activity but normal serum calcitonin levels. In the remaining 14 patients, both the serum calcitonin levels and the serum histaminase activities were elevated.

Serum calcitonin levels were normal in all of the clinically nonaffected individuals from families with the inherited disease. Twenty patients underwent surgery for removal of the thyroid carcinoma. Of 15 who had both pre- and postoperative determinations of basal serum calcitonin, all but one were elevated preoperatively. All showed a definite decrease and all but two decreased to normal levels as a result of the surgery. Of 19 who had similar determinations of serum histaminase activity, 6 patients continued to have high histaminase activity and 4 of these had local cervical metastases. It would therefore appear that, although determination of serum histaminase activity may be useful in the search for metastases and the detection of residual tumor after surgery, measurement of serum calcitonin offers a more sensitive criterion for the early detection of localized medullary thyroid tumor.

The normal response to the intradermal injection of histamine is the formation of a wheal, approximately 1 cm in diameter, surrounded by a flare, with an irregular erythematous area extending approximately 1–3 cm from the edge of the wheal (Lewis, 1927). The flare response seems dependent on an intact axon reflex along sensory fibers, and an absent flare response has been noted in a patient with the syndrome of pheochromocytoma, medullary carcinoma of the thyroid gland, and multiple mucosal neuromas (Baum, 1971). Several reports have now appeared to the effect that the axon flare is indeed absent in patients with medullary thyroid cancer and have suggested intradermal histamine injection as a convenient test for the presence of the tumor and, perhaps more particularly, for its metastatic spread (Gorlin, 1971; Jampol and Joison, 1972). Baum (1971) also raised the possibility that the absent flare might result from the degrading action of histaminase on histamine. Baylin (1971) has suggested that this relationship be further investigated by studying the flare response during the third trimester of pregnancy when circulating histaminase activity is high or by determining whether

the administration of aminoguanidine, a potent inhibitor of histaminase, would restore the response in cases of medullary carcinoma.

8. Diarrhea and Medullary Thyroid Carcinoma

In the course of reviewing 67 cases of thyroid medullary carcinoma, Williams (1966) noted that 14 of these had severe and prolonged diarrhea. This association has also been reported by Bernier *et al.* (1969) and by Clark's group (Ibanez *et al.*, 1967; Hill *et al.*, 1973). Of 53 patients with medullary carcinoma, 15 had persistent diarrhea. In a later, more complete study on 73 patients, 24 (32%) complained of diarrhea that was watery in consistency and contained undigested food particles.

In 1968, Williams *et al.* suggested that excessive prostaglandin secretion by the medullary thyroid carcinoma might be a cause of this diarrhea. The reader will recall that prostaglandins are C_{20} fatty acids containing a five-membered ring. A number of prostaglandins have been identified and differ from one another with respect to the number and positions of double bonds and hydroxyl group substituents. Prostaglandins have also been found in a bronchial carcinoid and two ileal carcinoids (Chapter 6, Section III,B,6).

Karim *et al.* (1967) observed that the concentrations of the prostaglandins E_2 and $F_{2\alpha}$ in human tissues, taken at autopsy 12–24 hours after death and macroscopically free of disease, were: in thyroid, 3.5–24.5 and 4.0–100 ng/gm of tissue, respectively; adrenal cortex, 2.5–3.0 and 3.0–6.3 ng/gm of tissue, respectively; and adrenal medulla, 22.5–45.0 and 25.5–64.0 ng/gm of tissue, respectively. Somewhat lower concentrations were present in such tissues as pancreas, adrenals, parotid gland, and cardiac muscle. No prostaglandins were detectable in 100 gm or 100 ml of the following human tissues and body fluids: spleen, liver, kidney, subcutaneous fat, milk, urine, and venous blood.

Williams *et al.* (1968) determined the prostaglandins in tumor specimens from 7 patients with medullary carcinoma. In 5 patients who had diarrhea, there were 3 with tumor concentrations of prostaglandin E_2, ranging from 36 to 231 ng/gm, and of prostaglandin $F_{2\alpha}$, ranging from 15 to 682 ng/gm. The remaining 2 had no prostaglandins. Of 2 patients without diarrhea, one tumor had no prostaglandins and the other extremely high levels of 674 ng/gm for E_2 and 844 ng/gm for $F_{2\alpha}$. Other types of thyroid carcinoma, such as papillary and anaplastic, and carcinomas of other tissues had no or very small concentrations of prostaglandins. Williams *et al.* (1968) were unable to find any in the blood of patients with ulcerative colitis, postgastrectomy steatorrhea, or jejunal diverticulitis—all of whom had diarrhea. In contrast, in 2 patients with medullary

thyroid carcinoma, the plasma concentrations of prostaglandin E_2 ranged from 0.8 to 3.5 ng/ml and of prostaglandin $F_2\alpha$ from 1.4 to 5.9 ng/ml. The venous blood from the tumor vein showed much higher concentrations. Although appreciable amounts of prostaglandins were present in the tumor tissue of 4 out of 7 cases of medullary carcinoma of the thyroid and elevated concentrations were observed in the plasma, there appeared to be no clear or specific relationship between these and the presence of diarrhea.

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14

Neoplasms of the Testis

I. Introduction*

A. Incidence and Mortality

The number of deaths in the United States from neoplasms of the testis ranged from 639 in 1960 to 711 in 1965 (World Health Organization, 1970). According to Blandy *et al.* (1970), various estimates of the incidence of testicular tumors average about 2–3 per 100,000 males per year and this is about 1–2% of all malignant neoplasms in men. However, there is some geographical, age, and even racial variation. Thus, for the years 1958–1962, the average annual rate per 100,000 males was 6.3 in Copenhagen, Denmark and 3.7 in rural Danish areas. For New York State, excluding New York City, the rate was 2.2 per 100,000 males for the years 1941–1960. A study in Alameda County, California for the years 1960–1964 revealed 60 cases among whites and none among blacks, incidences corresponding to annual rates per 100,000 males of 3.1 and 0.0, respectively. The incidence is also dependent upon age, so that testicular tumors are about the most common malignant tumor found in men 30–34 years of age (Blandy *et al.*, 1970; Ackerman and del Regato, 1970).

B. Structure of Normal Testis

The adult human testes are about 4 cm in the longer axis and 3 cm in width. The internal structure consists of compartments or lobules

* The following abbreviations are used most commonly in the present chapter: FSH = follicle-stimulating hormone; HCG = human chorionic gonadotropin; ICSH (LH) = interstitial cell-stimulating hormone (luteinizing hormone); 17-KS = 17-ketosteroids; 17-OHCS = 17-hydroxycorticosteroids; TSH = thyroid-stimulating hormone.

filled with coiled seminiferous tubules, each of which is about 30–70 cm long and 150–250 μm wide. Spermatogenesis occurs within these tubules, with the earliest generations of spermatic cells, the spermatogonia, lying near the basement membranes and the most mature cells, the spermatozoa, lying in the innermost layer, close to the lumen of the tubule. Two other types of cells are important in the function of the testis (Cruickshank *et al.*, 1968). The Sertoli cells, also situated within each tubule, are attached peripherally to the basement membrane, with an oval nucleus, 12 μm in length and 9 μm in width, placed some distance from the basement membrane. The plentiful cytoplasm of the Sertoli cell stretches from the basement membrane to the lumen of the tubule. Between the tubules, there is alveolar tissue in which are scattered the large interstitial cells of Leydig that, as we shall presently see in greater detail, constitute a kind of mesenchymal endocrine gland, since they are responsible for the secretion of testosterone and possibly other androgenic hormones under stimulation from the pituitary gonadotropic hormones.

C. Classification of Testicular Neoplasms

The detailed classification of testicular tumors has been the subject of considerable discussion and even disagreement. Blandy *et al.* (1970) have noted that the nomenclature has become chaotic as various authors have employed different terminologies without clarifying their criteria for defining and naming particular tumors. However, the classification of Dixon and Moore (1952), based on the study of 990 tumors, and of Collins and Pugh (1964), derived from a study of 995 cases, appear to have gained most acceptance. Blandy *et al.* (1970) have listed the equivalent terms in these two classifications for 9 main types of tumor arising from the specialized tissue and stroma of the testis.

In reviewing 1030 army cases of tumor of the testis, Dixon and Moore (1952) found 990 in which there was adequate tissue for histological study. Of these, 96.5% were germinal tumors that presumably arose from cells of the sex or germ series. The 35 nongerminal tumors which made up 3.5% of the tumors were diagnosed as follows: capsular fibromas, 14; interstitial cell tumors, 12; androblastomas, 4; adrenocortical-rest tumors, 2; neurilemma, 1; adenomatoid tumor, 1; and undifferentiated sarcoma, 1. Other testicular tumors, such as Sertoli cell tumors, were not encountered in this series. Dixon and Moore (1952) postulated that the germinal tumor patterns were related to each other. The germinal cell tumors were subclassified into 5 groups and their incidence was as follows: I, seminoma, 34.0%; II, embryonal carcinoma with or

without seminoma, 22.5%; III, teratoma with or without seminoma, 10.2%; IV, teratoma with embryonal carcinoma and/or choriocarcinoma and with or without seminoma, 31.1%; and V, choriocarcinoma with or without embryonal carcinoma and/or seminoma, 2.1%.

In the 995 cases of testicular tumor occurring in a civilian population referred to the Testicular Tumor Panel and Registry of Great Britain during the years 1958–1963, Collins and Pugh (1964) found the following distribution: seminoma, 40%; teratomas, 32%; and combined tumor, 14%. The remainder consisted of nongerminal cell tumors, and their incidence will be noted presently. The series of 244 testicular tumors seen at the London Hospital from 1927–1967 (Blandy *et al.*, 1970) included unclassified teratomas and some of mixed type, but 145, or 59%, of the group as a whole were seminomas. There were no choriocarcinomas and the remainder were teratomas of various types, including embryonal carcinomas.

Sertoli cell tumors and Leydig cell tumors quite clearly originate from the corresponding cells in the testis. The former arise from the Sertoli cells within the seminiferous tubules and are the rarest of testicular tumors. The incidence is less than 1% in most reported series (Collins and Pugh, 1964; Collins and Symington, 1964). They are rarely malignant, but they may be associated with gynecomastia and the excretion of large amounts of estrogens, aspects which we shall discuss in greater detail later. As has been already noted, the Leydig cells are situated in the tissue between the seminiferous tubules and the tumors arising from them are therefore known as interstitial cell tumors. The incidence is very low, comprising about 1.2% of the series of Dixon and Moore (1952), 1.4% in the series of Collins and Pugh (1964), and 2% of the testicular tumors reviewed by Ward *et al.* (1960). They are almost always benign, only about one in ten becoming malignant. The pathology has been described by Collins and Cameron (1964).

II. Biochemistry of the Normal Testis

A. Introduction

Like most tissues of the body, the testis is an actively metabolizing tissue. Except for the special emphasis involved in the endocrine function of secreting androgens and the germinal function of providing sex cells, the major biochemical aspects of metabolism are similar to those in other tissues. Limitation of space prevents us from discussing these in any detail, but the reader may be referred to the monograph edited by Johnson *et al.* (1970).

B. Biochemical Constituents of the Human Testis

The testicular fluid obtained by drainage from an incision made in the testes of 3 normal subjects dying in road accidents contained the following mean concentrations of electrolytes, expressed as milliequivalents per liter: sodium, 183; potassium, 7.8; chloride, 140; calcium, 3.78; and bicarbonate, 28.7 (Pande *et al.*, 1967). The concentrations of several other constituents, expressed as milligrams per 100 milliliters, were glucose, 21.1; glycogen, 20.2; lactic acid, 600; ascorbic acid, 30.9; total lipids, 281; free sterols, 42.1; sterol esters, 79; and phospholipids, 20. From this study, it appeared that the concentrations of glycogen, lactic acid, ascorbic acid, sodium potassium, and chloride were higher than those in serum. The concentration of total protein, 3.7 gm per 100 ml, and of glucose, lipids, calcium, and zinc were lower. The activities of several enzymes, glucose-6-phosphate dehydrogenase and of the acid and alkaline phosphatases were substantially higher than in the serum.

C. Lipid Metabolism

Lipids are an important constituent of the testis and are present in the germinal, the nongerminal, tubular, and the intertubular or interstitial structures. In man, the concentrations of several lipid components in the whole testis, expressed as milligrams per gram fresh tissue, are: total lipid, 15.9; total cholesterol, 3.4; and phospholipid, 8.9 (Bieri and Prival, 1965; Johnson, 1970). The content and, indeed, the localization of lipids in the testis are influenced by age, season, gonadotropic and steroidal hormones, mating habits, nutrition, ambient temperature, radiation, and antifertility agents (Johnson, 1970; Lynch and Scott, 1950). Starvation in rats leads to atrophy of the Leydig cells and the germinal epithelium, but marked accumulation of fat in the area occupied by the Sertoli cells. Deficiency of vitamin A or its precursors results in testicular degeneration with an increase in the total lipid and a change in the fatty acid pattern. Lipid synthesis and catabolism are active functions in the testis and are changed substantially with alterations of testis function (Johnson, 1970).

D. Protein Metabolism

Most of our information on testicular proteins is derived from studies on animals (Davis and Langford, 1970). The arginine content of most somatic proteins is about 3–4% and the lysine content is usually much higher, namely, from 10–20%. During the later stages of spermatogenesis,

the somatic-type lysine-rich type of histone is replaced in the germ cells by a new type of histone with a greater arginine content and higher basicity.

The effect of hormones on protein metabolism of testis is of particular interest. Using an *in vitro* system of various concentrations of a testosterone suspension and slices of adult rat testis, Morris and David (1966) observed that the incorporation of [U-^{14}C]L-lysine into protein was inhibited progressively with increasing concentrations of testosterone. This effect was specific for testicular tissue. Follicle-stimulating hormone (FSH) has been found to stimulate *in vitro* incorporation of [^{14}C]lysine into protein in the testis of immature rats (Means and Hall, 1967). No effect on protein labeling was obtained with interstitial cell-stimulating hormone (ICSH) or with prolactin, thyroid-stimulating hormone (TSH) or serum albumin. Pregnant mare serum and human chorionic gonadotropin (HCG) produced slight effects.

E. Endocrinological Aspects

1. Introduction

Both the hormonal and reproductive functions of the testis are under the control of gonadotropic hormones secreted by the anterior pituitary gland. FSH acts on germinal epithelium to promote the formation of spermatozoa from the germinal epithelium, whereas ICSH induces Leydig cells to secrete testosterone and other androgens.

The feedback mechanisms involved in the regulation of the secretion of the pituitary gonadotropins have not as yet been fully described. When Leydig cells are damaged so that the production of testosterone is decreased, ICSH levels increase. Conversely, when testosterone levels increase, ICSH levels decrease. Although it is known that FSH levels increase when the germinal epithelium and the Sertoli cells are damaged, no hormone has been isolated from the germinal epithelium to account for this action. Several theories have been suggested to explain the details of these regulatory actions (Paulsen, 1968).

2. Biosynthesis and Secretion of Androgens

As we have noted, the Leydig cells in the testis are involved in secreting hormones which are principally androgens. These are testosterone (4-androsten-17β-ol-3-one), androstenedione (4-androsten-3,17-dione), and dehydroepiandrosterone (5-androsten-3β-ol-17-one). Their biosynthesis has already been considered in Chapter 12.

The nature of the compounds secreted by an endocrine gland may be obtained by analysis of the venous blood draining this gland. Table 14-1 shows that the concentrations of testosterone and related compounds in the blood of the spermatic vein were considerably higher as the result of injecting 5000 units human chorionic gonadotropin daily for 5 days (Laatikainen *et al.*, 1971; Hudson *et al.*, 1967). It should be recognized that secretion of hormones from glands may be episodic in nature (Weitzman *et al.*, 1971; Rosenfeld *et al.*, 1971). This appears to be a minor factor for testosterone (Hudson *et al.*, 1967). The compounds shown in Fig. 14-1 were also excreted in conjugated form, as the mono- and disulfates. The relative potencies of testosterone, androstenedione, and dehydroepiandrosterone are 100:25:13 by the capon's comb method and 100:20:3 as judged by increase in weight of rat seminal vesicles (Hall, 1970).

The concentration of testosterone in the peripheral plasma of normal sexually mature males has been reported by several investigators as ranging between 0.6 and 0.7 μg per 100 ml. For example, Hudson *et al.* (1967) found the mean concentration in 59 normal males between 17 and 60 years to be 0.69 ± 0.15 (SD) μg per 100 ml. Table 14-2 shows values for the concentrations of the other androgens in the plasma. The secretion or production rate of a hormone may be determined by injecting intravenously a tracer of that hormone and measuring

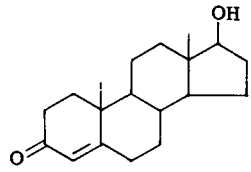
TABLE 14-1

Mean Concentrations of Unconjugated Neutral Steroids in Spermatic Venous Blood Plasma in Normal Males with and without HCG

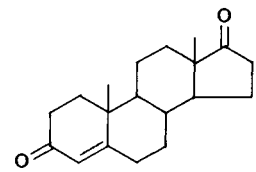
Steroid	μg per 100 ml plasma		
	Without HCG[a]	Without HCG[b]	With HCG[a]
Testosterone	74	47.9	272
Dehydroepiandrosterone	2.2	4.5	37
Androstenedione	2.5	2.9	7.4
5-Androstene-3β,17α-diol	4.0	—	9.2
5-Androstene-3β,17β-diol	18.5	—	78
Pregnenolone	4.8	—	14.0
17-Hydroxypregnenolone	3.9	—	34.0
17-Hydroxyprogesterone	6.2	—	23.0

[a] Data of Laatikainen *et al.* (1971). Mean values are for 5 normal males undergoing hernia repair.

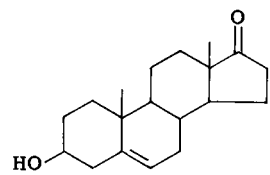
[b] Data of Hudson *et al.* (1967). Mean values are for 15 normal males undergoing hernia repair.



Testosterone



Δ^4-Androstenedione



Dehydroepiandrosterone

Fig. 14-1 Androgens secreted by testis.

the specific activity of any of its urinary metabolites (Gurpide *et al.*, 1965). The specific activity of the metabolite reflects the dilution of the tracer by the endogenous secretion of the hormone during the time of urine collection. In general, the secretory rate is equal to the counts per minute (cpm) injected, divided by the product of the specific activity of the metabolite and the time of urine collection. Gurpide *et al.* (1965) have described the conditions which modify this calculation and the precautions that are necessary in order to obtain valid calculations. Table 14-2 also shows the secretion rates for the three androgens. Testosterone

TABLE 14-2

Production and Metabolism of Androgens in Adult Male[a]

Androgen	Plasma steroid (μg/100 ml)	Secretion rate (mg/24 hours)	Metabolic clearance rate (liters/24 hours)
Testosterone	0.7	7.0	980
Androstenedione	0.03	0.55	2300
Dehydroepiandrosterone	0.4	7.0	1640

[a] Based on Table II of Hall (1970). Reproduced by permission of Academic Press. The value for plasma concentration of dehydroepiandrosterone, 0.04 μg per 100 ml as given in the original article, was a misprint and should be, as given here, 0.4 μg per 100 ml. (Personal correspondence with Dr. P. F. Hall, Nov. 8, 1972.) The mean values for metabolic clearance rate are the means of the values obtained by dividing the individual value for secretion rate by the individual value for plasma steroid.

and dehydroepiandrosterone are secreted in approximately the same amounts, 7 mg/day, but the secretion of androstenedione is less than 10% of this value.

The metabolic clearance rate (MCR) is defined as the volume of blood irreversibly cleared of the steroid in unit time and is equal to the secretion rate divided by the plasma concentration.

The adrenal cortex also produces testosterone and other androgens, and the question arises concerning the relative testicular and adrenocortical contributions. As has just been noted, the secretion or production rate of testosterone is about 7 mg/day (Hudson *et al.*, 1967; Hall, 1970). Other tissues, such as liver, prostate gland, and skeletal muscle, also have the ability to transform steroid precursors to testosterone, but it has been estimated that normally in males the entire extratesticular contribution to the plasma pool is less than 5% of that produced by the testes (Paulsen, 1968). The testicular contribution is 0.4–0.5 mg/day to androstenedione production and 0.6–0.7 mg/day to dehydroepiandrosterone production. The adrenocortical contributions are much higher, namely, 2–3 mg/day to androstenedione and 9–10 mg/day to dehydroepiandrosterone. The sums of the testicular and adrenal productions for these two androgens are somewhat higher than that shown in Table 14-2 but, as has been pointed out (Gurpide *et al.*, 1965; Hudson *et al.*, 1967), the calculations are based on certain assumptions which may hold in only an approximate manner.

Since the testis is a major source of testosterone, it might be expected that the concentration in the blood plasma and the secretion rate of testosterone are considerably lower in females than in males. Although some of the methods used have been imprecise for low concentrations, the following mean values for the plasma concentration have been reported as micrograms per 100 ml : 0.034 ± 0.015 (SD) in 9 women (Lobotsky *et al.*, 1964); 0.07 ± 0.03 (SD) in 10 women (Kirschner *et al.*, 1965); and 0.07 with a range of 0.01–0.12 in 10 women (Korenman *et al.*, 1965). The production rate in 8 women averaged 1.7 mg with a range of 0.8–2.9 mg per 24 hours (Korenman *et al.*, 1965). Additional evidence that there are extratesticular sources of testosterone comes from suppression studies with prednisone, in which basal plasma concentrations and production rates are decreased by as much as 30–60% in some normal women.

The testis is also capable of secreting estrogens. Fishman *et al.* (1967) found that the estradiol production rates in 6 young men averaged 65 μg/day and were increased three- to fourfold by the daily injection of 4000 IU of chorionic gonadotropin for 5 days. As may be noted, this is about 1% of the production rate of testosterone. In the male,

the conversion of testosterone and androstenedione to blood estrogens results in the formation of about 25 μg estradiol per day, or about 40% of the total production.

3. Metabolism and Urinary Excretion of Steroids

The metabolism of the steroids secreted by the testis and the nature of the metabolites excreted into the urine have been studied for many years. However, as will be noted more fully presently, many of these metabolites also arise from steroids secreted by the adrenal gland; thus, it has been difficult to evaluate the extent to which these metabolites arise from the testicular hormones. Butenandt and Dannenbaum (1934) first isolated the androgenic compound, androsterone (5α-androstan-3α-ol-17-one), from male human urine. This early study was soon followed by others in which additional ketosteroids were obtained from the urine. Indeed, in 1948, Lieberman and his associates reported the presence of as many as 42 different ketosteroids in the urine.

Initial studies on the metabolism of testosterone and other testicular steroids were carried out with large doses of testosterone. For example, the intramuscular administration of 90 mg testosterone daily for a period of 45 days increased substantially the urinary excretion of androsterone and etiocholanolone but did not affect the excretion of other ketosteroids. The increase in excretion of these two ketosteroids amounted to 24 and 19%, respectively, of the administered dose (Dobriner and Lieberman, 1950). These results were confirmed by Fukushima *et al.* (1954) with the use of small doses of labeled testosterone. In two studies, the urinary recovery of both steroids within 48 hours after intravenous infusion of testosterone amounted to about 48% of the administered dose.

The ketosteroids which we have been discussing are 17-ketosteroids and form a major portion of the 17-ketosteroids that are excreted into the urine. Of the total ketosteroid production, the testes account for only about 30% of these steroids; the remaining fraction is the result of production by the adrenal cortex (Paulsen, 1968). In either the adrenal cortex or the testis, cholesterol is metabolized by side chain cleavage to Δ^5-pregnenolone; the further metabolism can take either of two pathways. The first leads to the successive formation of 17α-hydroxypregnenolone, dehydroepiandrosterone, Δ^4-androstenedione, and testosterone. The second pathway leads to the successive formation of progesterone, 17α-hydroxyprogesterone, Δ^4-androstenedione, and testosterone. It may be seen that dehydroepiandrosterone and androstenedione are the immediate precursors of testosterone. Androstenedione and testosterone are also metabolized in the liver and end-organ tissues to androsterone,

epiandrosterone, and etiocholanolone. Thus, a number of 17-ketosteroids can be secreted into the blood from the adrenal gland, be excreted into the urine, or lead to the formation of other 17-ketosteroids which are excreted in the urine (Paulsen, 1968). The structures of the main urinary metabolites of testosterone are shown in Fig. 14-2.

Camacho and Migeon (1963) obtained an average value of 87 μg with a range of 46–106 μg per 24 hours for the urinary excretion of testosterone in 5 young male adults. The average value for 4 young women was 5.9 μg with a range of 3.5–7.5 μg per 24 hours. Values for the daily urinary excretion in the normal male of the various metabolites of testosterone as well as of other steroids will be noted later (see Table 14-8).

4. Urinary Excretion of 17-Ketosteroids

The daily urinary excretion of 17-ketosteroids has been used for many years as an indicator of diseases of the testis and the adrenal cortex. The chemical methods for the quantitative determination depend essentially upon Zimmermann's observation in 1935 that certain substances with an active methylene grouping, —CH_2—CO—, interact with *m*-dinitrobenzene and potassium hydroxide to yield a red-colored compound. A great variety of substances that are found in the urine therefore yield the Zimmerman reaction, but some specificity for the reaction has

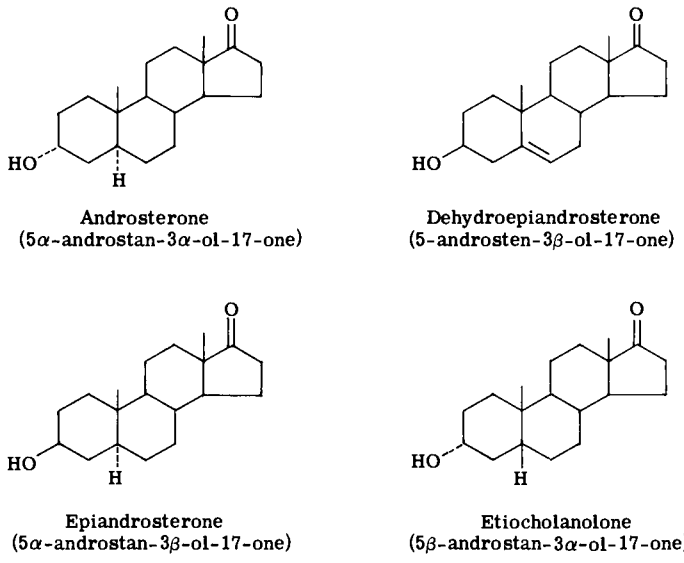


Fig. 14-2 Structures of main metabolites of testosterone in urine.

been gained by extraction procedures and by reading the resulting color in a narrow wave band of the spectrum. Most of the procedures which have aimed at the determination of urinary steroids have started by heating an aliquot of the 24-hour urine with acid in order to hydrolyze the conjugated form of the steroids.

Table 14-3 shows that the mean values and the ranges obtained by various investigators from 1940–1968 for the daily urinary excretion of 17-ketosteroids differ only slightly from each other. Albert *et al.* (1968) give a mean value of 14 mg with a range of 6–21 mg for men and a mean value of 10 mg with a range of 6–12 mg for women. Precise methods for determining the quantitative excretion of the individual 17-ketosteroids have evolved more slowly. Some of the values obtained in this connection, expressed as milligrams excreted per 24 hours, were: androsterone, 1-3; etiocholanolone, 2-4; dehydroepiandrosterone, 0.2-2.0 (Lipsett *et al.*, 1966).

5. Gonadotropins

As we have noted in Chapter 11, the anterior pituitary contains several hormones which are involved in the regulatory action of other hormonal effects. Of these, the gonadotropic hormones, the follicle-stimulating hormone (FSH), and the luteinizing or interstitial cell-stimulating hormone (LH or ICSH) are of immediate relevance to our present subject. The FSH and LH extracted from pituitary glands are both glycoproteins and contain approximately 26 and 19% of carbohydrate, respectively (Stevenson and Loraine, 1971). The molecular weights of human ICSH is 28,500 and that of FSH has been listed as 34,000 (White *et al.*, 1973). In addition to these two pituitary gonadotropins, the placenta in the pregnant female produces a hormone, designated as human chorionic gonadotropin (HCG). It is excreted in the urine in large amounts during

TABLE 14-3

Daily Urinary Excretion of 17-Ketosteroids in Urine of Normal Men

Investigator	No. of subjects	Range (mg)	Mean (mg)
Engstrom and Mason (1943)	13	9.8–20.8	14.2
Fraser *et al.* (1941)	9	8.1–22.6	13.8
Scott and Vermeulen (1942)	10	11.6–17.5	14.3
Baumann and Metzger (1940)	11	10.5–19.0	14.1
Albert *et al.* (1968)	—	6–21	14

pregnancy, but is present in very small amounts in normal female urine and is absent from normal male urine.

The actions of FSH and LH in the female will be discussed in Chapter 15. In the male, FSH stimulates the epithelium of the seminiferous tubules, causing the appearance of large numbers of spermatocytes in various stages of development, including mature spermatozoa. FSH also acts on the Leydig cells which secrete testosterone.

During the past few years specific radioimmunoassays capable of measuring LH and FSH in small samples of human serum have been developed (Peterson *et al.*, 1968). Although there were hour-to-hour and day-to-day fluctuations in the serum concentrations, there was no diurnal variation or other evidence of rhythmicity or regular peaking. Table 14-4 shows the mean concentrations of LH and FSH in the sera of 4 healthy adult males, 22–35 years of age. As may be seen, the serum concentrations were essentially the same for the 4 subjects.

Several factors have been found to influence the concentrations of these gonadotropins. Intramuscular injection of 25 mg of testosterone proprionate each day for 3 days resulted in a decrease of about 40–50% in the serum LH levels in all 3 subjects tested but no consistent decrease in FSH levels. Oral ingestion of 0.5 mg of ethinyl estradiol 3 times per day for 2–3 days resulted in a consistent fall in serum concentrations of both FSH and LH. The oral administration of 200 mg of clomiphene citrate, a presumable pituitary activator, daily for 3 days, resulted in an elevation in serum concentrations of both FSH and LH in all 4 subjects tested.

The correlation of the concentrations of plasma gonadotropins and

TABLE 14-4

Mean Concentrations of LH and FSH in Sera Drawn Once Per Day for Periods Ranging from 3 to 10 Weeks in Normal Male Adults[a]

Subject No.	Age	No. of samples	Mean serum LH (milli IU/ml ± SD)	Mean serum FSH (milli IU/ml ± SD)
1	23	27	8.0 ± 1.4	6.4 ± 1.6
2	32	19	6.1 ± 1.2	10.0 ± 1.7
3	24	29	7.0 ± 2.0	8.3 ± 1.1
4	23	57	7.8 ± 1.6	7.6 ± 1.0

[a] From Peterson *et al.* (1968). Reproduced by permission of The Endocrine Society and of Dr. Mortimer B. Lipsett, Editor in Chief, *Journal of Clinical Endocrinology and Metabolism.*

TABLE 14-5

Correlation of the Concentration of Plasma LH, FSH, and Testosterone with Sexual Development[a,b]

Stage of puberty in bone age (years ± SE)	Testosterone (ng/100 ml ± SE)	FSH (ng/ml ± SE)	LH (ng/ml ± SE)	TVI[c] (cm^2 ± SE)
P1 (7.7 ± 0.3)	18 ± 4.4	0.8 ± 0.05	1.1 ± 0.06	1.7 ± 0.1
P2 (12 ± 0.2)	71 ± 19	0.96 ± 0.15	1.5 ± 0.15	4.3 ± 0.6
P3 (13.7 ± 0.2)	248 ± 46	1.7 ± 0.30	1.6 ± 0.07	8.2 ± 0.5
P4–5 (15.7 ± 0.3)	482 ± 27	2.5 ± 0.50	1.7 ± 0.11	10.4 ± 0.7
Adults —	625 ± 28	3.1 ± 0.49	2.0 ± 0.18	—

[a] From August *et al.* (1972). Reproduced by permission of The Endocrine Society and of Dr. Mortimer B. Lipsett, Editor in Chief, *Journal of Clinical Endocrinology and Metabolism*.

[b] The mean values were usually based on determinations in approximately 10–20 individuals in each group, with the exception of the values for TVI and bone age in P1 which were each based on 73 individuals.

[c] TVI is testicular volume index.

testosterone with sexual development in boys, as measured by bone age and testicular volume, is shown in Table 14-5 (August *et al.*, 1972). The presence of the standard errors of the mean, SE, permits determination of whether statistical significance exists between the mean values for the various groups. For example, the values for the concentration of plasma testosterone at each stage of puberty differ significantly from each other. Again, with regard to the concentrations of plasma FSH, there were significant differences between groups P1 and P3 ($p < 0.01$) and between P2 and P4–5 ($p < 0.01$).

III. Biochemistry of Testicular Tumors

A. Introduction

Earlier in this chapter, we discussed the incidence and classification of the various testicular tumors (Sections I,A and C). As we noted, the subject of classification has been invested with some controversy, particularly with regard to the dominant group of germinal cell tumors. It may be recalled in this connection that, according to Dixon and Moore (1952), germinal cell tumors were subclassified into seminoma and vari-

ous combinations of seminoma with embryonal carcinoma, teratoma and choriocarcinoma (Section I,C). In contrast, Collins and Pugh (1964) considered seminoma, teratoma, and combined tumors, with seminoma and teratoma coexisting in the same tumor, as 3 separate classes. For our purposes, we shall not attempt to subclassify this group but use the terms for which various investigators have presented biochemical findings.

B. Germinal Cell Tumors

1. Androgens and 17-Ketosteroid Excretion

Some of the older literature indicates that, in seminoma, the excretion of 17-ketosteroids (17-KS) is slightly increased, whereas the excretion of androgens is decreased. For example, Warren (1945) observed the following excretions of 17-KS, expressed as milligrams per 24 hours, in 4 patients with seminoma: 15.4, 18.0, 20.0, and 32.2. These represented an average increase of approximately 50% over normal basal levels. In a series of 19 patients with seminoma who had been under treatment in the radium station at Copenhagen, Hamburger and Godtfredsen (1941) observed that, prior to surgical operation, the excretion of androgens, as determined by biological assay, was markedly diminished. Thus, whereas the excretion in normal men ranged from about 20 to 100 and averaged 50 IU/day, only 6 of 27 determinations in the series of 19 patients were above 20 IU and the average androgen excretion was 10 IU/day, or about one-fifth of the normal average.

2. Gonadotropin Excretion in Germinal Cell Tumors

The excretion of gonadotropin is increased greatly in some types of testicular tumors, but the question has come up whether this gonadotropin arises from the pituitary or is chorionic in nature. Hamburger (1941) attempted to differentiate between these two sources by the shape of the dose–response curve, as measured by increase in weight of the ovary or uterus of the infantile rat as test animal. He presented several cases of testicular tumors such as "mixed epithelioma" and chorionepithelioma. These patients were subjected to hemicastration for removal of their tumors. In these cases, the excretion of HCG increased from a few thousand IU/day at the time of the operation to very high levels of 80,000 to 1,000,000 IU/day, presumably in association with the development of metastases. In about 75% of cases of seminoma and small cystic tumor of the testis, FSH was also excreted in the urine,

albeit in small amounts. This urinary excretion did not cease after orchiectomy and was independent of the development or absence of metastases. In their review of the U.S. Army cases, Dixon and Moore (1952) found that, in those patients with germinal cell tumors who had had urinary gonadotropin tests, the incidences of "positive" tests had been as follows: group I, 20 of 24 cases; group II, 85 of 135 cases; group IV, 69 of 116 cases. These groups were defined earlier in this chapter (Section I,C). No information was available concerning the incidences in groups III and V.

These early results have been confirmed in more recent years with either bioassay or radioimmunoassay of HCG. Using the mouse uterus test, Loraine (1960) found that, in a group of 9 patients with seminoma, 63 serial determinations for periods ranging from 2 weeks to 5 months following treatment by unilateral orchiectomy yielded a mean value of 32 human menopausal gonadotropin (HMG) units. This mean was significantly higher than that, 12 HMG units, obtained from 18 determinations on a control group of 5 patients subjected to unilateral orchiectomy for conditions other than seminoma. One "HMG unit" represented the amount of activity present in 1 mg of a reference preparation.

The bioassay which employs increase in weight of the immature mouse or rat uterus measures the effect of both hypophysial and chronic gonadotropins. In contrast, the female toad, *Xenopus laevis*, lays eggs when injected with chorionic gonadotropin, but does not respond to gonadotropins of hypophysial origin. Using this procedure, Hobson (1965) obtained the results on men with various types of testicular tumor shown in Table 14-6. One case may be used to illustrate the effects of treatment and of the development of metastases. A 44-year-old man with a tumor of the right testicle excreted 300,000 IU of HCG per liter of urine 2 days before orchiectomy. Histological examination of the neoplasm revealed that this was a "mixed" tumor with choriocarcinoma in some areas and seminoma in others. Two days after orchiectomy, the excretion of HCG had fallen precipitously to 24,000 IU per liter. Despite intensive x-ray therapy, the excretion of HCG increased and attained a level of 420,000 IU/liter by the seventeenth postoperative day. Lung metastases had developed and neither radiotherapy in this connection nor pituitary radiation affected the level to any degree. The patient died 41 days after operation.

Table 14-6 shows the extent to which men diagnosed as having various types of testicular tumor excrete HCG (Hobson, 1965). Incidentally, it may be noted that the listing does not strictly follow either the classification of Dixon and Moore (1952) or that of Collins and Pugh (1964). Although the greatest incidence, 90%, and the highest amounts of HCG

TABLE 14-6
The Excretion of HCG by Men with Testicular Neoplasms[a]

Type of testicular tumor	No. of patients	No. with negative tests	No. of patients with IU of HCG per liter of urine					Fraction of patients with elevated excretion of HCG (%)
			>3,000 <30,000	>30,000 <300,000	>300,000 <450,000	>600,000 <900,000	>1,500,000 <2,000,000	
Seminoma	115	107	2	4	2	—	—	7
Embryonal carcinoma	19	15	2	2	—	—	—	21
Mixed	13	11	0	2	—	—	—	15
Teratoma	83	59	16	6	1	1	—	29
Choriocarcinoma	10	1	1	2	1	4	1	90

[a] From Hobson (1965). Reproduced by permission of Periodica, Copenhagen.

were found in the group of patients with choriocarcinoma of the testicle, a lower percentage of the other types also showed elevated excretion. Hobson (1965) pointed out forcibly that the routine pathological examination may have missed areas of chorionic tissue present in the other types of tissue or failed to differentiate such tissue from the multiple and varied tissues of some testicular tumors. Indeed, he has stated: "A biological test using female *Xenopus laevis* will establish whether HCG is being excreted. If it is, chorionic tissue must be present."

Kirschner *et al.* (1965) had found that the administration of HCG to normal men caused Leydig cell stimulation with increased secretion of testosterone. For example, the daily injection of 1000–2000 IU of HCG to each of 6 men for periods ranging from 3 to 10 days caused a rise in plasma testosterone from a mean control value of 0.86 μg per 100 ml to a mean value of 1.77 μg per 100 ml. The level of plasma dehydroepiandrosterone was not affected.

In contrast, patients with metastatic trophoblast-containing testicular tumors had normal concentrations of plasma testosterone in spite of the large amounts of HCG produced by these tumors. In a group of 7 patients, consisting of 4 with choriocarcinoma, 2 with teratocarcinoma, and 1 with embryonal cell carcinoma, the plasma testosterone concentrations ranged from 0.4 to 1.09 μg per 100 ml at a time when the urinary gonadotropin excretion ranged from 2×10^2 to 5×10^5 IU/day (Kirschner *et al.*, 1970). The plasma testosterone levels were within the normal range, 0.4–1.2 μg per 100 ml, but the gonadotropin excretion was far in excess of the normal range of 5–50 IU/day (Kirschner *et al.*, 1970). The daily administration of the androgen, fluoxymesterone, for 3–5 days led to a decrease of the plasma testosterone in each patient, but the plasma HCG concentrations remained unchanged. These findings suggested that Leydig cell function was maintained by pituitary LH and that the patients were unresponsive to their own tumor gonadotropin.

Kirschner *et al.* (1970) explored several possible mechanisms to explain this unresponsiveness. These were (a) gonadotropin produced by the tumor was biologically inactive, (b) the plasma of the tumor patients contained antigonadotropin antibodies, and (c) a "burned out" phase develops in the testis so that testosterone is not released in response to HCG. This is similar to the posited existence of such phases in acromegaly where acral changes do not progress in spite of continued excessive production of growth hormone, or in patients with thyroid medullary carcinoma who are not hypocalcemic in the presence of production of excessive amounts of calcitonin by their tumors. However, experiments designed to test these various possibilities failed to provide conclusive proof for any of them.

3. Estrogen Excretion and Follicle-Stimulating Hormone in Germinal Cell Tumors

There are several reports in the literature showing that chorionic gonadotropin-producing tumors of the testis (Martin and Garden, 1963; Kirschner *et al.*, 1970) and, indeed, at other sites (Fusco and Rosen, 1966) are associated with increased excretion of estrogens. For example, in 2 patients with teratocarcinoma of the testis, Kirschner *et al.* (1970) found estrone production rates five- to tenfold the normal level, as was the urinary excretion of estrone and estradiol.

As we have previously noted (Section II,E,5), the administration of exogenous estrogen to normal men results in a decrease in the serum concentrations of FSH and LH. Using the same 7 male patients with gonadotropin-producing testicular tumors who had been the subjects of previous studies (Kirschner *et al.*, 1970), Reiter and Kulin (1971) found that when these patients were excreting large amounts of chorionic gonadotropin the plasma concentration and urinary excretion of FSH were very low, indeed, significantly less than in normal men. During the first month following chemotherapy, the excretion of chorionic gonadotropin in the patients with testicular tumors decreased markedly, indeed, to levels within the normal range. At this point, the urinary and plasma FSH had risen to normal levels.

It would appear reasonable then to postulate, as Reiter and Kulin (1971) have done, that decreased FSH levels in patients with chorionic gonadotropin-secreting testicular tumors may result, at least in part, from estrogen-induced suppression of pituitary FSH synthesis and/or release. This constitutes a negative feedback related to estrogen, but the possibility also exists that chorionic gonadotropin may act directly upon the hypothalamus to reduce FSH levels.

Several groups of investigators (Fine *et al.*, 1962; Martin and Garden, 1963; Fusco and Rosen, 1966; Reiter and Kulin, 1971) have observed that patients with chorionic gonadotropin-producing tumors exhibit a damaged germinal epithelium, the changes consisting of peritubular fibrosis, basement membrane hyalinization, and focal or generalized disturbance of spermatogenesis. It is possible that these changes may be the consequences of lowered FSH production and action.

C. Interstitial Cell Tumors

1. Introduction

As we have already pointed out (Section I,C), tumors of the interstitial cells of the testis, also known as Leydig cell tumors, are very rare, com-

prising about 1.2–2.0% of all testicular tumors. In 1957, Dalgaard and Hesselberg reviewed a total of 94 cases in the literature and, in 1970, Hopkins reported a total of approximately 170 cases. The tumor usually consists of a solitary, palpable nodule, about 2–3 cm in diameter, with sharp borders and an impressive, brown-yellow cut surface (Blandy *et al.*, 1970). Masses of compact cells are seen on microscopic examination. The cytoplasm is eosiniphilic, generally granular, but occasionally vacuolated or foamy.

The systemic clinical manifestations of the disease depend on the age of the patient. Before puberty, the outstanding effect is the occurrence of precocious sexual development. Later in life, the most common presenting symptom is gynecomastia, and other symptoms are reduced libido, failing potency, loss of body hair, and diminution in size of the contralateral testis (Collins and Cameron, 1964; Blandy *et al.*, 1970).

2. Interstitial Cell Tumors and Isosexual Precocious Development

Perhaps the symptoms of this type of precocious sexual development can best be illustrated by a description of the earliest recorded case in the literatue—that of Sacchi in 1895, cited by Lisser and Escamilla (1962). The patient was a boy, aged 9 years, 7 months, whose symptoms were evident at 5 years of age. At the time of his first visit, the boy had a deep voice, a heavy beard, luxuriant pubic hair, chest hair, and muscular thighs and biceps. His left testicle had enlarged to 10 cm in diameter and his penis was 9 cm in length and 9 cm in circumference. There were frequent erections, but no ejaculations. The patient weighed 97 lb and was 56.5 in. in height, parameters which were more characteristic of a boy about 11–12 years of age. His appearance was that of a short, 30-year-old man. Left orchiectomy was performed and, within 1 month after operation, the beard and body hair had begun to fall out and his voice became higher in pitch. Four months postoperatively, his beard had completely disappeared. The penis had decreased to 7.5 cm in length and the erections had become much less frequent.

Since this first description of interstitial cell tumor and precocious sexual development, additional cases have continued to be reported at the rate of one or two a year. In 1965, Wilkins noted that there were 33 such cases in the literature and, in 1967, Johnstone listed a total of 39 cases. Subsequently, new cases of this syndrome have been described by Allibone *et al.* (1969), Gittes *et al.* (1970), and Root and his associates (1972).

Rare as this condition is, its biochemical aspects are of unusual interest.

As may be seen from Fig. 14-3, the urinary excretion of 17-ketosteroids (17-KS) was increased clearly above the normal level in 10 of the 15 cases collected from the literature by Wilkins (1965).

A more detailed analysis of the steroid metabolism in an interstitial cell tumor was attempted in 1960 by Savard *et al.* The patient was a 5-year, 7-month-old white male who had a right enlarged testis and displayed the typical symptoms of precocious sexual development. The urinary 17-KS excretion was 13.6 mg per 24 hours, well above the normal range for that age (Fig. 14-3). The right testis was removed. Postoperatively, the 17-KS excretion dropped precipitously to 1.4 mg per 24 hours and remained essentially at this level when tested 1½ years later.

Of particular interest was the comparison of the levels of excretion of other 17-ketosteroids preoperatively and 2 days postoperatively. These, expressed as milligrams per 24 hours, were as follows: androsterone, 2.4 and 0.0; etiocholanolone, 2.1 and 0.1; dehydroepiandrosterone, 1.4 and 0.0; and 11β-hydroxyandrosterone, 1.6 and 0.0. Several other 17-ketosteroids, which were excreted in fractions of a milligram preoperatively, showed a decrease to even lower levels postoperatively. In the case of pre-

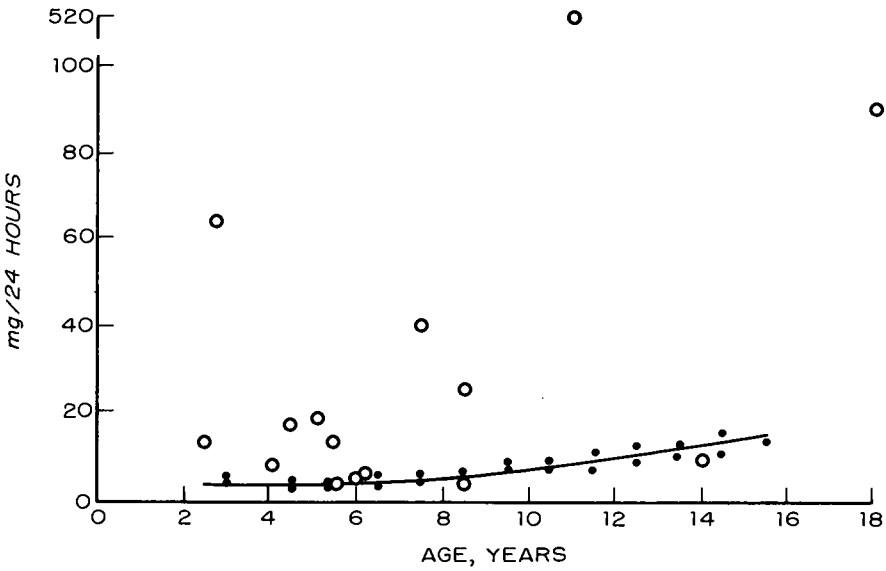


Fig. 14-3 17-Ketosteroid excretion in boys with interstitial cell tumor of the testis: (●) normal values and (○) interstitial tumor of the testis. Normal values are based on data obtained by Oesting and Webster (1938) and by Nathanson *et al.* (1941). Values in interstitial tumor of the testis are based on summary of literature by Wilkins (1965).

cocity in a 6-year-old boy reported by Root *et al.* (1972), the urinary 17-KS before and 6 weeks after operation were, respectively, 7.9 and 1.9 mg per 24 hours. In addition, the 24-hour excretions of pregnanetriol were 1.9 preoperatively and <0.5 mg postoperatively. The values for the 24-hour urinary excretion in the normal 6-year-old male are about 2.0 mg 17-KS and 0.1–0.8 mg pregnanetriol.

The concentrations of testosterone and various 17-ketosteroids in the serum of the case reported by Root *et al.* (1972) are shown in Table 14-7. It may be seen that these concentrations were much higher than those of a normal prepubertal male. Removal of the right testis containing the tumor resulted in decreases of the concentration of all of these steroids to levels well within the normal range of the normal prepubertal male. The blood taken from the right spermatic vein during the operation contained very high concentrations of the various steroids as, for example, 17,100 ng testosterone and 46,100 ng androstenedione per 100 ml.

The preceding results show that an interstitial cell tumor is capable of secreting *in vivo* androstenedione, testosterone, and dehydroepiandrosterone. Some other attempts have been made to determine whether there are any characteristic metabolic properties that can be ascribed to interstitial cell tumors. Savard *et al.* (1960) sectioned the tumor removed from their patient into thin slices and incubated aliquots of these slices with trace amounts of various ^{14}C-labeled steroid precursors in a suitable medium for 3 hours. Some typical results may be given in illustration. Incubation with labeled testosterone yielded the following

TABLE 14-7

Concentrations of Testosterone and Steroid Metabolites in Serum of 6-Year-Old Boy with Interstitial Cell Testicular Tumor and Isosexual Precocity[a]

Subject	Testosterone (ng/100 ml)	Androstenedione (ng/100 ml)	Dehydroepiandrosterone (ng/100 ml)	Dehydroepiandrosterone sulfate (μg/100 ml)
Normal adult male	238–1000	39–199	137–1261	42–256
Normal prepubertal male	0–13	0–50	24–109	0.3–19.8
Patient–preoperative	384	695	109	16.1
Patient—1 week postoperatively	4	58	36	6.5

[a] From Root *et al.* (1972).

metabolites, expressed as percent of the original: testosterone, 66.0; androstenedione, 5.0; 11β-hydroxyandrostenedione, 1.7; and 11β-hydroxytestosterone, 0.8. Similarly, incubation with progesterone left 58.0% as progesterone, and resulted in the following metabolites: 17α-hydroxyprogesterone, 32.6%; testosterone, 3.1%; and androstenedione, 8.6%. [^{14}C]Acetate, which is a precursor for many metabolites, yielded very small but definite amounts of testosterone and androstenedione. On the basis of these studies, Savard and his associates (1960) concluded that the virilizing properties of the interstitial cell tumor could be ascribed to the formation of two principal androgenic steroids, testosterone and 4-androstenedione, by the tumor. However, it must be noted that there were no control quantitative data on the extent of formation of these steroids by normal testes under the same *in vitro* conditions.

According to Wilkins (1965), no gonadotropins were detectable in the urine of boys with interstitial cell tumors. However, later studies, utilizing more sensitive radioimmunoassay methods (August *et al.*, 1972; Root *et al.*, 1972), not only revealed the presence of gonadotropins in the urine and serum of normal boys but also evaluated the changes which followed orchiectomy in those with interstitial cell tumors. In their study of a 6-year-old boy with testicular interstitial tumor, Root *et al.* (1972) obtained the following values for the urinary excretion of luteinizing hormone (LH), expressed as IU per milliliter: 2.3 and 2.7, preoperatively; 3.9, 1 day postoperatively, and 6.5, 1 year postoperatively. The normal values were 2.6 ± 1.1 IU/ml for the prepubertal male and 31.3 ± 10.3 IU/ml for the adult male. The urinary excretion of follicle-stimulating hormone (FSH) also rose—from 2.5 and 1.9 IU/ml preoperatively to 4.4 IU/ml 1 day postoperatively and 3.5 IU/ml 1 year postoperatively. Tumor secretory activity was autonomous and did not alter preoperatively in response to either HCG or hydrocortisone. The concentration of LH in the serum tended to be higher and that of FSH lower than in normal prepubertal males. A decrease in the concentration of the serum LH and a decrease followed by an increase in the concentration of serum FSH appeared to occur within the 7-month interval following operative removal of the tumor.

3. Interstitial Cell Tumor in the Adult Male

As we have already pointed out (Section III,C,1), the clinical manifestations of interstitial cell tumor in the adult male usually differ from those of the prepubertal male in that they are not characterized by excessive or even moderate androgenicity. Of the 94 cases at all ages reviewed by Dalgaard and Hesselberg (1957), 23 were below 15 years of age and the others showed a maximum at 30–35 years of age, but

ranged up to the age of 82 years. All of the 23 boys displayed precocious puberty and virilism, but most adults showed no dysendocrine symptoms. Thirteen of the 71 adults revealed either hypervirilism, feminism, or both.

A relatively small percentage of interstitial cell tumors are malignant. Dalgaard and Hesselberg (1957) listed 8, or about 10% of the number then recorded in the literature. In 1963, Short and Coe stated that 13 cases had been reported, but accepted only 7 as authentic. In 1969, Tamoney and Noreiga again reviewed the literature, concluded that there were only 9 documented cases and added one of their own.

Objective biochemical evidence of altered hormonal function has been obtained in several instances of interstitial cell tumor in adults and these appear to have been chiefly in those with metastatic disease. Thus, Masson in 1943 reported a case, 32 years of age, and Ohlsen and Rønn in 1956 reported a case, 80 years of age, both of whom had metastases and both of whom had increased urinary excretion of 17-KS and of estrogens. The first patient had increased urinary excretion of gonadotropin, but the latter exhibited normal excretion.

Some adult patients with interstitial cell tumor may show signs of feminization. This is well illustrated by 2 cases with gynecomastia reported by Ward *et al.* (1960). Detailed biochemical studies were carried out on one of these patients who was being treated for postnasal drip and who was found to have an enlarged left testicle at the time of his second admission when he was 58 years of age. Orchiectomy was performed. Metastases to the renal pedicle and perirenal area were observed at this time, but no attempt was made to remove these. During the remaining 5 years of his life, the urinary gonadotropins were repeatedly found to be low. In contrast, the 24-hour excretions of 17-KS were extraordinarily high, increasing from 302 mg about 2 years postoperatively to 1860 mg several months before death. On the two occasions when this determination was performed, the 24-hour urinary excretion of estrogens was also very high, 700 and 480 μg, and the individual estrogens were also substantially elevated. The daily excretion of 17-hydroxycorticosteroids was within the normal range or slightly elevated until the terminal stage, when it rose to 44 mg.

Similar biochemical findings have been obtained in other cases of interstitial cell tumor by several groups of investigators (Abelson *et al.*, 1966; Hopkins, 1970; Selvaggi *et al.*, 1973). For example, Selvaggi *et al.* (1973) recently reported 2 cases, one of whom, a 27-year-old man, had bilateral gynecomastia and enlargement of the left testicle. The preoperative urinary excretion of total estrogens was elevated moderately to 47 μg per 24 hours, but the excretions of 17-OHCS and 17-KS were normal. Operation revealed an interstitial cell tumor and, postoperatively, the patient underwent a marked increase in libido and regression of breast tender-

ness and size. In the 6 weeks following operation, the plasma testosterone, estradiol, estrone, and FSH in the peripheral blood were within normal limits; LH was slightly elevated. No signs of metastases occurred during the year following operation.

The possible effect of therapeutic agents was studied by Abelson *et al.* (1966). The patient was a 55-year-old man with a large tumor of the right testicle which had been present for 5 years. This was removed and found to be an interstitial cell tumor. Four years later, the patient began to complain of sharp pains in the upper abdomen. His liver was greatly enlarged, but no gynecomastia was present. Routine laboratory determinations of the sera revealed the following possibly abnormal values: low cholesterol, 101 mg per 100 ml; total protein, 6.5 mg per 100 ml with an albumin–globulin ratio of 2.7:3.8 gm per 100 ml; and bromsulfophthalein retention, 16%. Urinary 17-KS excretions on three occasions were 338, 738, and 1060 mg per 24 hours. Pregnanetriol was less than 0.1 mg/day, and the level of Δ^5,3β-hydroxysteroids was 1791 mg/day. Laporatomy revealed that the left lobe of the liver was almost completely replaced by tumor. The right lobe was also involved, but to a lesser degree. Metastases were present on the abdominal wall and omentum. Biopsies from all of these sites showed the histological appearance of interstitial cell tumor. Biochemical determinations performed 2 months later showed the following: elevated urinary 17-KS excretion, 896 mg/day; urinary estrogen excretion, more than 200 mouse units per day as compared with a normal value of 6–24 mouse units. The daily urinary excretions of the individual 17-KS components were dehydroepiandrosterone, 43 mg; etiocholanolone, 90 mg; androsterone, 41.5 mg; 11-ketoetiocholanolone, 31 mg; 11-ketoandrosterone, 14 mg; and 11-hydroxyandrosterone, 10.4 mg. The concentrations in the plasma, in micrograms per 100 ml, were: dehydroepiandrosterone sulfate, 1108; androsterone, 435; and testosterone, 1.9. On the whole, therefore, there was a high androgen output, and this was reflected in a mild growth of abdominal hair and preservation of appetite and muscular strength.

The administration of 2,2-bis-[2-chlorophenyl-4-chlorophenyl]1,1-dichlorethane (*o,p'*-DDD) was at first followed by an increased excretion of 17-KS. This was succeeded by a temporary fall, concurrent with the administration of dexamethasone, and again a rebound to high excretions, approximately 1000–1250 mg per 24 hours, somewhat higher than the pretreatment levels. However, when the dexamethasone was eliminated and the dose of *o,p'*-DDD raised to 6 gm daily, the 17-KS excretion decreased to lower levels, oscillating between about 50 and 200 mg/day, and persisting for several months until the death of the patient. Although biochemical improvement is not necessarily associated with clinical remission,

this particular patient achieved temporary improvement during the treatment with *o,p'*-DDD.

A more comprehensive study of the metabolism of testosterone and related steroids in interstitial cell carcinoma of the testis in adults appears to be that of Lipsett and his associates (1966). The patient was a 67-year-old man who had had a left orchiectomy for a testicular mass in 1963 and a year later developed a lower abdominal mass which showed the typical histology of interstitial cell tumor.

As in the studies we have already cited, the urinary excretion of 17-KS was increased substantially. It was not suppressed by either dexamethasone or fluoxymesterone. The administration of human chorionic gonadotropin in doses of 4000 IU daily for 5 days increased the 17-KS excretion from a control level of 200–400 mg/day to an average level of 690 mg/day. Of particular interest were the parameters involving testosterone. The daily urinary excretion ranged from 500 to 1500 μg, about ten- to thirtyfold the normal excretion. The metabolic clearance rate was 2700 liters/day, about 3 times the normal rate, as may be seen from Table 14-2. The production rate was 53 mg/day, or 8 times normal (Table 14-2). The plasma testosterone level was 4 times normal, and it was shown that dehydroepiandrosterone sulfate was the unique precursor of essentially all of the plasma testosterone and the urinary testosterone glucuronide.

The 24-hour urinary excretions of various steroids in this patient are shown in Table 14-8. It may be seen that pregnenediol, pregnenetriol, androstenediol, and dehydroepiandrosterone were excreted in amounts much greater than in normal subjects. These findings were similar to those described for adrenocortical carcinoma, as was the high excretion of 16-hydroxydehydroepiandrosterone. Since the corresponding Δ^4-3-ketosteroids were not found in the urine, the possibility existed that tumor 3β-hydroxysteroid dehydrogenase activity was limited. This pattern, too, was pointed out by Lipsett *et al.* (1966) as being similar to that found in adrenocortical carcinoma. It may also be noted that the excretions of the three 11-oxo-17-KS were also increased, a finding similar to that obtained by Abelson and his associates (1966) and by Savard *et al.* (1960). Since this patient's remaining testis was atrophic, the various steroid synthetic activities resulted from the metastatic interstitial cell carcinoma.

D. Androblastoma (Sertoli Cell) Tumors

1. Introduction

It will be recalled that Sertoli cells are the sustentacular cells which lie within the limiting membranes of the seminiferous tubules. The adult

TABLE 14-8

Urinary Steroid Excretion in Interstitial Cell Carcinoma of the Testis[a]

Steroid	Patient (sulfate + glucuronide) (mg/24 hours)[b]	Normal (sulfate + glucuronide) (mg/24 hours)[b]
Androstenol	1.2	1–2
Dehydroepiandrosterone	48.5	0.2–2.0
16-Hydroxydehyroepiandrosterone	11.8	0.1–0.2
Androstenediol	9.0	0.1–0.2
Pregnenediol	6.0	0.1–0.4
Pregnenetriol	4.9	0.2–0.4
Pregnanediol	2.1	0.4
Pregnanetriol	6.1	0.5
17-Hydroxypregnanolone	2.8	0.2
Androsterone	72.3	1–3
Etiocholanolone	120.8	2–4
11-Ketoetiocholanolone	1.4	0.5
11-Hydroxyetiocholanolone	3.1	0.4
11-Hydroxyandrosterone	6.7	0.5

[a] From Lipsett *et al.* (1966). Reproduced by permission of the American Society for Clinical Investigation, Inc.

[b] These values were not corrected for losses during isolation.

Sertoli cells have been considered to be direct descendants of those cells which, in the embryo, condense out of the primitive gonadal blastema as the male sex cords (Teilum, 1946, 1971; Collins and Symington, 1964; Scully, 1970). The granulosa cells of the ovary also arise from the primitive blastema. Teilum (1946, 1971), who pointed out this relationship in 1946, has recently defined the term "androblastoma and Sertoli cell tumors" as a "histogenetic and morphologic designation for neoplasms, in either sex, which are derived from the undifferentiated gonadal mesenchyme and which histologically may reflect various phases in the development of the male gonad." This tumor has also appeared under the names of granulosa cell tumor and tumors of specialized gonadal stroma. Considering those tumors in which connection with the Sertoli cell line was obvious, Collins and Symington (1964) distinguished only 6 such cases of the 995 cases of testicular tumors summarized by the British Testicular Tumor Panel and Registry (Collins and Pugh, 1964)—an incidence of 0.6%. Dixon and Moore (1952) did not encounter any Sertoli cell tumors, but recorded 4 cases of "androblastoma" in their series of 990 cases of testicular tumor—an incidence of 0.4%. The number

of cases that have been recorded from the first description until 1971 was placed at approximately 70 by Teilum (1971) and at about 60 by Siller and Farah (1971). The incidence is probably about 1–1.5% of all testicular tumors. The number of reported cases with metastases has been variously estimated at 7–10 (Hopkins and Parry, 1969; Talerman, 1971; Koppikar and Sirsat, 1973; Teilum, 1971).

2. Hormonal Studies in Androblastoma

In his review of the literature, Teilum (1971) found gynecomastia in 16 of 59 benign and in 3 of 10 malignant androblastomas, and this finding has been considered as the chief evidence of the feminizing capacity of the tumor. In most cases, the gynecomastia decreases gradually following semiorchiectomy.

There are only a few studies on gonadotropins or steroid metabolism and these are rather cursory in nature. Seymour *et al.* (1968) described the case of a 48-year-old man with benign Sertoli cell tumor (androblastoma) of the right testis and gynecomastia. No hormonal determinations were performed before orchiectomy, and postoperative quantitative determinations of urinary gonadotropin and 17-KS were found to yield results within normal limits. Essentially normal values have also been more recently reported in a series of case reports of androblastoma: preoperative urinary 17-ketogenic steroids and estrogens in a 63-year-old man with metastasizing androblastoma (Hopkins and Parry, 1969), preoperative values for chorionic gonadotropin in a 25-year-old man (Siller and Farah, 1971), and postoperative values for urinary chorionic gonadotropins and estrogens in a 79-year-old man (Talerman, 1971). Two cases of androblastoma have been reported which have been considered as predominantly feminizing, presumably on the basis of the presence of gynecomastia. In one of these (Teilum, 1946), no hormonal studies were presented. In the other (Fuglsang and Ohlsen, 1957), neither the urinary excretion of gonadotropin nor estrone, as determined by biological assay, was elevated above normal. Androgen excretion was raised slightly.

E. Granulosa Cell Tumor of the Testis

A closely related feminizing tumor is the granulosa cell tumor of the testis. Teilum (1971) has pointed out that there is an indistinct borderline between androblastoma and granulosa cell tumors in the undifferentiated stages. This is consistent with the common origin of these neoplasms from the mesenchymal cord of both ovary and testis. Granulosa

cell tumor of the testis is extremely rare. In 1971, Teilum summarized 3 cases in the literature.

Another case which was supported by hormonal studies is that reported in the case records of the Massachusettes General Hospital in 1955. A 53-year-old man entered the hospital with the relevant complaints and findings of impotency and absence of libido for the preceding 2 years, a large stony-hard mass in the right testis, bilateral enlargement of the breasts, and hyperpigmentation of the areolae. The 17-KS excretion ranged from 4.6 to 9.5 mg and averaged 6.1 mg per 24 hours, somewhat below the normal level. Before operation, the 24-hour urinary excretion of total estrogens was slightly elevated at 90 μg and decreased postoperatively only slightly to 70 μg. The preoperative 24-hour excretions of estrone and estriol were 13 and 7.5 μg, respectively. After operation, the excretion of estrone decreased to 7 μg and that of estriol to 6 μg. The preoperative 24-hour urinary excretion of FSH was then being performed by biological assay and was found to be negative for 6.5, 13, and 52 mouse units (normal excretion, 7–50 mouse units per day; Bodansky and Bodansky, 1952). The histopathological diagnosis of the 100 gm solid tumor removed at operation was granulosa cell tumor of testis. Extraction of the tumor and analysis of the extract yielded no evidence of estrogens.

As may be seen from our review, only a few studies have been performed on the hormonal aspects of androblastoma and granulosa cell tumors of the testis. Although feminizing effects are evident clinically, study of estrogen and gonadotropin excretion have not always given unequivocal biochemical evidence of such feminization.

F. Other Tumors of the Testis

1. Lymphomas

In their series of 995 cases of testicular tumors, Collins and Pugh (1964) classified 66, or 6.6%, as malignant lymphoma. Additional cases have appeared in the literature since then (Abell and Holtz, 1968; Kiely *et al.*, 1970; Tanenbaum *et al.*, 1972). Broadly, this classification includes leukemia, lymphosarcoma, and reticulum cell sarcoma. The tumor may develop at any age, but 80% occur after the age of 50 (Teilum, 1971). There is a marked tendency for bilateral involvement (Collins and Pugh, 1964). The first clinical manifestation is frequently the enlargement of the testis, and this lesion is usually the presenting sign of generalized lymphomatous disease already present or about to become clinically active (Tanenbaum *et al.*, 1972). There appear to be no data on clinical

manifestations of hormonal derangements and no biochemical studies which would aim to reveal these.

2. Adrenal Rest Tumors

The concept has existed that certain tumors of the testis result from stimulation by continuous excessive ACTH production of "adrenal rest" cells existing within the testes (Hamwi *et al.*, 1963). Clinically, the patients resemble those with interstitial cell tumors and the question has arisen of differentiating between the 2 types of tumors. It has been stated, not without contradiction, that the intracellular presence of certain crystalloids, known as Reinke crystalloids, is characteristic of Leydig cells and, therefore, of interstitial cell tumors (Bishop and Sommerville, 1970).

3. Questionably Classifiable Testicular Tumors

In 1947, Östergaard reported a feminizing "presumably aberrant adrenocortical tumor" of the testis which Teilum (1971) later classified as an androblastoma (Sertoli cell tumor). The patient was a 28-year-old man with the presenting complaint of marked gynecomastia. Physical examination revealed an abnormal mass in the left testis. Utilizing biological assays available at that time, Östergaard (1947) found that removal of the tumor-bearing testis resulted in a substantial decrease in the excretion of estrogens and an increase in the excretion of gonadotropin and androgen. Lewis and Stockard (1950) studied a 68-year-old man who complained of enlargement of the breasts and was discovered to have a tumor of the right testis. Determinations of the 24-hour excretion of ketosteroids, estrogen, and gonadotropin before operation were stated to yield values within the normal range and not to be altered postoperatively. The urinary excretion of pregnanediol was increased. However, the published values for 24-hour urinary excretion for 17-KS were far below the generally accepted normal range and the estrogen excretion was higher. Lewis and Stockard (1950) considered the condition to be a benign feminizing tumor of the testis.

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15

Neoplasms of the Ovary

I. Introduction*

A. Incidence and Mortality

The incidence and mortality rate of cancer of the ovary are substantial. The 1947 cancer survey for the United States showed a sex–age-adjusted incidence of 14.7 for white and 9.9 for nonwhite females per 100,000 population, and surveys in both New York and Connecticut indicated an approximately 15% increase from the later 1940's to the early 1960's (Ackerman and del Regato, 1970). Recorded mortality rates for deaths from malignant neoplasms of the ovary also include the small fraction resulting from neoplasms of the Fallopian tubes and broad ligament. The numbers in the United States during 1966 and 1967 were 9,142 and 9,489, respectively. The death rates per 100,000 females are dependent to some extent on geography. For example, in the United States, the rates during the 1966–1967 period were 9.62 and 5.45 for the white and nonwhite populations, respectively. These can be compared with a low rate of 1.94 in Japan and a high rate of 16.77 in Denmark (Segi and Kurihara, 1972).

* The following abbreviations are used most commonly in the present chapter: ACTH = adrenocorticotropic hormone; FSH = follicle-stimulating hormone; HCG = human chorionic gonadotropin; LH (ICSH) = luteinizing (interstitial cell-stimulating hormone).

B. Structure of the Normal Ovary

Since the neoplasms of the ovary arise from various types of cells in this organ, it is desirable to describe briefly its anatomy and histology (Leeson and Leeson, 1970). The ovaries in the adult female are each about 4 cm in length, 2 cm in width, and 1 cm in thickness. One lies at each side of the uterus on the lateral wall of the pelvic cavity and is attached at one of its margins to the uterus. Embryologically, the ovaries develop from a thickening of cells, known as the germinal ridge, which appear medial to the Müllerian and Woolfian bodies at about the sixth week after conception.

Two layers, the germinal epithelium and tunica albriginea, cover the ovary in the prepubertal stage. In the postpubertal stage, the inner layer or the medulla of the ovary consists of loose, fibroelastic, connective tissue containing blood vessels, lymphatics, and nerves. The outer layer or cortex consists of a compact cellular stroma that contains the ovarian follicles. About 400,000 follicles are present at birth; the number decreases progressively during life until none exists after menopause and the cortex becomes a narrow zone of connective tissue.

Each primordial follicle consists of an oocyte or primordial ovum and its surrounding single layer of epithelium. The oocyte is a large, generally round cell, about 33 μm in diameter with a fairly large nucleus, approximately 20 μm in diameter. During the reproductive years and, in childhood to a lesser degree, the oocyte increases in size, the follicular cells become cuboidal and several rows of these cells appear. As the follicular growth increases, the oocyte tends to assume an eccentric position. Fluid gathers between the cells and a vesicle is formed with the ovum on one side. In this initial stage, a clear elastic membrane, the zona pellucida, forms and envelops the ovum.

The mature follicle is known as the graafian follicle. The follicular or granulosa cells that immediately surround the zona pellucida of the ovum are known as the cumulus oophorus or discus proligerus. As the graafian follicle grows, the surrounding stromal cells in the ovary grow larger and gather more closely about the follicle in a layer known as the theca interna. As we shall note later, this layer is the site of estrogen formation. Surrounding this layer, the ordinary ovarian stroma arranges itself concentrically to form another layer, known as the theca externa.

In the course of each normal menstrual cycle, one graafian follicle makes its way to the surface, appearing there as a transparent vesicle and protruding above the surface of the ovary. Certain changes which have occurred both in the graafian follicle and at the surface of the ovary facilitate the discharge of the ovum, follicular fluid, and granulosa cells. The corpus luteum then begins to form at the site of the ruptured

follicle. Four stages are discernible in the event that no pregnancy results. These are proliferation, vascularization, maturity, and regression and are usually complete with 28 days, the normal period for the menstrual cycle. The end stage of regression is the formation of the corpus albicans, which is chiefly connective tissue resembling the surrounding ovarian stroma. This structure is, however, gradually invaded by the surrounding stroma, broken up into increasingly small hyaline masses and ultimately resorbed (Hellman *et al.*, 1971). The correlation between these changes and the endometrium is depicted in Table 15-1. We shall presently describe the changes in secretion of estrogen, progesterone, and the pituitary gonadotropins during the normal menstrual cycle.

C. Classification of Ovarian Neoplasms

Several types of classification of ovarian tumors have been proposed. Novak and Woodruff (1974) grouped about 30 different types of ovarian tumors into benign and malignant tumors, of which the former included cystic and solid types. The malignant tumors were comprised of 5 groups: cystic, solid, rare malignant lesions such as teratoma, tumors with endocrine potential such as arrhenoblastoma, and metastatic tumors. Teilum (1971) has classified 27 different types into 5 large classes as follows: germ cell tumors, gonadal stromal or sex cord-mesenchyme tumors, "mesonephric" tumors, endometrial tumors of the ovary, and miscellaneous tumors. In discussing later in this chapter the various types of ovarian tumors for which biochemical and endocrinological data are available, we shall attempt to follow Teilum's classification.

II. Biochemistry of the Normal Ovary

A. The Hypothalamic-Hypophysial-Ovarian Axis

The pituitary gonadotropins play an essential role in the release of ova and in the secretion of various steroid hormones from the ovary. As the name indicates, follicle-stimulating hormone (FSH) stimulates follicle growth in all mammalian species that have so far been studied, including the human. Luteinizing or interstitial cell-stimulating hormone (LH or ICSH) causes ovulation of the follicle that has been brought to maturity by FSH. The factors involved in the maintenance of the human corpus luteum after ovulation are not known precisely, but it would appear that they might result from an intrinsic secretory activity initiated by LH at the time of ovulation (Lloyd, 1968).

The synthesis of gonadotropins in the pituitary and their release from it are, in turn, regulated by centers in the hypothalamus. Neurohumoral

TABLE 15-1

Correlation of Ideal 28-Day Ovarian and Endometrial Cycles[a]

	Phase						
	Menstrual	Early follicular	Advanced	Ovulation	Early luteal	Advanced luteal	Premenstrual
Days	1–3 to 5	4 to 6–8	9 to 12–16	12–16	15–19	20–25	26–28
State of ovary	Involution of corpus luteum	Growth and maturation of graafian follice			Active corpus luteum		Involution of corpus luteum
State of endometrium	Menstrual desquamation and involution; bleeding	Reorganization and proliferation	Further growth and watery secretion	—	Active secretion and glandular dilation	Accumulation of secretion and edema	Regression; premenstrual state

[a] From "Williams' Obstetrics" by Hellman *et al.* (1971). Reproduced by permission of Appleton-Century-Crofts, New York.

substances are transported from these centers through vessels that originate in the median eminence of the hypothalamus and terminate in the anterior lobe of the pituitary (Lloyd, 1968). But the formation and secretion of the gonadotropins are subject to a negative feedback control. Estrogens inhibit FSH secretion, and postmenopausal women who have no ovarian function usually have elevated plasma levels and increased urinary excretions of FSH and LH. These effects may be mediated either through the pituitary or the hypothalamus. The various control and feedback pathways in the hypothalamic-hypophysial-ovarian axis are shown in Fig. 15-1.

B. Plasma Levels of FSH and LH during the Menstrual Cycle

Until the past few years, the determination of these parameters was handicapped by the use of biological methods that required large quanti-

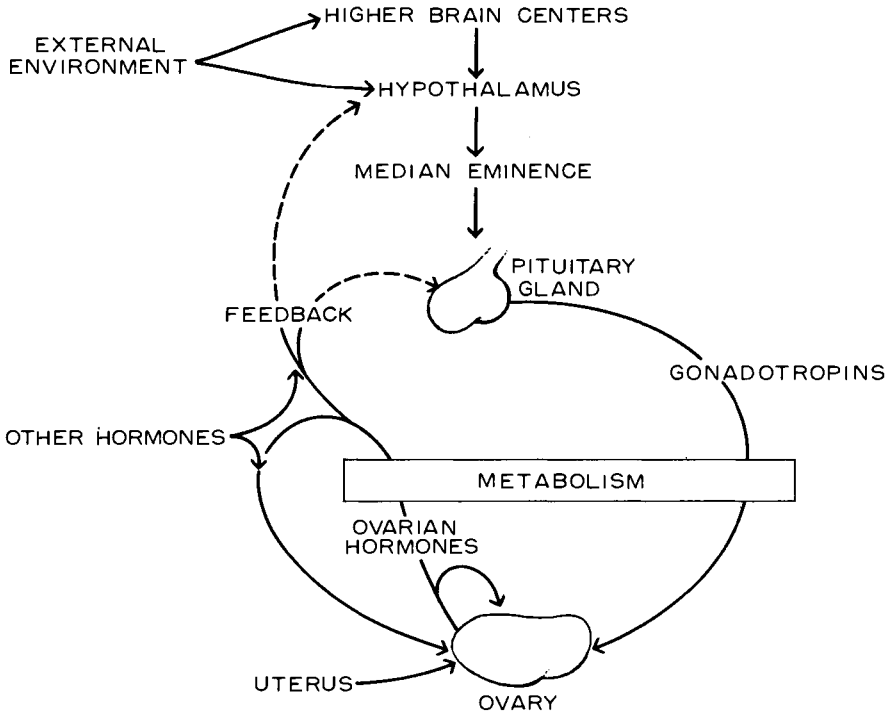


Fig. 15-1 Hypothalamico-hypophysial-ovarian circuit. After Lloyd (1968). Reproduced by permission of W. B. Saunders and Company.

ties of urine or plasma for accurate measurement (Becker and Albert, 1965). The advent of radioimmunoassay methods made it possible to collect small samples of blood or urine at daily intervals for the assay of both gonadotropins as well as of various steroid metabolites (Ross *et al.*, 1970). We shall later discuss the possibility that, even within the same day, the secretion of FSH and LH may be episodic in character, a situation that, as we pointed out in Chapter 12, was demonstrated so exquisitely by Gallagher, Hellman, and their associates (Weitzman *et al.*, 1971) for the secretion of cortisol.

Employing a radioimmunoassay, Ross *et al.* (1970) found that the estimation of plasma FSH and LH concentrations in blood samples taken at 8:00 A.M., 4:00 P.M., and 10:00 P.M. showed no consistent pattern of variation. Accordingly, blood, usually taken in the fasting state before 9:00 A.M., was used for determining FSH and LH values during the cycle. Figure 15-2 shows the mean daily plasma LH and FSH concentrations during normal ovulatory cycles synchronized around the day of the LH peak, presumably the time of ovulation. The LH pattern is characterized by several features. The values do not vary much during most of the preovulatory or follicular phase, oscillating about a level of 18 milli-International Units (mIU)/ml. During the last 2 days of this phase, they begin to rise markedly, culminating in a marked peak that has a mean value of 84 mIU/ml. The concentration drops sharply during the first day of the postovulatory or luteal phase and then very slowly during the remainder of this phase.

With respect to the plasma FSH pattern, the concentration increases progressively early in the follicular phase, thus constituting an early follicular rise. This is followed by a decline during the second half of the follicular phase which reaches a low point, designated by Ross *et al.* (1970) as the preovulatory nadir. About a day or two before the LH midcycle peak, the concentration of FSH begins to rise, attaining its peak at the same time as the LH values. During the luteal phase, the values gradually decrease to the lowest level observed during the cycle (luteal nadir). These features of the FSH and LH patterns were not changed when the day of onset of menstrual flow was used as a reference point (Ross *et al.*, 1970).

It has been assumed that ovulation occurs at the midcycle peak of LH or FSH plasma concentration because of such presumptive secondary signs as a rise in the basal body temperature. Yussman and Traymor (1970) have attempted to relate the serum levels of the gonadotropins more closely to ovulation by direct examination of the corpus luteum obtained at laparotomy. Normally cycling patients were admitted to the hospital 4–5 days prior to scheduled surgery and blood samples were

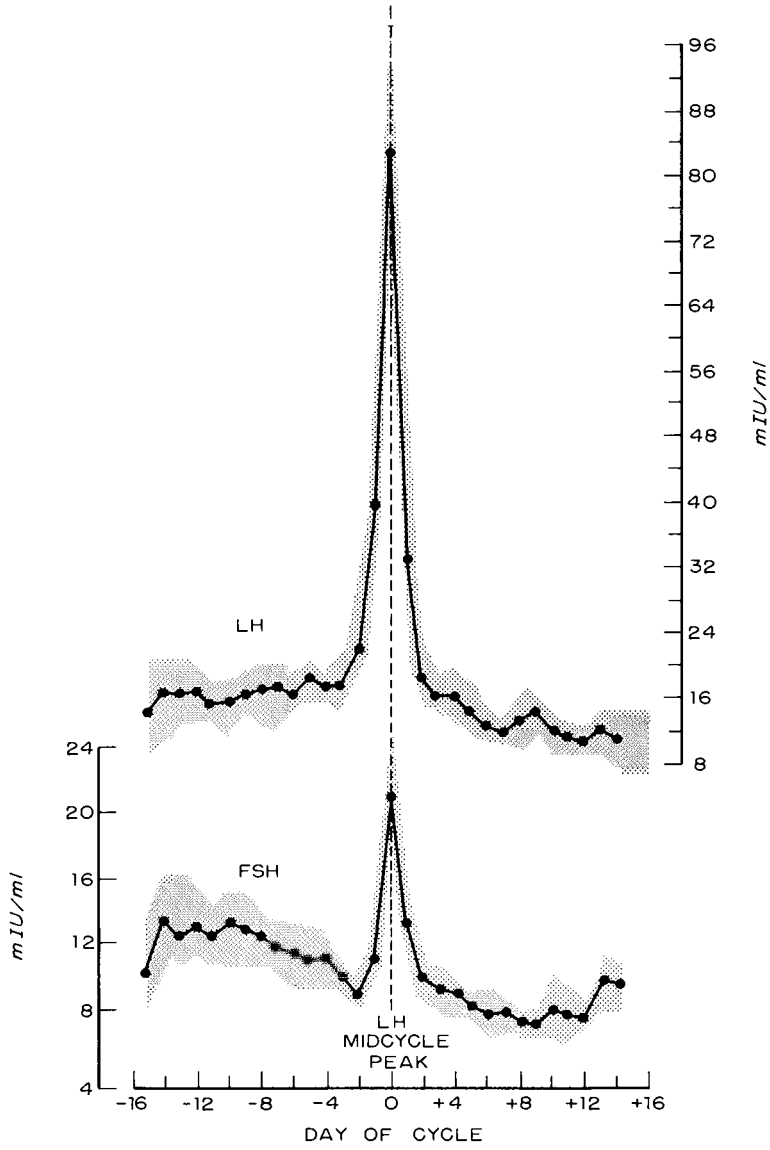


Fig. 15-2 Mean (bold lines) daily plasma LH and FSH concentrations in presumptively ovulatory cycles from 16 women synchronized around the day of LH peak and distributed in a cycle with a mean value of 29 days. Results of estimations of the two gonadotropins were expressed in terms of units of biological activity of the Second Reference Preparation for Human Menopausal Gonadotropin (IRP 2 HMG). After Ross *et al.* (1970). Reproduced by permission of Academic Press.

obtained at 8-hour intervals. When the basal body temperature suggested that ovulation had occurred, laparotomy was performed and the corpus luteum searched for. A sample of endometrium was also obtained by dilation and curettage. The dating of the corpus luteum was determined by the procedure of Corner (1956), which may be in error by 12 hours. The time of ovulation, as determined by the dating procedure, was plotted against the concentrations of serum LH, serum FSH, plasma progesterone, or basal body temperature. The level of serum LH rose significantly 24 hours prior to ovulation and peaked at 16 hours prior to ovulation. Follicle-stimulating hormone showed a similar but less marked pattern. The levels of progesterone will be discussed presently.

The forms of the curves for LH and FSH in Fig. 15-2 were based on fasting morning samples of blood. We have already indicated the possibility that, like other hormones, FSH and LH may be secreted episodically during the day. Midgeley and Jaffe (1971) observed that analysis of serum LH in samples drawn at intervals of 1 hour showed considerable fluctuation in consecutive samples. The largest LH excursions took place during the ascending and descending phases of the major LH peak at midcycle and during the luteal phase of the cycle. For example, on days 11–12 of a cycle, the concentration of the serum LH fluctuated between 19 and 23 mIU/ml, showing no particular trend. On days 12–13, the concentration rose in an oscillatory but stepwise fashion from 25 to about 90 mIU/ml. The concentrations of FSH showed very little fluctuation in successive 1-hour samples. It did not deviate by more than 1 or 2 mIU/ml from a level of about 7 mIU/ml. The fluctuations in serum LH indicate the possibility that serum concentrations of LH are maintained by brief periods of release followed by periods of little or no release; in other words, that the secretion is episodic in nature. Midgeley and Jaffe (1971) suggested that sampling at shorter intervals as, for example, $\frac{1}{2}$ hour might contribute further information on the episodic character of LH secretion.

C. Steroid Formation and Metabolism in the Ovary

1. Biosynthesis of Estrogens

The biosynthesis of adrenocorticosteroids and of androgens has been previously described (Chapter 12, Section II,C; Chapter 14, Section III,E,1). Estradiol-17β, the hormone normally secreted by the ovarian follicle, is synthesized from testosterone by any of several pathways as, for example, that shown in Fig. 15-3 (White *et al.*, 1973). Lloyd (1968) has presented a scheme in which Δ^4-androstenedione, formed

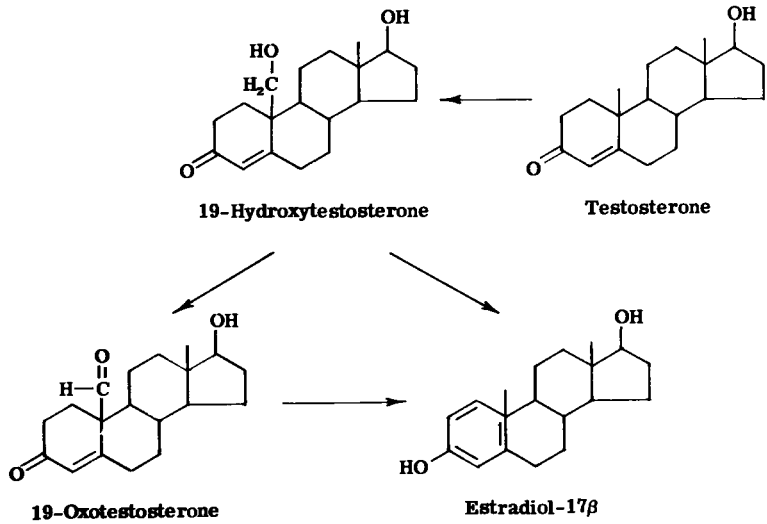


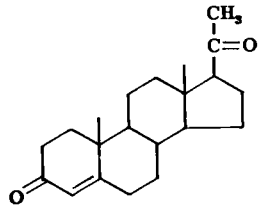
Fig. 15-3 Pathways of estrogen biosynthesis.

reversibly from testosterone, is converted to 19-hydroxyandrostenedione. The latter compound is converted directly or through 19-oxoandrostenedione to estrone [1,3,5(10)-estratrien-3-ol-17-one] which is then reduced reversibly to 17β-estradiol. It should be noted that an essential feature of this biosynthesis is the aromatization of Ring A in the perhydrocyclopentanophenanthrene skeleton of the steroids. Estrone and estradiol may be metabolized to many other related compounds, as manifested by their occurrence in urine. One of these is the conversion of estrone to 16α-hydroxyestrone and then to estriol [1,3,5(10)-estratrien-3,16α,17β-triol].

Several methods are available for determining the relative biological potency of the estrogens. Using as a criterion the increase in weight of the uterus resulting from administration of various estrogens to the immature mouse, the following relative activities were obtained: estrone, 100; β-estradiol, 1000; α-estradiol, 7.5; and estriol, 40 (Pearlman, 1948).

2. Biosynthesis and Metabolism of Progesterone

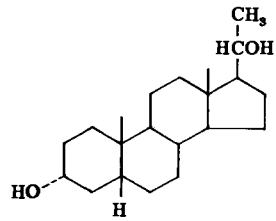
Progesterone (4-pregnen-3,20-dione) is synthesized by several tissues such as the adrenal cortex, placenta, testis, and corpus luteum. In the adrenal cortex, cholesterol is cleaved to form isocaproic acid and Δ^5-pregnenolone, and the latter is then oxidized to progesterone (see Chapter 12, Section II,C).



Progesterone

As has been shown, both Δ^5-pregnenolone and progesterone act as precursors of the androgens and estrogens.

Progesterone is secreted by the corpus luteum during the latter half of the menstrual cycle and exerts a number of important effects. As we have seen, estrogen mediates the growth of endometrium, rendering it thicker and relatively dense. As progesterone begins to act, thickening and proliferation of the endometrium stops and secretory effects are initiated. The glycogen content of the epithelial cells increases and passes into the fluid filling the glands. The stroma accumulates water. All these changes prepare the endometrium to nurture the fertilized ovum. Progesterone may also exert some extragenital effects such as ameliorating the sequelae of adrenalectomy or causing naturesis in normal subjects or in patients with Addison's disease who are also receiving aldosterone (Lloyd, 1968). Pregnanediol (5β-pregnan-3α,20α-diol), formed chiefly in the liver, is a quantitatively significant metabolite of progesterone.



Pregnanediol

It is coupled with glucuronic acid to form the chief urinary metabolite, pregnanediol glucosiduronate, which serves as a good index of progesterone formation and metabolism.

3. Concentration of Progesterone and Its Metabolites in Plasma and Urine of Normal Women

As may be expected, the plasma levels and urinary excretions of estrogens and progesterone are dependent on the state of the menstrual cycle.

A considerable number of studies have been carried out in this field with constantly changing and hopefully improving techniques, and only a few studies in each area will be cited.

Employing electron capture detection of the chloroacetate derivative following gas–liquid chromatography, van der Molen and Groen (1965) obtained the following mean values for the concentration of progesterone in plasma, expressed as micrograms per 100 ml, at various stages of the menstrual cycle before the onset of menstruation: 0–22 days, 0.049; 21–15 days, 0.13; 14–8 days, 1.52; and 7–1 days, 0.71. It may be seen that plasma progesterone is very low during the early part of the follicular phase, begins to rise toward the midcycle peak, reaches a maximum several days later during the luteal or postovulatory phase, and then begins to recede. Using a competitive protein-binding (CPB) method (Murphy, 1967), Yoshimi and Lipsett (1968) studied this relationship in several normal females. One of these is shown in Fig. 15-4. In this particular subject, the level of plasma progesterone was very low, namely, 0.43 ± 0.37 (SD) ng/ml during the follicular phase, began to increase immediately following the LH peak, reached a maximum 7 days after the LH peak, and returned to normal by the onset of menses. This maximum was about 17 ng/ml of plasma and agreed fairly well with the maximal mean value, 1.52 μg per 100 ml or 15.2 ng/ml, obtained

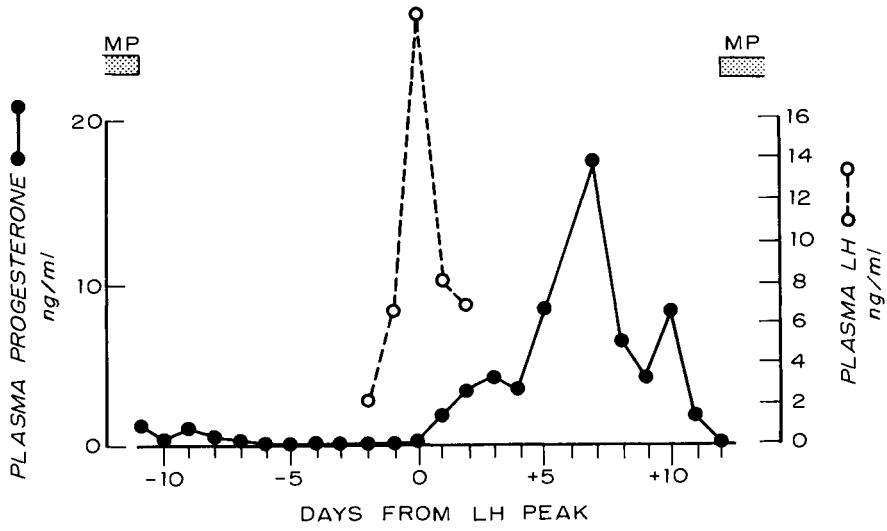


Fig. 15-4 Relation of the plasma concentrations of progesterone to LH levels during the menstrual cycle. MP indicates menstrual period. From Yoshimi and Lipsett (1968). Reproduced by permission of Holden-Day, Inc.

by van der Molen and Groen (1965) for the 14 to 8-day period before onset of menstruation. Yoshimi and Lipsett (1968) found the mean level of plasma progesterone in 5 normal men to be 0.33 ± 0.17 (SD) ng/ml, essentially the same as that obtained in a series of 10 ovariectomized women, namely, 0.34 ± 0.20 (SD) ng/ml.

The first step in the metabolism of progesterone to the androgens and estrogens is the formation of 17-hydroxyprogesterone (Figs. 12-3 and 15-3). The concentration of plasma 17-hydroxyprogesterone averages about 0.3 ng/ml or 30 ng per 100 ml during the first part of the follicular phase, then rises during the second part as the concentration of plasma LH surges to a maximum and the FSH decreases slightly (Strott *et al.*, 1969; Ross *et al.*, 1970). The concentration of 17-hydroxyprogesterone reaches a peak of about 2.0 ng/ml or 200 ng per 100 ml at the same time as the plasma LH and FSH concentrations attain their peaks (Fig. 15-5). During the day or two following the peak, the concentration of 17-hydroxyprogesterone falls by about 40%, only to rise again and, with some fluctuation, reaches another maximum in the luteal phase at the same time that progesterone does, both at about the eighth postovulatory day of the cycle. These values for plasma 17-hydroxyprogesterone correspond only fairly well with those obtained by others such as Abraham *et al.* (1971): 40 ± 21 (SD) ng per 100 ml for the follicular phase and 156 ± 74 (SD) ng per 100 ml for the luteal phase. Incidentally, the value for normal males was 87 ± 53 (SD) ng per 100 ml and that for postmenopausal women was 24 ± 11 (SD) ng per 100 ml.

Progesterone is present in only very small amounts in the urine but, as we have already indicated, it gives rise to a number of metabolites that are excreted in greater amounts, the most important of which is pregnanediol. As long ago as 1937, Venning developed a simple gravimetric method for the determination of pregnanediol and thus opened up a means of studying the metabolism of progesterone (Venning and Browne, 1937; Wilson *et al.*, 1939). Venning and Browne (1937) found that pregnanediol was not detectable in the urine until a few days after ovulation. Then the excretion began to average about 1 mg/day and rose to a maximal 24-hour excretion of 4–5 mg about 6–7 days before the menses.

More recent studies have confirmed these observations and have defined more precisely the relationship of pregnanediol excretion to various stages of the menstrual cycle in normal women. Figure 15-6 shows that the plasma progesterone and urinary pregnanediol excretion are at a low level during the follicular stage; the plasma concentrations of progesterone are less than 1 ng/ml, and the urinary excretions of pregnanediol are less than about 0.5 mg per 24 hours. After the FSH and LH peaks,

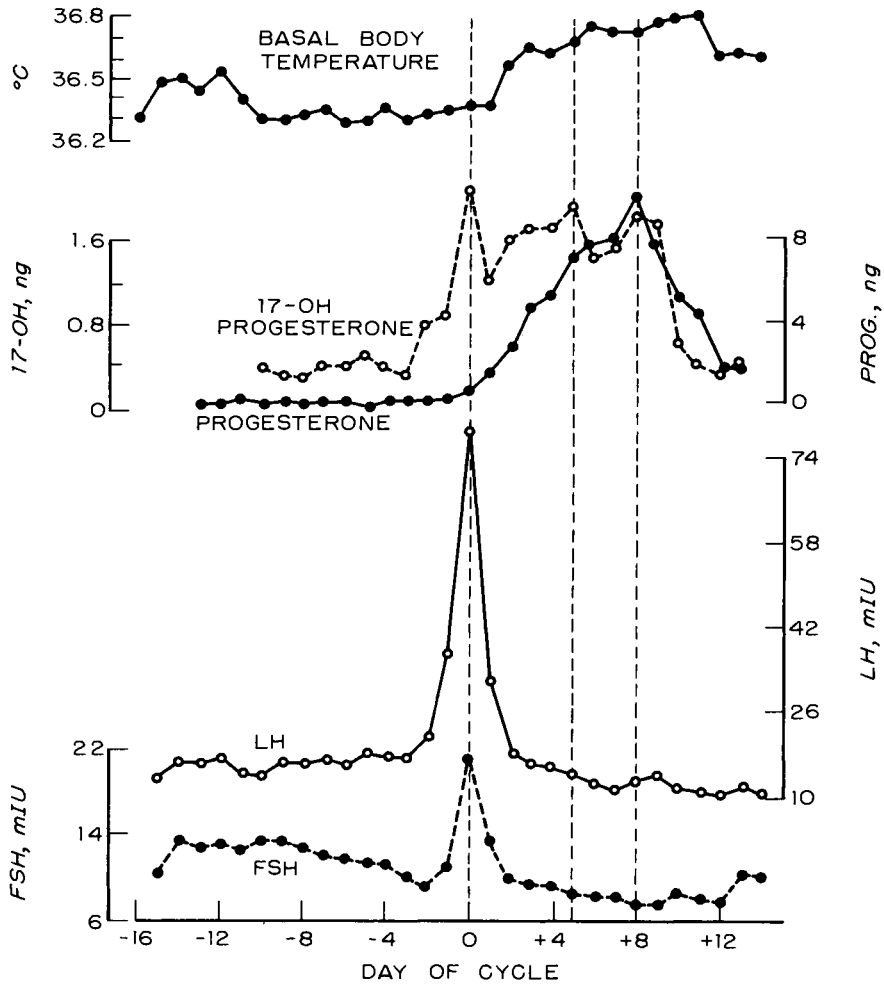


Fig. 15-5 Mean daily basal body temperature (upper), daily plasma 17-hydroxyprogesterone and progesterone concentrations, as nanograms per milliliter plasma (middle), and mean daily plasma LH and FSH concentrations (lower) as mIU per milliliter during 16 presumptively ovulatory cycles synchronized around the day of the LH midcycle peak. After Ross *et al.* (1970). Reproduced by permission of Academic Press.

the levels of both the plasma concentration of progesterone and excretion of pregnanediol begin to increase, the former reaching maximum levels of 12–14 ng/ml 4–7 days after the LH peak and the pregnanediol attaining maximal excretions of about 4 mg/day during the same period. Thereafter, the levels of both parameters begin to decrease until they reach

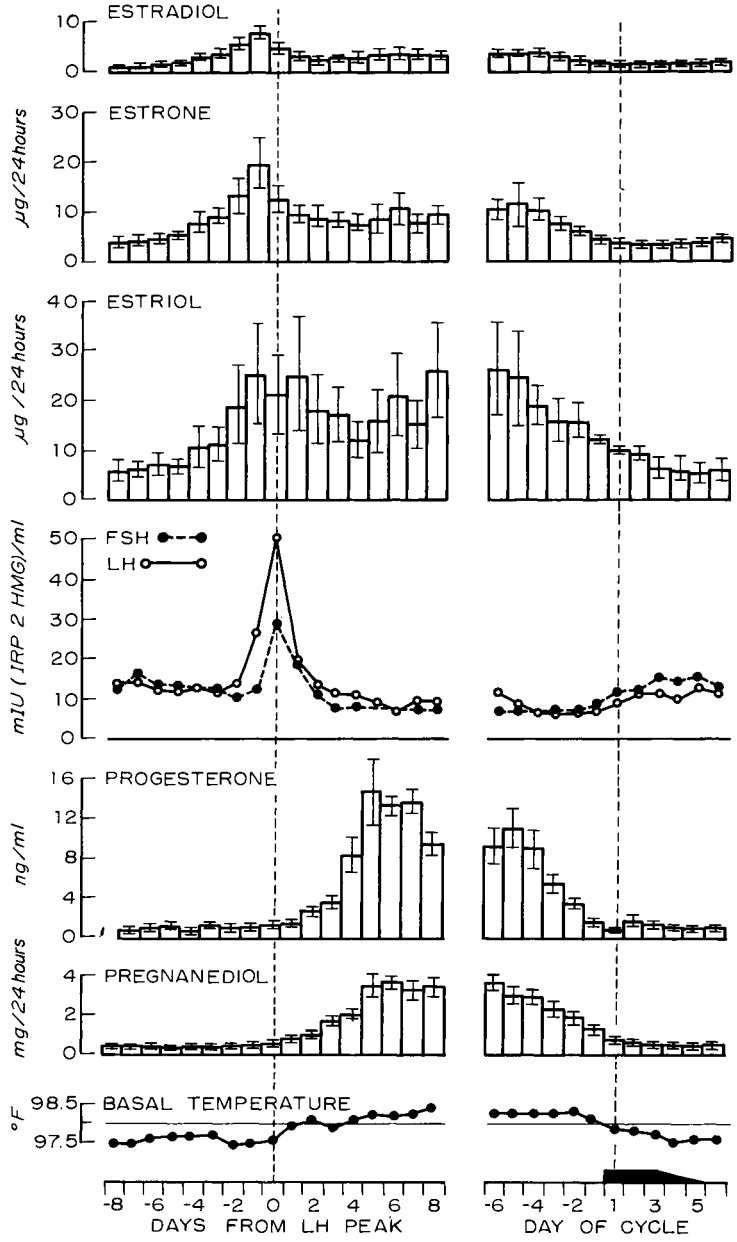


Fig. 15-6 Plasma levels of FSH, LH, and progesterone, and urinary excretion of estrogens and pregnanediol during menstrual cycle. After Jaffe, Discussion in Ross *et al.* (1970). Reproduced by permission of Academic Press.

minimal levels toward the end of the first cycle and the beginning of the next (Jaffe, 1970). The urinary excretions of the estrogens, also shown in Fig. 15-6, will be discussed later.

4. Estrogen Concentrations in Plasma and Excretion in Urine in Normal Women

Early studies in this area usually utilized biological methods such as the capacity to produce cornification in the otherwise quiescent vaginal epithelium of the ovariectomized mouse or rat. The international unit (IU) was defined as the estrogenic activity of 0.1 μg of a standard preparation of estrone kept at the National Institute for Medical Research at London under the auspices of the Health Organization of the League of Nations. One international unit was equivalent to 2 mouse units or 0.17 rat unit (Bodansky and Bodansky, 1952).

Several chemical methods were also developed which not only evaluated the estrogens as a group (Kober, 1938) but which were also capable of determining each of the three main estrogens, namely, estrone, estradiol, and estratriol, in human urine (Brown, 1955). For example, the latter method involved acid hydrolysis, ether extraction, a new phase-change purification procedure for the phenolic fraction depending on methylation of the phenol group, separation of the estrogen methyl esters by chromatography on alumina columns, development of color by use of a quinol-sulfuric acid reagent, and spectrophotometric measurement with correction for interfering chromogenic material.

The early methods were capable of measuring excretion of urinary estrogen and eliciting the existence of a peak excretion at midcycle (Bodansky and Bodansky, 1952). However, they were not sensitive enough to determine very low estrogen levels in blood or urine. Later, several other more sensitive methods were introduced, and some data on the levels of estrogens in blood were obtained (Icchi *et al.*, 1963; Svendsen and Sørensen, 1964).

More recently, by means of a double-isotope derivative, the plasma concentrations of free estrone and estradiol-17β have been more precisely evaluated throughout the entire course of the menstrual cycle (Baird and Guevara, 1969). Table 15-2 shows these values in menstruating, castrated, and postmenopausal women and in men. The plasma concentration of unconjugated estrone rose significantly from values of 4.0 ± 0.4 (SE) ng/100 ml during menstruation to a peak of 17.0 ± 1.3 (SE) ng/100 ml at ovulation and midcycle, then declined again during the luteal phase. The concentration of unconjugated estradiol showed a biphasic curve, rising to a peak value at around midcycle, falling during the few

TABLE 15-2

Concentration of Unconjugated Estrone and Estradiol in Plasma in Women During the Normal Menstrual Cycle, in Castrated and Menopausal Women and in Men[a]

Group	Number	Estrone mean value + SE (ng/100 ml)	Estradiol mean value + SE (ng/100 ml)
Women			
Days from ovulation			
−17 to −12	8	4.0 ± 0.4	2.9 ± 0.7
−11 to −8	9	6.0 ± 0.6[b]	5.5 ± 0.7[b]
−7 to −4	6	6.7 ± 0.8	8.5 ± 0.8[b]
−3 to 0	13	17.0 ± 1.3[c]	31.8 ± 2.7[c]
+1 to +4	9	9.2 ± 1.1[c]	11.7 ± 2.0[c]
+5 to +9	6	12.1 ± 1.1	19.1 ± 2.0[d]
Castrate	6	11.6 ± 4.5	3.6 ± 1.3
Postmenopausal	6	7.1 ± 2.7	1.3 ± 0.2
Men	7	8.6 ± 3.5	2.6 ± 0.6

[a] Based on the tabulation by Baird and Guevara (1969). Reproduced by permission of Dr. M. B. Lipsett, Editor-in-Chief, *Journal of Clinical Endocrinology and Metabolism* and The Endocrine Society.

[b] Significant difference from the preceding value $p < 0.02$.

[c] Significant difference from the preceding value $p < 0.001$.

[d] Significant difference from the preceding value $p < 0.05$.

days that followed ovulation, and undergoing a smaller secondary rise in the midluteal phase.

Many studies have been performed on the urinary excretion of estrogens, particularly in relation to the menstrual cycle. Several may be cited in illustration. In 1935, Smith and Smith observed that the 24-hour excretion of "total" estrogens rose from a level of 10–15 rat units during the first 5 or 6 days of the cycle to a maximum of 50 rat units on the fifteenth day, after which it receded to the original level at the end of the cycle and the beginning of the menses. The reader is referred to Fig. 15-6 for a detailed pattern obtained from the average excretion values for 5 subjects (Jaffe, 1970). It may be seen that the 24-hour excretions of estradiol and estrone rise from low values at the time of menstruation and, in the follicular phase, to maxima of about 7 and 20 μg, respectively, at 1–2 days before the LH peak. They then decrease slowly, but with some fluctuation, during the luteal or postovulatory phase until the end of the cycle. The 24-hour excretion of estriol rises from about 5μg to a level of 25 μg 1 day preceding the LH peak. The decrease in the luteal phase is slow and, indeed, appears to be interrupted by an increased excretion at about the ninth postovulatory day.

The relationship of the plasma level of progesterone and the urinary excretion of pregnanediol to the FSH and LH peaks has previously been noted, but may be examined again in Fig. 15-6 with regard to the excretion of the various estrogens.

III. Biochemistry of Ovarian Neoplasms

A. Introduction

At the beginning of this chapter, we considered briefly the incidence, mortality, and classification of ovarian neoplasms. We also pointed out that there were several types of classification (Novak and Woodruff, 1974; Teilum, 1971) and that, for our purposes, it would be most convenient to follow the outline proposed by Teilum (1971). It may be noted that a certain degree of parallelism exists between some tumors of the testis and ovary.

B. Gonadal Stromal Tumors (Sex Cord-Mesenchyme Tumors)

1. Introduction

The gonadal stromal tumors include the following types: (a) granulosa cell tumor; (b) thecoma or theca cell tumor; (c) androblastoma, also known as arrhenoblastoma or Sertoli–Leydig cell tumor; (d) androblastoma tubulare lipoides; (e) gynandroblastoma; (f) ovarian Leydig (hilus) cell tumor; (g) lipoid cell tumor, also designated as adrenal-rest tumor, luteoma, or virilizing lipoid cell tumor; and (h) luteoma of pregnancy. They have been considered as having low malignant potential (Novak and Woodruff, 1974), but they are of interest here because of their androgenic or estrogenic production and, consequently, because of the masculinizing or feminizing effects which they exhibit clinically.

2. Granulosa Cell Tumors

Most cases of granulosa as well as the theca cell tumors and androblastoma are derived directly from the mesenchyme. They usually affect only one ovary and exhibit great variations in size, with the smallest being only a few millimeters in diameter and the largest reported case filling the abdominal cavity and weighing as much as 34 lb (Dockerty and MacCarty, 1939; Novak and Woodruff, 1974). The microscopic pat-

tern varies widely, not only from tumor to tumor, but also within the same tumor. Most commonly, the granulosa cells tend to arrange themselves in small clusters or rosettes around a central lumen or cyst. Occasionally, there may be a large central cystic cavity surrounded by several layers of granulosa cells with a distinct likeness to large graafian follicles. However, other patterns such as trabecular, cylindromatous, or gyriform may be seen.

A substantial number of the feminizing tumors exhibit varying combinations of the epitheloid or granulosa cell and the connective tissue or theca cell, together with an admixture of fibromatosis. Such tumors are designated as granulosa-theca cell tumors. Novak and Woodruff (1974) recently reviewed the last 1800 specimens that have been added to the Ovarian Tumor Registry. Of these, 307 could be classified as belonging to the granulosa-theca categories or, more generally, to the group of feminizing gonadal stromal tumors. Seventy-one specimens, or 23%, fell into the fairly pure granulosa cell tumor type; 114, or 37%, into the combination of granulosa-theca cell tumor type; and 87, or 29% into the theca cell tumor subgroup. A number showed varying degrees of luteinization; indeed, 35, or 11%, fell into this group. Because of considerations such as these, Novak and Woodruff (1974) raised the question whether it is advisable to draw too sharp a division between granulosa cell and theca cell tumors.

Nonetheless, such a division has usually been drawn. Granulosa cell tumors constitute about 5–10% of all solid ovarian cancers (Teilum, 1971). According to Dockerty, more than 600 cases of this tumor type had been reported by 1945, and additional substantial series have continued to be reported since then. For example, in 1958, Morris and Scully stated that a total of 1000 cases of this tumor had been recorded.

The tumor causes feminizing symptoms as a result of excessive estrogen production. In the young child, clinical manifestations of precocious puberty are seen; these consist of precocious menstruation, premature hypertrophy of the breasts and uterus, appearance of axillary and pubic hair, and pubertal development of the external genitalia. In the adult and reproductive female, the increment of estrogen formation has little systemic effect. In some patients, menstrual irregularities with prolonged or profuse bleeding or with intervals of amenorrhea may occur. In postmenopausal women, the effect of excessive estrogen formation may become evident in resumption of uterine bleeding, enlargement of the uterus, and sometimes of the mammary glands.

The excessive production of estrogens and its high urinary excretion in patients with granulosa cell tumor have been documented for over 30 years. For example, in 1939, Palmer described the case of a 19-year-old

female who had had constant menstrual bleeding for about a year. Biological assay showed that the urinary excretion ranged from about 40 to 87 μg of estrone equivalent per day, as compared with the excretion in normal women of about 25 μg/day. Anderson and Sheldon (1937) reported the case of a 3-year- and 9-month-old girl with a granulosa cell tumor. The preoperative excretion was 17,390 mouse units per liter. Within a week after operation, the excretion had fallen to 40 units per liter, and 3 months after the operation, none could be detected. By the method of bioassay then in use, no urinary estrogen was present in normal children of either sex under the age of 10 or 11 years. Other cases of granulosa cell tumor showing clinical signs of feminization and biochemical evidence of increased urinary excretion of estrogen have been reported by Marsh *et al.* (1962) in a 26-month-old girl and by Besch *et al.* (1966).

A more precise and sensitive evaluation of estrogen production in a 9-year-old girl with granulosa cell tumor has recently been submitted by Jenner *et al.* (1972), using a radioimmunoassay. The concentrations of estradiol in plasma were first determined in a series of 98 normal girls at various prepubertal stages (Fig. 15-7). The patient had experienced vague right lower quadrant abdominal discomfort, breast development, and vaginal spotting 1 month prior to admission. On physical examination, lower abdominal distention was evident, and a firm nontender mass in the right lower quadrant was located. Preoperatively, she was considered to have stage 3 breast development, and stage 2 pubic hair. As may be seen from Fig. 15-7, a normal girl at this stage of prepubertal development would be about 11 years of age and would have a plasma estradiol concentration of 20 pg/ml. The 9-year-old patient had a preoperative estradiol level of 413 pg/ml (41.3 ng per 100 ml), substantially higher than the levels at various stages of the cycle in adult females (Table 15-2).

The tumor was removed and diagnosed as a granulosa cell tumor. By the seventh postoperative day, the plasma estradiol had decreased precipitously to 22 pg/ml. Two months postoperatively, breast tissue was no longer palpable, and the plasma estradiol was 12 pg/ml, well within the normal range for a 9-year-old girl.

The normal biosynthesis of estrogens has been discussed earlier in this chapter. Several groups of investigators (Marsh *et al.*, 1962; Griffiths *et al.*, 1964; Kase, 1964; Besch *et al.*, 1966) have considered these pathways *in vitro* in granulosa cell tumors removed at operation. In general, the tumor was homogenized, chopped or sliced, and incubated for the period shown in Table 15-3, with the labeled steroid precursor, buffer, and various cofactors. At the end of the incubation period, the mixture

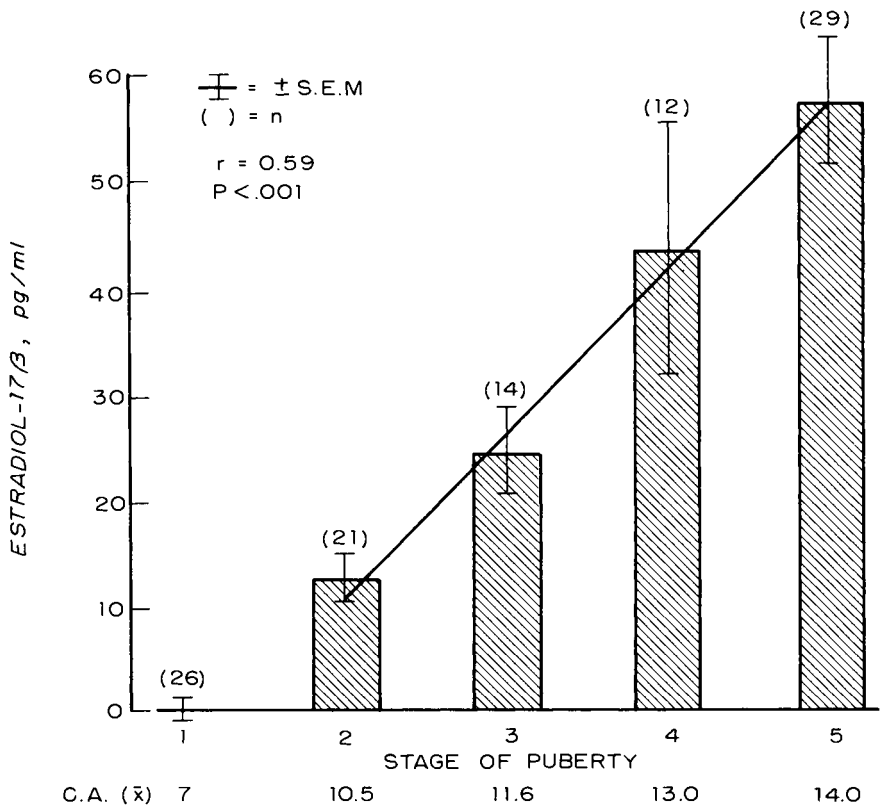


Fig. 15-7 Relationship between concentration of plasma estradiol-17β (pg/ml) and stage of puberty: P1, no clinical signs of puberty; P2, breast budding and no more than sparse labial hair, minimal maturation of the vaginal mucosa, labia majora and minora; P3, further breast enlargement with palpable glandular tissue, Montgomery follicles, extension of the public hair over the mons pubis, sparse axillary hair, dullness of the vaginal mucosa; P4, further breast enlargement with projection of the areola to form a secondary mound, lateral extension of pubic hair, moderate axillary hair, and well-developed external genitalia but no menses; and P5, postmenarchial girls. C. A., chronological age in years. From Jenner *et al.* (1972). Reproduced by permission of Dr. M. B. Lipsett, Editor-in-Chief, *Journal of Clinical Endocrinology and Metabolism* and The Endocrine Society.

was analyzed with addition of various nonradioactive steroids as carriers for the anticipated transformation products.

Before the results in Table 15-3 obtained from several studies are considered, it may be realized that, because of technical considerations, these are not strictly comparable. Limited amounts of tumor material made it difficult to evaluate the optimal effect of pH, time of incubation,

TABLE 15-3

Estrogens and Other Steroids Formed by Incubation of Various Labeled Substrates with Granulosa Cell Tumor Preparations; Comparison of Several Studies[a]

Investigator:	Besch *et al.* (1966)			Kase (1964)		Griffiths *et al.* (1964)		Marsh *et al.* (1962)
Type of preparation:	Homogenate (3 hours)[b]			Homogenate (3 hours)[b]		Chopped tissue (2 hours)[b]		Slices (3 hours)[b]
Substrate:	Progesterone	17-Hydroxyprogesterone	Androstendione	Pregnenolone	Progesterone	Progesterone	Androstendione	Testosterone
Products (as % of substrate)								
Progesterone	26.7	—	—	26	39	31.5	—	—
17-Hydroxyprogesterone	39.6	8.1	—	±	14	—	—	—
17,20-Dihydroxyprogesterone	—	8.5	—	—	—	—	—	—
Androstenedione	5.4	33.0	7.4	1.4	4.2	—	21.5	—
Testosterone	1.8	13.1	2.7	0.6	0.74	—	40.8	—
19-Hydroxyandrostenedione	2.6	17.6	3.7	—	—	—	—	—
Total estrogens	8.3	7.8	70.0	1.3	±	None	2.0	80.0

[a] From Besch *et al.* (1966). Reproduced by permission of C. V. Mosby Company.
[b] Parentheses after type of preparation contains time of incubation.

and the presence of various cofactors in the incubation. This lack in some studies has led to concern chiefly with the final stages of estrogen formation and has minimized the determination of the earlier biosynthetic pathways (Besch *et al.*, 1966). Control studies on normal ovarian tissue in the tumor-bearing patient have necessarily been difficult if not impossible.

Table 15-3 shows the products, including estrogens, that are formed from incubation with various precursors such as progesterone, 17-hydroxyprogesterone, androstenedione, pregnenolone, and testosterone. Of particular note is the conversion of about 70% androstenedione (Besch *et al.*, 1966) and 80% testosterone (Marsh *et al.*, 1962) to estrogens. As control, Marsh *et al.* (1962) cited the study of Baggett *et al.* (1956), indicating a conversion of only 1.2% of testosterone to estrogens.

It may be seen from Table 15-3 that the extent of conversion to estrogen was negligible in the studies by Kase (1964) and by Griffiths *et al.* (1964). This may result from technical factors such as have already been mentioned, namely, the most favorable pH for action or the time necessary to produce maximal action on a steroid. On the other hand, the differences may result from the cellular compositions and nature of the tumor. The tumor studied by Marsh *et al.* (1962) was reported to be a luteinized granulosa cell tumor, as was the tumor investigated by Besch *et al.* (1966), although part of such luteinization may have resulted from the administration of chorionic gonadotropin 2 days preoperatively. In contrast, the material studied by Kase (1964) was diagnosed as a nonluteinized granulosa cell tumor and that investigated by Griffiths *et al.* (1964) was diagnosed as a luteinized granulosa-theca cell tumor.

3. Theca Cell Tumor of the Ovary (Thecoma)

Like the granulosa cell tumor, the theca cell tumor is also feminizing. In 1958, Morris and Scully noted that about 300 cases of theca cell tumor had been recorded in the literature up to that time, as compared with 1000 cases of granulosa cell tumor. However, the more recent report of Novak and his associates (1971) indicates that the incidence of theca cell tumor is slightly higher than that of granulosa cell tumor and somewhat less than the incidence of the mixed granulosa-theca cell type. Microscopically, the tumor is composed of "plump, oval or spindle cells arranged in intermingling bundles or anastomosing trabeculae. The nuclei are small and oval-shaped, and the abundant cytoplasm is pale" (Teilum, 1971).

The clinical symptomatology in patients with this tumor indicates its estrogenic nature. Thus, in patients over 50 years of age, the most

prominent symptom is uterine bleeding. Younger patients with thecomas may exhibit amenorrhea, polymenorrhea, and menorrhagia. In spite of these symptoms, there do not appear to be any detailed studies in which the urinary excretion of estrogens has been measured in the patient in any detail or in which the biochemical activities of the excised tumor have been explored. In 1948, Knight reported the case of a 55-year-old woman with cyclic vaginal spotting from whom a 17-lb theca cell tumor was removed. Analysis showed an estrogen content equivalent to 0.2 μg per 100 gm of tissue. Recently, Brandau (1969) has studied various enzyme activities in the tumor and found that the absolute glycolytic activity was 5 times higher in the tumor than in the ovarian stroma.

A considerable number of instances have been reported in which granulosa or theca cell tumors are associated with undue proliferative activity of the endometrium and, indeed, with adenocarcinoma of the uterus (Novak and Woodruff, 1974; Teilum, 1971). It has been speculated that more or less continuous endogenous estrogen from these tumors may exert a carcinogenic effect on the endometrium, and that thecoma has a much greater effect in this connection (Teilum, 1971). Gusberg and Kardon (1971) have recently reviewed this problem by studying the endometrium from 115 patients with so-called feminizing ovarian tumors recorded in their institution and in the Ovarian Tumor Registry. Of these 115 patients, 46 had thecoma, 34 had granulosa cell tumors, and 35 had theca-granulosa cell tumors. For the group as a whole, 24 patients, or 21%, had adenocarcinoma of the endometrium, 5 had carcinoma *in situ*, and 45 had adenomatous hyperplasia. These latter 2 subgroups were considered to be cancer precursors and constituted 43% of the 115 patients. However, the percentage of these precursor subgroups among the patients with thecoma was not significantly different from that among the patients with granulosa cell tumors.

4. Arrhenoblastoma

The synonyms and related terms for arrhenoblastoma are androblastoma, andreioblastoma, Sertoli–Leydig cell tumor, and adenoma testiculare ovarii. It will be recalled from our discussion in Chapter 14 (Section II,D) that the term "androblastoma" has been defined by Teilum (1971) as a "histogenetic and morphologic designation for neoplasms, in either sex, which are derived from the undifferentiated gonadal mesenchyme." In the male, the testicular tumors are generally feminizing, and in the female, the ovarian tumors of this group are generally virilizing. According to Teilum (1971), there are three principal histological patterns which correspond to various stages in gonadogenesis. These are, first, the most undifferentiated type which is composed of a diffuse

blastema of closely packed cells with occasional areas of imperfect tubule formation and of islands of Leydig cells. In the second or intermediate type, differentiated Sertoli cells form solid cords or nests. Leydig cells may also be present. In the third or well-differentiated tubular form, numerous tubules resembling seminiferous tubules lined by Sertoli cells are present. Arrhenoblastomas may contain any combination of these 3 types. By 1960, 240 cases had been reported in the literature (Pedowitz and O'Brien, 1960).

The age incidence of arrhenoblastoma has ranged from 2½ to 70 years of age, although the greatest incidence is in the decade between 20 and 30 years. The clinical symptomatology indicates the onset of defeminization followed by masculinization. It consists of the development of amenorrhea, flattening of breasts, appearance of facial and body hirsuitism, deepening of voice, and hypertrophy of the clitoris.

The levels of urinary 17-ketosteroid and estrogen excretion and of plasma values of testosterone and estrogens are not consistently altered in patients with arrhenoblastoma, but a relationship does exist between these alterations and the stage of the disease. In their 1960 review of the literature, Pedowitz and O'Brien reported that, despite the marked virilization manifested by the patients, the 17-ketosteroid levels were increased preoperatively in only 13 of 35 cases tested. Postoperatively, these levels rapidly decrease to the normal range and become elevated again only in those with recurrence of the disease. Kase and Conrad (1964) reported the case of a 43-year-old female with progressive masculinization of 3 years' duration whose preoperative 17-ketosteroid urinary excretions were 25 and 19 mg/day on two occasions, slightly higher than the range of 4–17 mg and average of 10 mg/day for normal women (Albert *et al.*, 1968). A 32-year-old woman with amenorrhea of 4 years' duration, excessive hair growth on several parts of the body, deepening of voice, and enlarged clitoris had normal urinary excretions of 17-ketosteroid, 17-hydroxycorticosteroids, pregnanediol, pregnanetriol, estrone, estradiol, and estriol (Sato *et al.*, 1969). The urinary FSH was stated to be low. Operation revealed a large arrhenoblastoma in the right ovary, but no steroid studies were carried out postoperatively.

Although the urinary excretion of 17-ketosteroids as a group may not be elevated or elevated only slightly, the pattern of steroid excretion may be altered substantially (Table 15-4). In the case of arrhenoblastoma studied by Mahesh *et al.* (1970), the removal of the tumor led to some decrease in the 17-ketosteroids but to greater decreases in the 11-deoxy-17-ketosteroids. The administration of dexamethasone preoperatively did not cause any decrease in the fraction of 11-deoxy-17-ketosteroids, whereas other subgroups of neutral steroids such as the 11-oxyge-

TABLE 15-4

Urinary Steroid Excretion Patterns in a Patient with Arrehenoblastoma prior to Operation and after Removal of the Tumor[a]

Steroid	Preoperative[b]	1 Week postoperative[b]	5 Months postoperative[b]
Routine steroid determinations			
17-Ketosteroids	30.9	12.6	17.1
17-Ketogenic steroids	11.6	27.4	2.7
Pregnanetriol	1.8	0.3	—
11-Deoxy-17-ketosteroids			
Dehydroepiandrosterone	0.8	<0.1	<0.1
Etiocholanolone	4.0	0.6	0.9
Androsterone	4.1	0.6	0.5
11-Oxyenated 17-ketosteroids			
11-Hydroxycholanolone	0.7	0.5	0.3
11-Hydroxyandrosterone	<0.1	0.1	<0.2
11-Ketoetiocholanolone	0.2	0.3	0.1
Tetrahydrocorticosteroids			
Tetrahydrocortisol	1.5	2.7	1.4
Allotetrahydrocortisol	1.1	0.4	0.8
Tetrahydrocortisone	2.2	3.2	1.0
Estrogens			
Estrone	2.8	3.8	0.5
Estradiol	2.7	2.9	0.7
Estratriol	1.6	1.9	3.5

[a] Based on data of Mahesh *et al.* (1970).

[b] All values are expressed as milligrams per 24 hours, except for estrogens where the results are in micrograms per 24 hours.

nated 17-ketosteroids and tetrahydrocortisol went down to exceedingly low levels.

Frequently, the urinary excretion of 17-ketosteroids or related steroids in arrhenoblastoma may be normal or only slightly elevated, yet the blood plasma may show more profound changes. For example, the 24-hour urinary excretions of 17-ketogenic steroids were within normal limits before operation in each of 3 cases reported by Greenblatt *et al.* (1972), and were not altered postoperatively. The 24-hour 17-ketosteroid excretion was high, 30.8 mg, in only 1 of the 3 cases, and was reduced to a normal level postoperatively, namely, 12.8 mg. In the other 2 cases, the preoperative levels were within normal ranges, although removal of the tumors resulted in definite decreases. The preoperative plasma testosterone levels in peripheral vein blood were stated to be elevated for females and within the normal range for normal males

in all 3 cases, and to be reduced postoperatively to normal levels for females. Data were presented for 1 case in which the preoperative plasma testosterone was 0.36 μg per 100 ml and the postoperative level 0.04 μg per 100 ml. Mahesh *et al.* (1970) reported the following values for the preoperative blood plasma concentrations of androgens in μg per 100 ml: dehydroepiandrosterone, 0.85; Δ^4-androstenedione, 0.98; and testostrone, 0.45. Postoperatively, the values were all less than 0.05 μg per 100 ml.

During operation, the concentrations of androgens in the ovarian vein blood were quite high: testosterone, 11.4 and 18.2 μg per 100 ml in the cases reported by Greenblatt *et al.* (1972) and Mahesh *et al.* (1970), respectively. In the latter case, the ovarian vein blood at operation contained 21.7 and 24.3 μg per 100 ml of dehydroepiandrosterone and Δ^4-androstenedione, respectively.

The basis for the higher levels of plasma testosterone in arrhenoblastoma has been explored further. Loriaux *et al.* (1970) found that the maximum quantity of plasma testosterone derived from circulating dehydroepiandrosterone sulfate (DS) in a 32-year-old female with arrhenoblastoma was 640 μg/day preoperatively and 310 μg/day postoperatively. The preoperative DS production rate was 80 mg/day and decreased to 15.5 mg postoperatively.

That the urinary excretion of 17-ketosteroids and 17-ketogenic steroids in patients with arrhenoblastoma may frequently be within normal limits, yet that the plasma reveals abnormal deviations is brought out particularly in a case reported by Laatikainen *et al.* (1972). The urinary excretion of 17-ketosteroids and 17-ketogenic steroids and the response to ACTH administration were normal. The plasma steroids were analyzed in some detail and showed a pattern quite different from that seen in normal subjects (Table 15-5).

As with other tumors that are involved in steroid metabolism, attempts have been made to define the derangement in human arrhenoblastoma by studying preparations of the excised tumor *in vitro*. Savard *et al.* (1961) observed that the incubation of arrhenoblastoma slices with [4-^{14}C]progesterone resulted in the formation of labeled 20α-hydroxy-4-pregnene-3-one, 20β-hydroxy-4-pregnene-3-one, 17-hydroxyprogesterone, 4-androstene-3,17-dione, and testosterone. However, no labeled estrone, estradiol, or estrone could be detected. It would seem probable, from the considerations we presented earlier in this chapter (Section II,C), that the metabolism of androgens to estrogens in arrhenoblastoma tissue is blocked at the stage where androstenedione is converted to 19-hydroxyandrostenedione or, alternately, testosterone to 19-hydroxytestosterone.

Other investigations also indicate greater formation of testosterone.

TABLE 15-5

Plasma Neutral Steroid Sulfates in Patient with Arrhenoblastoma and in Normal Women[a]

Steroid	Concentration of steroid in plasma (μg/100 ml ± SE)	
	In normal subjects	In patient with arrhenoblastoma
Monosulfates		
Androsterone	8.6 ± 1.6	92
Dehydroepiandrosterone	36.5 ± 10.4	59
Epiandrosterone	3.4 ± 0.9	41
5-Androstene-3β,17β-diol	6.7 ± 1.6	25
Pregnenolone	5.3 ± 0.7	12
5-Pregnene-3β,20α-diol	17.1 ± 3.8	29
Disulfates		
5-Androstene-3β-17α-diol	8.0 ± 1.5	4
5-Androstene-3β-17β-diol	18.2 ± 5.0	17
5-Pregnene-3β-20α-diol	9.9 ± 1.7	20

[a] Adapted from Table 1 of Laatikainen *et al.* (1972).

For example, after 2 hours' incubation of arrhenoblastoma slices with labeled androstenedione, 4.2% of the count was left in the initial substrate and 75% was present in the conversion product, testosterone. In contrast, after incubation with slices of normal ovary in two different experiments, much higher amounts, 65 and 37%, were in the androstenedione, and much lower amounts, 4.9 and 5.7%, were present in testosterone. Similarly, when [4-^{14}C]dehydroepiandrosterone was used as substrate, the extent of conversion to testosterone was greater in the arrhenoblastoma tissue than in normal ovarian tissue (Sato *et al.*, 1969). The presence and overproduction of testosterone appeared to be significant factors in explaining the degree of masculinization in cases of arrhenoblastoma.

5. Gynandroblastoma

As the name indicates, gynandroblastoma refers to those tumors in which there is a combination of granulosa or thecal elements (feminizing) together with those which are typically arrhenoblastomous (masculinizing) (Novak and Woodruff, 1974; Teilum, 1971). Although either may be predominant clinically, Novak and Woodruff (1974) pointed out that, in view of the innumerable variations of both granulosa cell tumors and arrhenoblastoma, care should be taken in making the micro-

scopic diagnosis of gynandroblastoma. Few cases of this tumor have been reported (Teilum, 1971). Neubecker and Breen (1962) studied 5 patients, 2 of whom showed masculinization, 2 manifested hyperestrogenism, and 1 had no endocrine symptoms.

The nature of the plasma steroids in a 52-year-old woman with this tumor has been recently reported by Laatikanen *et al.* (1972). Extensive hirsuitism, masculine constitution, frontal baldness, and deepening of the voice were present, but there was no clitoral enlargement. The histological features of the removed tumor were consistent with those of a gynandroblastoma. As in the case of arrhenoblastoma similarly studied (see Table 15-5), the plasma concentrations of the monosulfates of androsterone and epiandrosterone were substantially elevated above normal to 195 and 41 μg per 100 ml, respectively. At operation, the ovarian vein blood contained a number of the unconjugated steroids. The concentration of unconjugated testosterone was very high, namely, 244 μg per 100 ml.

6. Ovarian Leydig (Hilus) Cell Tumor

As we have noted in the preceding chapter, the Leydig cells in the interstitial area of the testis are involved in the formation and secretion of androgens. The presence of Leydig cells in the hilus of the normal ovary was first recognized in 1922, and the possibility of excessive proliferation of these cells with the formation of a masculinizing tumor was recognized some 20 years later (Berger, 1942). Hilus cell tumors are usually small, ranging in diameter from 0.3 to 5.0 cm. They are brownish, yellow, or orange in color and form unencapsulated tumors situated in the hilus region (Teilum, 1971). Microscopically, they are composed of uniform, closely packed polyhedral cells with distinct, round nuclei and eosinophilic, finely granular cytoplasms (Novak and Woodruff, 1967; Teilum, 1971). The rod-shaped Reinke crystals are present in about 50% of the cases (Novak and Woodruff, 1967). A total of about 60 cases had been reported in the world literature by 1972 (Anderson, 1972).

Most of the patients who have been reported in any detail show clinical and biochemical signs of virilism. However, Novak and Mattingly (1960), in a review of 18 cases from the Ovarian Tumor Registry, observed endometrial hyperplasia in most cases in which the endometrium was available for study. As has been pointed out earlier in this chapter (Section III,B,3), endometrial hyperplasia is presumably a manifestation of an estrogenic effect.

Although prior to 1965, determination of urinary excretion of 17-keto-

steroids in a few patients with ovarian hilus cell tumor had been performed, these showed no distinct elevations preoperatively nor any consistent change as a result of operation. No results on estrogen excretion were available. In 1965, Hawkins and Lawrence studied in some detail the excretion of 17-ketosteroids and various estrogens in a 25-year-old female who had menstrual irregularities, hirsutes, and primary infertility. Upon subsequent operation, a hilus cell hyperplasia was revealed, and the ovary was subjected to bilateral wedge resection. Table 15-6 shows the preoperative values for hormone excretion and values at two to four occasions during the year following operation. Although the preoperative 17-ketosteroid and 17-ketogenic steroid excretions were not above normal, a consistently decreased excretion occurred postoperatively. As may be seen by comparison with Fig. 15-6, the preoperative pregnanediol excretion was very high for the proliferative phase of the cycle as indicated by the vaginal smear. Pregnanetriol was also increased. Most striking were the excretions of the total and individual estrogens, which were much above the normal levels at any stage of the menstrual cycle, and which decreased markedly after operation. These results indicated that hilus cells may produce estrogens as well as androgens.

A similarly detailed study in which the estrogen excretion was studied but showed no elevation is that provided by Mori *et al.* (1970). The urinary excretion of 17-ketosteroids in a 27-year-old Japanese patient with a virilizing hilus cell tumor was 84 mg per 24 hours as contrasted with a range of 5–12 mg per 24 hours in normally menstruating Japanese women. The urinary production rates of testosterone, dehydroepiandro-

TABLE 15-6

Hormonal Urinary Excretions, Pre- and Postoperatively in a Patient with Virilizing Hilus Cell Hyperplasia[a]

Steroid	Preoperatively	Postoperatively[b]
17-Ketosteroids (mg/24 hours)	15.6	3.7–5.3 (4)
17-Ketogenic steroids (mg/24 hours)	14.6	2.1–4.8 (4)
Pregnanetriol (mg/24 hours)	2.48	0.18–1.04 (4)
Pregnanediol (mg/24 hours)	8.0	0.44–1.84 (3)
Total estrogens (μg/24 hours)	147.2	22.5–61.8 (4)
Estrone (μg/24 hours)	34.0	0.6–8.8 (4)
Estradiol (μg/24 hours)	14.2	0.4–7.5 (4)
Estriol (μg/24 hours)	99.0	20.5–45.5 (4)
Gonadotropin (mouse units/24 hours)	500	20 (1)

[a] Based on Table 2 of Hawkins and Lawrence (1965).

[b] Number in parentheses denotes postoperative determinations.

sterone, and dehydroepiandrosterone sulfate were 1970, 98.5, and 31.9 mg/day, respectively. These were all substantially higher than the corresponding values of 5.99 ± 1.66 mg testosterone per day for normal Japanese men and of 11.1 ± 5.4 mg dehydroepiandrosterone and 8.9 ± 3.8 mg dehydroepiandrosterone sulfate per day for normal Japanese women. The urinary excretion of 17-hydroxycorticosteroids was normal. The excretions of estrone, estradiol, estriol, and total gonadotropin were within the range of the normal follicular phase and the excretion of pregnanediol was within the range of the normal luteal phase. Postoperatively, the androgenic manifestations subsided, the menstrual cycle returned to normal, and the urinary 17-ketosteroids decreased to 4.4 mg/day.

The large majority of hilus cell tumors are local, but Echt and Hadd (1968) reported the case of a 60-year-old woman who had developed symptoms of masculinization 5 years earlier. One year previously, she had had a total abdominal hysterectomy and a right salpingo-oophorectomy revealing the presence of a metastatic hilus cell tumor of the ovary. Abdominal recurrence began to be noted 6 months postoperatively. Study begun 1 year postoperatively showed impressively high urinary excretions of 17-ketosteroids, ranging from 60 to 745 mg per 24 hours over a 2-year period of observation. The excretion of 17-hydroxycorticosteroids ranged from about 5 to 28 mg per 24 hours.

Most impressive was the extent and mode of excretion of testosterone in this patient. As will be recalled from Chapter 14 (Section II,E,3) and as noted by Echt and Hadd (1968), the urinary excretion of testosterone ranges from about 50 to 150 μg/day in the normal male, with at least 90% being excreted as the glucuronide and the remainder as the sulfate. In the normal female, the total urinary excretion of testosterone is less than 10 μg/day. In the patient studied by Echt and Hadd (1968), the urinary excretion of testosterone was very high, ranging from 3437 to 5184 μg/day on several occasions with the major fraction, from 75 to 92% now being in the sulfate-conjugated form. In other words, testosterone sulfate excretion had been increased from its normal level almost 10,000 times. The excessive production of testosterone and other androgens in patients with Leydig cell tumor has been substantiated by others (Anderson, 1972).

7. Luteoma of Pregnancy

In 1966, Sternberg and Barclay designated as a diagnostic entity a specific, benign lutein cell tumor of the ovary which the senior author had had occasion to observe for several years (Sternberg, 1963). Sternberg and Barclay (1966) presented 12 cases of this tumor and 6 cases described by other authors. In a more recent study, Thomas *et al.* (1972)

noted that 34 full cases had been reported in the literature and reported 1 case of their own. Grossly, these tumors are ovoid, soft, fleshy, yellowish, and frequently hemorrhagic. The microscopic pattern reveals small masses of eosinophilic, polyhedral cells with pink finely granular cytoplasm. The question has arisen whether these tumors constitute a distinct entity or an exaggerated physiological response to pregnancy (Sternberg and Barclay, 1966; Novak and Woodruff, 1974).

Of the 34 cases reviewed by Thomas *et al.* (1972), 8, or 24%, were associated with maternal virilization and 3, or 9%, with temporary virilization of the female fetus. Prior to the study of Thomas *et al.* (1972), there do not appear to be any determinations of 17-ketosteroid excretion before delivery in the patients with maternal virilization. Four of these had such determinations on or after the day of delivery, and the highest recorded values for the urinary 17-ketosteroids was 125.8 mg per 24 hours on the day of delivery. The case reported by Thomas *et al.* (1972) is most instructive, for sequential urinary and plasma steroid values both antepartum and postpartum were obtained.

The patient was an 18-year-old black gravida 4, para 3 who had noted deepening of her voice and increase of facial hair during the fourth month of her third pregnancy. She was admitted at 38 weeks' gestation of her fourth pregnancy because of a cul-de-sac mass which had been noted 4 weeks previously. The patient had a deep voice, coarse and hirsute facial features, hair on the upper and lower extremities, a male escutcheon and a slightly enlarged clitoris. The urinary steroid excretions on the day before delivery, expressed as milligrams per 24 hours, were as follows: 17-ketosteroids, 230 (normal 6–15); 17-ketogenic steroids, 25 (normal 3–15); dehydroepiandrosterone, 186 (normal 0–1.4); and pregnanetriol, 7.2 (normal 0–4.0). At delivery, the right ovary was found to be enlarged and to occupy the cul-de-sac. Sections of these were taken for microscopic study, but the ovaries were conserved. The diagnosis was luteoma of pregnancy. During the immediate postpartum period, the various biochemical parameters decreased with some fluctuation and reached normal levels by the tenth day. The plasma testosterone was over 600 ng per 100 ml on the second postpartum day, but decreased steadily to 204 ng per 100 ml on the eighth day. The normal value used by Thomas *et al.* (1972) was 9–150 ng per 100 ml. Stimulation with human chorionic gonadotropin (HCG) in the postpartum period resulted in significantly increased excretions of 17-ketosteroids and ovarian enlargement, supporting the theory that luteoma of pregnancy is an HCG-dependent tumor.

The steroid content and biosynthetic pathways of a luteoma of pregnancy that caused virilization in mother and infant were first studied by O'Malley *et al.* (1967). The tumor, weighing 690 gm, was removed

at the time of delivery. The steroid content, expressed as micrograms per gram of tissue, was: progesterone, 0; androstenedione, 0.46; dehydroepiandrosterone, 3.1; dehydroepiandrosterone sulfate, 4.3; testosterone, 7.6; and testosterone sulfate, 0.37. It is interesting to compare these values with those obtained by various investigators for the normal corpus luteum of pregnancy (Zander *et al.*, 1958; Simmer and Voss, 1960). Also expressed in micrograms per gram of tissue, these were progesterone: 3–40; androstenedione, 0.34; dehydroepiandrosterone, 0.04; dehydroepiandrosterone sulfate, 0.0; and testosterone, <0.03. It may be seen, therefore, that the progesterone content is considerably lower and the dehydroepiandrosterone and testosterone contents considerably higher in the luteoma of pregnancy than in the normal corpus luteum.

O'Malley *et al.* (1967) incubated slices of luteoma with a mixture of [^{14}C]progesterone and [^{3}H]17α-hydroxypregnenolone, and also with a second mixture of [^{14}C]dehydroepiandrosterone and dehydroepiandrosterone [^{3}H]sulfate, both in phosphate buffer for 1 and 3 hours at 37°C. After isolation and purification, the ^{3}H:^{14}C ratios of the various steroids in the first incubaton were: 17-hydroxyprogesterone, 5.0; androstenedione, 3.5; 5-androstene-3β,17β-diol > 100; and testosterone > 100. In the second incubation, the ratios were: androstenedione, 1.8; testosterone > 18.

Figure 12-6 has shown that, normally, testosterone is formed by either of two pathways: (a) from progesterone via 17α-hydroxyprogesterone and Δ^4-androstenedione; (b) from Δ^5-pregnenolone via 17α-hydroxypregnenolone, and dehydroepiandrosterone. This last compound may go either to Δ^4-androstenedione or to Δ^5-androstenediol and, through either precursor, to testosterone.

The results obtained from the incubation studies of O'Malley *et al.* (1967), together with the findings of high concentrations of dehydroepiandrosterone and its sulfate conjugate in tumor tissue, indicated that testosterone was synthesized preferentially through the pathway with androstenediol as the key intermediate. In their studies of another luteoma of pregnancy, Rice *et al.* (1969) found that the major Δ^4-3-ketosteroid detected by their methods was the androgen, Δ^4-androstenedione, which was present in a concentration of 2.6 μg/gm of luteoma tissue, and was also formed upon incubation of slices of tumor tissue with acetate.

8. Lipoid Cell Tumors

The lipoid cell tumor has also been designated as "adrenal-like tumor, adrenal rest tumor, luteoma, masculinovablastoma, and virilizing lipoid

cell tumor" (Teilum, 1971). It is among the rarest of ovarian tumors, 30 specimens having been contributed to the Armed Forces Institute of Pathology during a 28-year period ending in 1964 (Taylor and Norris, 1967). In 1962, Pedowitz and Pomerance reviewed 56 cases reported in the literature. Some of the synonyms represent the opinion that this group of tumors is of adrenal origin or at least of a common progenitor that also gives rise to adrenal tumors (Novak and Woodruff, 1974). According to Teilum (1971), the characteristic microscopic picture is that of masses or sheets of large, rounded or polyhedral, lipoid-containing clear cells separated by vascular sinuosids and thus resembling cells of the adrenal cortex but also such structures of the ovary as vacuolated Leydig cells or theca-lutein cells. Taylor and Norris (1967) noted that both adrenocortical-like and hilus-like cells are present in most instances, but decried the attempt to separate them objectively into adrenal-like tumors, hilus cell tumors, or stromal luteomas. They preferred to regard lipoid cell tumors as a specific entity and not a collection of different entities. Of the 30 cases reviewed by Taylor and Norris (1967), 77% of the patients showed clinically virilizing effects, and 23% had clinical evidence of estrogenic activity.

Two studies may be cited to illustrate the biochemical disorders in this condition. Osborn *et al.* (1969) reported the case of a 35-year-old female whose menses had ceased 7 years previously and who thereafter began to develop facial and body hair whose growth accelerated, accompanied by development of frontal scalp alopecia. The patient had gained considerable weight. Physical examination revealed abnormal muscularity, centripetal distribution of fat with pinkish striae on the abdomen, an escutcheon with a male pattern, and a slightly enlarged clitoris.

The values for steroid excretion, expressed as milligrams per 24 hours, were as follows: 17-hydroxycorticosteroids, 17 and 20 on two occasions; 17-ketosteroids, 81 and 112. The values for the 17-hydroxycorticosteroids were substantially above the upper level and those for 17-ketosteroids greatly so. The values for the plasma unconjugated steroids, expressed as micrograms per 100 ml, were: testosterone, 3.18; androstenedione, 6.44; and dehydroepiandrosterone, 2.19. These were much higher than the corresponding normal values for these components obtained by Osborn *et al.* (1969), namely, 0.013 ± 0.009 (SD), 0.104 ± 0.066 (SD), and 0.608 ± 0.40 (SD). Adrenal stimulation with 25 units ACTH given intravenously over 8 hours on each of 2 successive days raised the 17-ketosteroid excretion slightly and the 17-hydroxycorticosteroid excretion considerably, but did not clearly affect the plasma levels of testosterone, androstenedione, and dehydroepiandrosterone. Adrenal suppression tests, carried out by the administration of dexamethasone, slightly decreased

the urinary excretion of 17-hydroxycorticosteroids but increased the excretion of 17-ketosteroids to 228 mg per 24 hours and the levels of plasma steroids, expressed as micrograms per 100 ml, to 7.41 for testosterone, 9.92 for androstenedione, and 6.27 for dehydroepiandrosterone. Since normal adrenals would have responded to the stimulation and suppression tests, these results indicated the possibility of an adrenal neoplasm. However, at operation, 2500 ml of ascitic fluid was removed, and the left ovary was found to be replaced by a large yellow-tan and gray tumor. Both adrenals were of normal size and consistency. After the removal of the tumor, the urinary 17-ketosteroids and 17-hydroxycorticosteroids, and the plasma testosterone, androstenedione, and dehydroepiandrosterone were found to be decreased to normal levels.

A later study by Lipsett and his associates (1970) on the malignant (metastatic) form of lipoid cell tumor has not only furthered our biochemical knowledge of this tumor but has also presented evidence that it does not cause Cushing's syndrome and that its origin is the ovarian stromal cell. The patient was a 37-year-old woman who had developed clinical evidence of virilization at the age of 16. Seven years later, at the age of 23, laparatomy was performed and a left ovarian tumor, diagnosed as benign masculinovoblastoma, was removed. Virilization diminished but began to recur 14 years later when an abdominal mass was noted, and abdominal exploration revealed extensive metastatic disease. Biopsy of a metastasis showed a histological picture identical with the original tumor. After 2 weeks of x-ray therapy, the patient was admitted and studies begun.

Administration of ACTH for 2 days led to more marked effects than had been produced in the benign lipoid cell tumor studied by Osborn *et al.* (1969). The urinary 17-ketosteroid excretion rose from 74 to 112 mg per 24 hours, and the already elevated plasma levels, 0.2–0.4 μg per 100 ml for testosterone and 2.6–3.4 μg per 100 ml for androstenedione, rose still further. The levels for normal women used by Lipsett *et al.* (1970) were 0.02–0.07 μg per 100 ml for testosterone and 0.07–0.15 μg per 100 ml for androstenedione. The administration of 4000 units HCG daily for 4 days led to much more dramatic increases, namely, to a maximal urinary excretion of 240 mg 17-ketosteroid per 24 hours, and to plasma testosterone and androstenedione levels of 2.0 and 15 μg per 100 ml, respectively. The administration of 2,2-bis-(*p*-chlorphenol-1,1-dichlorethane) (*o,p'*-DDD) resulted in decreases in 17-ketosteroid excretion of normal levels, of plasma testosterone to the normal female range, and of androstenedione to 0.28 μg per 100 ml, or about twice the normal level.

At the beginning of the study, the plasma levels of 17-hydroxyprogesterone and 17-hydroxypregnenolone were 0.6 and 0.85 μg per 100 ml,

respectively, and were higher than the corresponding normal values, namely, 0.05 and 0.4 μg per 100 ml. After the administration of HCG, the plasma concentrations of the two components rose approximately tenfold, 17-hydroxyprogesterone to 6.4 μg per 100 ml and 17-hydroxypregnenolone to 8.7 μg per 100 ml. The abnormal levels of several plasma steroids were paralleled by high urinary excretions of their metabolites. Androsterone and etiocholanolone, the metabolites of testosterone and androstenedione, respectively, accounted for approximately 80% of the 17-ketosteroids. Pregnanetriol and pregnenolone, the metabolites of 17-hydroxyprogesterone and of 17-hydroxypregnenolone, were also excreted in high amounts. On the basis of this pattern of plasma steroid levels and urinary steroid excretion, Lipsett *et al.* (1970) concluded that the important secretory product of the tumor was androstenedione. A consideration of the biosynthetic properties of this tumor, as determined by their work as well as that in the literature, also led them to conclude that lipoid cell tumor does not cause Cushing's syndrome but that its origin is the ovarian stromal cell.

C. Germ Cell Tumors

1. Introduction

Tumors in this class, which may be subdivided into several groups, are considered to arise from germ cells going back to the undifferentiated stage of gonadal development, before these cells have gained any sexual characteristics. Such ovarian tumors should have testicular counterparts and, indeed, Teilum (1971) has depicted graphically the histogenesis and interrelationship of ovarian and testicular tumors of germ cell origin. In general, these tumors should have no endocrinological manifestations, but actually such aspects have been uncovered, and it has been found that, in these instances, these tumors contained cells of sex-cord and mesenchymal origin.

2. Dysgerminoma

The name "dysgerminoma" was derived on the assumption that the tumor arises from "neuter" or "disgerminal" cells which do not have a capacity for sexual differentiation in either sex. A number of synonyms and related terms have also been used. These are disgerminoma, germinoma, gonocytoma, and ovarian seminoma (Teilum, 1971). The size of dysgerminomas range from a few centimeters to 30 or 40 cm in diameter, the latter large enough to fill the abdominal cavity. They are surrounded by a smooth dense capsule and may contain areas of necrosis,

degeneration, and hemorrhage. Microscopically, the tumor resembles testis seminoma. The cells are vesicular, round, or polygonal in shape, with a diameter ranging between 12 and 18 μm, and arranged in strands or nests, separated by fibrous septa which show extensive lymphocytic infiltration (Novak and Woodruff, 1974; Teilum, 1971). By 1971, approximately 700 cases had been reported (Kofler and Spona, 1971).

In 1953, Scully reported 2 cases, and, in 1956, Usizima reported 1 case of dysgerminoma which exhibited clinical evidences of masculinization. An 8-year-old girl showed heavy growth of pubic hair and enlarged clitoris, while the other 2 patients, a 19-year-old girl and a 20-year-old girl, had never menstruated, had experienced deepening of the voice, and had developed excess hair on the face, body, and legs.

As has been indicated, only a very small proportion of ovarian dysgerminomas show clinical evidence of hormonal disturbance, particularly of masculinization. Supporting biochemical evidence is available in some of these reports. For example, in the case of the 19-year-old girl reported by Scully (1953), the preoperative urinary excretion of 17-ketosteroids ranged between 7.1 and 9.0 mg per 24 hours. During the first 2 postoperative weeks, the excretion dropped to levels ranging between 1.1 and 3.0 mg per 24 hours. In the case of the 14-year-old girl reported by Kofler and Spona (1971), masculinization was accompanied by a preoperative 17-ketosteroid urinary excretion of 24.0 mg per 24 hours. The removal of a 3000-gm tumor of the right ovary was followed by a decrease in the excretion of 17-ketosteroids to approximately 2–3 mg per 24 hours. Removal of the tumor in these cases as well as others (Williamson and Pratt-Thomas, 1972) was followed by regression of virilism.

A fairly complete biochemical evaluation of masculinization as well as isosexual precocity in a $7\frac{1}{2}$ year-old girl with a mixed dysgerminoma has been submitted by Drobnjak *et al.* (1971). At the age of 7 years, this girl began to exhibit growth of breasts, appearance of pubic and axillary hair, and onset of menses. Physical examination showed an enlarged clitoris and hypertrophic labia majora. At operation, a tumor, $24 \times 20 \times 15$ cm, which had replaced the right ovary, was removed. Microscopically, most of the tumor was composed of classic dysgerminoma cells, but Leydig cells, Sertoli-granulosalike cells, trophoblastic and teratoid portions were also present. The biochemical findings (Table 15-7) corresponded fairly well with the clinical manifestations and pathological findings. The high excretion of estriol, well within the range of an adult woman, betokened maturity of isosexual development, whereas the elevated excretion of testosterone and androstenedione corresponded to the clinical and pathological manifestations of masculinization.

Excessive secretion of gonadotropin also characterized patients with

TABLE 15-7

Urinary Excretion of Hormones in 24 Hours in Patient with Dysgerminoma Containing Sex-Cord and Mesenchymal Cells[a]

Hormonal component	Before operation	After operation
Estriol (μg)	51.4[b]	18.4[b]
17-Ketosteroids (mg)	9.2[b]	12.9[b]
17-Hydroxycorticosteroids (mg)	8.2[b]	13.0[b]
Dehydroepiandrosterone (mg)	0.2	0.2
Testosterone (μg)	9.1	0.5
Androstenedione (μg)	3.4	0.2
Chorionic gonadotropin (IU/liter)	4000	0

[a] Based on Table I of Drobnjak *et al.* (1971).
[b] Represents midpoint of range given.

dysgerminoma. We may see from Table 15-7 that the patient reported by Drobnjak *et al.* (1971) excreted 4000 IU/liter prior to operation, and that this excretion became negligible after removal of the tumor. Similar cases have been reported by others (Hobson and Baird, 1966; Kofler and Spona, 1971). Because of the magnitude of the gonadotropin excretion and the enlargement of the abdomen, the first inclination is to diagnose pregnancy when the patient is of suitable age. However, such a diagnosis is contravened by x-ray examination which fails to reveal fetal parts.

The question naturally arises concerning the source of the HCG. In the case of the 13-year- and 10 month-old girl reported by Kofler and Spona (1971), laparotomy revealed an encapsulated solid tumor of the right ovary, $26 \times 18 \times 11$ cm in dimension and weighing 3.8 kg. Preoperatively, the urinary excretion of HCG, performed by radioimmunoassay, was 25,000 IU/liter. Postoperatively, the excretion of HCG began to drop, reaching a level of 2000 IU/liter at 7 days. Forty-six days after operation, the patient began to complain of breathing difficulties and chest pain. These symptoms became more severe and, after readmission 66 days postoperatively, x-ray of the chest showed large areas of metastases. The urinary excretion of HCG rose to 320,000 IU/liter 75 days postoperatively and 2,560,000 IU 96 days postoperatively. The patient died 105 days postoperatively, and autopsy revealed large areas of metastatic tissue in the lungs, brain, liver, and kidneys. Further analyses showed that the HCG contents of several tissues, expressed as IU per gram, were: ovarian dysgerminoma, 111; lung metas-

tases, 3453; and pituitary, 0.2. Several analyses of the serum at about the eightieth postoperative day showed levels of about 990–1030 IU/ml.

Utilizing the capacity of urinary HCG to stimulate the ovulation by the female toad, *Xenopus laevis*, Hobson and Baird (1966) found that, prior to the removal of a typical dysgerminoma of the ovary from a 16-year-old girl, the 24-hour urinary excretions of HCG ranged between about 1000 and 4000 IU, averaging 1625 IU. After removal of the tumor, the urinary excretion of HCG decreased and reached an undetectable level, probably less than 20 IU, by the seventh postoperative day. Female *Xenopus laevis* do not ovulate when they are injected with follicle-stimulating hormone.

The results of these two studies indicated that the dysgerminoma and, in the first case, its metastases were the source of HCG. The question obviously arises whether the typical cell of the dysgerminoma is the sole source or whether there may not be an admixture of chorionic tissue. The sectioning of the tumor in the case reported by Hobson and Baird (1966) failed to show the presence of any cytotrophoblastic elements and indicated that it was the cells of the dysgerminoma which were secreting a luteinizing type of gonadotropic hormone. But the possibility must be considered that more extensive sectioning would have shown that the tumor was not a "pure" dysgerminoma and that HCG-secreting chorionic tissue was also present. In the case studied by Kofler and Spona (1971), histological study of the original tumor showed that, in addition to areas of typical dysgerminoma, there were also areas which partly contained dysgerminomalike tissue mixed with other structures of the germ cell type. Indeed, these areas were suggestive of embryonal carcinoma resembling the adenomatous structure of choriocarcinoma. In this case, the possibility must be considered that HCG was being produced by chorionic tissue in the ovarian tissue and later by the metastases.

3. Cystadenomas of the Ovary

Cystadenomas as a group are of biochemical interest because of the composition and nature of the cystic fluid. Cystadenomas of the ovary account for approximately 60% of all ovarian tumors and are about equally divided between the mucinous and serous types (Novak and Woodruff, 1967). Originally, the fluid in mucinous types was considered to contain chiefly "pseudomucin," but the contents were generally similar to mucin elsewhere and consisted, therefore, of mucopolysaccharides and glycoproteins. The contents of the serous cystadenomas were considered to contain chiefly serum proteins.

More detailed information about the contents have been supplied by Fisher (1954), Odin (1959), Mettler and Mäder (1971), and Ekindjian *et al.* (1972). Odin (1959) distinguished between 2 types of contents in a series of 30 mucinous ovarian cysts, termed by him as "pseudomucinous" cysts. The first type of contents was usually fluid, although sometimes extremely viscous. The contents mixed homogeneously with water. The characteristic components were neutral glycoproteins, containing galactose and fucose in approximately equal quantities. Sialic acid was present in about one-tenth of this quantity, and mannose in small or trace amounts. Serum proteins were present in varying amounts. In general, there was no basic difference between the contents of these pseudomucinous cysts and most mucous secretions of other origins.

The contents from the second type of pseudomucinous cysts as well as pseudomyxomatous material from the peritoneum and appendix as the result of ruptured ovarian cysts were elastic, water-insoluble gels. The characteristic components were also glycoproteins, but the sialic acid was higher than in the first type, and the fucose content was comparatively low. These gels contained considerable amounts of serum protein.

Serous cystadenomas contained fluids which varied greatly in appearance, but were usually watery, clear, and colorless or straw-colored (Odin, 1959). Amino acid chromatography of a few specimens showed the presence of the same amino acids as the pseudomucinous material, and, in addition, cysteic acid. Where the pseudomucinous material had galactose and fucose in approximately equal amounts as the major components of the carbohydrate, galactose and mannose were the main components in the serous material and fucose was present in trace or small amounts. Sialic acid was also present in the serous cyst material, perhaps in somewhat smaller amounts than in the pseudomucinous material. Two glycoproteins have been isolated from a gelatinous peritoneal effusion by Ekindjian *et al.* (1972), and their properties have been described in some detail.

The patterns of the proteins in a series of 51 benign and malignant cystic tumors of the ovary were studied by Mettler and Mäder (1971) by means of vertical disc electrophoresis. Benign cystic tumors (cystomas) with ciliated epithelium and malignant cystic tumors (cystic malignomas) had a higher protein content than polycystic ovaries and parovarian cysts. In general, the electrophoretic pattern in ovarian cysts resembled that of serum proteins. Albumin represented the highest fraction in all cases except in the parovarian cysts. In these, the transferrin C fraction was predominant. In 3 cases, pre-albumin, and in 2 cases, transferrin C were the only fractions of protein present.

It is possible that mucinous cystadenomas may contain stromal elements which become androgenic, and several such instances have been reported (Scully, 1963; Novak *et al.*, 1970; Federman and Scully, 1970). Novak *et al* (1970) have described the interesting case of a 21-year-old woman who became markedly virilized during the eighth month of gestation. The value for plasma testosterone was 5300 ng per 100 ml 1 day antepartum, greatly elevated above the normal values for the pregnant, 114 ± 38 ng per 100 ml, or nonpregnant adult female, 20–76 ng per 100 ml, and even much above that of normal adult males, 230–740 ng per 100 ml. The 17-ketosteroid excretion, 43 mg/gm creatinine, was substantially elevated above the normal levels for female, 3–12 mg/gm creatine, or male adults, 8–20 mg/gm creatinine. Estrogen excretion in this case, 7700 mg/gm creatinine, was much higher than the values, 7–104 mg per 24 hours for nonpregnant adult females or 8–23 mg per 24 hours for normal males, and even exceeded the values occurring in normal late pregnancy.

On the sixth postpartum day, a multilocoluated, thin-walled ovarian cyst, measuring 30 cm in diameter, was removed through the performance of a left salpingo-oophorectomy. Microscopically, the epithelial component of the tumor was diagnosed as a proliferating papillary mucinous cystadenoma without stromal invasion, possibly malignant. The stromal component of the tumor was identified as stromal cell hyperplasia with Leydig cell metaplasia. The plasma testosterone and 17-ketosteroid excretion fell to normal levels by the fourth postoperative day. The estrogen excretion also decreased to levels of 7–15 mg/gm creatinine.

4. Carcinoid Tumor (Argentaffinoma, Argentaffin Carcinoma)

Primary carcinoid of the ovary is classified by Teilum (1971) under germ cell tumors. We have already discussed carcinoid tumors at other sites in Chapter 6. Although these occur chiefly in the gastrointestinal tract, they may also develop in other areas such as the lower respiratory tract mucosa. The ovarian tumor is rare and almost always arises in benign dermoid cysts or in the potentially malignant ovarian teratoma (Kelley and Scully, 1961). The dermoid cyst and the teratoma contain various fetal tissues and elements, and it is considered that the carcinoid is usually derived from gastrointestinal or respiratory epithelium in the wall of the dermoid cyst or teratoma (Teilum, 1971). More recently, Haines (1971) reported 8 cases from his institution of which 4 arose in ovarian teratomatous cysts, 2 were metastatic from

carcinoid of the bowel, and 2 had no discernible teratomatous or extrinsic source.

It may be expected that carcinoid tumors in the ovary may show clinical and biochemical findings similar to those reported for gastrointestinal carcinoids. Because of the rarity of the tumor, little information concerning these points is available, but the case reported by Kephart *et al.* (1960) may be cited as an illustration. This 69-year-old woman complained of episodic flushing of the face, shortness of breath, and "nervousness" lasting a few seconds. Her preoperative 24-hour urinary excretion of 5-hydroxyindoleacetic acid (5-HIAA) was 10 mg, just above the normal range of 2–9 mg, but after the removal of the tumor, this excretion decreased to 4.9 mg and later to 2.4 mg. The tumor was a teratoma which showed carcinoid elements in close proximity to respiratorylike epithelium and cartilage.

D. Other Tumors of the Ovary

1. Brenner Tumor

In 1967, Novak and Woodruff estimated that about 500 cases of Brenner tumor had been reported in the literature. Several other series have since been described (Ehrlich and Roth, 1971; Silverberg, 1971). Microscopically, the Brenner tumor consists of nests or elongated strands of uniform epithelial cells, sharply demarcated from the fibromatous connective tissue surrounding these nests. The histogenesis of this tumor still appears unsettled, and various possible sources such as the ovarian surface epithelium, the ovarian hilus, and the remnants of mesonephric tubules have been explored (Novak and Woodruff 1967; Teilum 1971). Although it was originally considered that Brenner tumors displayed no endocrine effect, it was later held, particularly by Morris and Scully (1958), that the tumor cells might stimulate adjacent stromal cells to produce endocrine effects.

This latter thesis has been supported by several studies. Hamwi *et al.* (1963) reported the case of a 21-year-old woman who, at the end of the first trimester of pregnancy, began to develop signs of masculinization—excessive growth of hair on face, body, and extremities; deepening of voice, and decrease in size of breasts. Masculinization continued to develop after delivery, and she was hospitalized. The 24-hour excretions of 17-ketosteroids and 17-hydroxycorticosteroids were normal. The routine blood biochemical parameters were also found to be normal, except for a flat blood glucose tolerance curve. A right ovarian mass was re-

moved and identified as a Brenner tumor. Although no details were given, it was stated that incubation of tumor slices with progesterone resulted in the formation of testosterone only in the presence of HCG. Punnonen *et al.* (1971) reported a series of 6 cases, of which at least 4 were accompanied by increased estrogenic activities, as manifested by postmenopausal bleeding and hyperplasia of the endometrium. However, there appears to be no biochemical data to implement these clinical observations.

2. Krukenberg Tumor

The Krukenberg tumor, a rare tumor, consists of a firm solid growth, retaining the general shape of the ovary. It is usually of moderate size and is almost always bilateral (Novak and Woodruff, 1967). The microscopic picture shows considerable variation. Epithelial elements may occur as clusters of well-marked acini that have varying degrees of mucoid epithelial change. The stroma may be firm and richly cellular in some areas and edematous in others. Approximately 20% are primary, and the rest are usually secondary to gastrointestinal cancer, lodging in the ovary through direct implantation on the ovarian surface, extension, or transport through the blood or lymphatics. A small proportion exhibit estrogenic or androgenic activity.

In 1955, Turunen observed 2 cases of Krukenberg tumor in which the excretions of urinary gonadotropin and estrogens were abnormally high before removal of the tumor and decreased to normal levels after operation. The preoperative 17-ketosteroid excretion was 6.2 mg/day and decreased to levels of 1.9 and 2.6 mg/day on two occasions following operation. A few years later, Ober and his associates (1962) studied a 55-year-old woman with a metastatic tumor to the ovary from the sigmoid colon. Clinically, evidence of masculinization was present, and morphological evidence of progestational stimulation was observed. The signs and symptoms of masculinization regressed following removal of the tumor. Only sparse biochemical data were available. No preoperative studies were performed. The 17-ketosteroid excretion decreased from 13.1 mg/day on the first postoperative day to 3.5 mg/day 4 weeks later. The excretion of follicle-stimulating hormone during this period rose.

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16

Neoplasms and Ectopic Humoral Syndromes

I. Introduction*

As we have had occasion to observe in the past several chapters, the biochemical aspects of human cancer may be quite clearly perceived in the neoplasms of various endocrine glands. These neoplasms give rise to arrays of biochemical effects which are appropriately related to the hormones secreted by these glands. In addition, during the past 10–15 years, there has developed a substantial body of information showing that conventional hormones may be produced by neoplasms arising from a variety of nonendocrine tissues. The term "ectopic" or "inappropriate" has been applied to this phenomenon, and the conditions have been termed "ectopic humoral syndromes."

This area has been reviewed by several investigators, among them

* The following abbreviations are used most commonly in the present chapter: ACTH = adrenocorticotropic hormone; ADH = antidiuretic hormone; FSH = follicle-stimulating hormone; HCG = human chorionic gonadotropin; HCS = human chorionic sommatotropin; 17-KS = 17-ketosteroids; LH (ICSH) = luteinizing (interstitial cell-stimulating) hormone; M/A = ratio of melanocyte-stimulating to adrenotropic hormone activities; MSH = melanocyte-stimulating hormone; 17-OHCS = 17-hydroxycorticosteroids; PTH = parathyroid hormone; TSH = thyroid-stimulating hormone.

Lipsett (1965) and Liddle and his associates (1969). Current evidence indicates that tumors may produce any of the following different ectopic hormones: adrenocorticotropic hormone (ACTH), melanocyte-stimulating hormone (MSH), parathyroid hormone (PTH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), antidiuretic hormone (ADH), human chorionic gonadotropin (HCG), chorionic somatomammotropin (HCS), gastrin, erythropoietin, a thyroid-stimulating factor, and insulin. The ectopic production of parathyroid or parathyroidlike hormone has already been discussed in Chapter 10 (Section VII), and will not be considered here. Similarly, the possible ectopic production of insulin or insulinlike material was noted in Chapter 8 (Section VII).

There are several studies in the literature which indicate quite directly that each cell in an adult organism contains all and, indeed, the same genetic information, although this information is expressed differently in different tissues. For example, Gurdon (1968) showed that the highly specialized epithelial cell from the gut of tadpoles contained a nucleus which could be transplanted to an enucleated frog egg and was then capable of inducing a full-grown fertile egg. This shows that these cell nuclei contain a complete spectrum of genetic information and that specialization in various tissues may be considered an expression of mechanisms which remain active after "suppression" of most of the other possible functions of the cell. In a similar way, it may be considered that the genome in neoplastic cells of nonendocrine tissues become derepressed, resulting in the transcription of DNA and the synthesis of various hormones, which are repressed in normal cells. As we have already noted (Chapter 3, Section III,C,3), the production of a placental-like isoenzyme of alkaline phosphatase by bronchogenic and other neoplasms and the appearance of serum α-fetoprotein in hepatocellular cancer have also been considered as instances of the derepression of the genome in the cancer cell (Fishman *et al.*, 1968; Smith and O'Neill, 1971).

The thesis has also been submitted by Thorling (1972) that in ectopic humoral syndromes the neoplastic cells might be characterized by deranged protein synthesis and thus synthesize abnormal protein pieces or polypeptides. Although it would appear remote, the possibility exists that these polypeptides might, by pure chance, bear a structural resemblance to some of the polypeptide hormones, and thus acquire hormonal activity without being identical with the natural hormone. We shall note the extent to which this lack of identity exists in various ectopic syndromes, particularly in the case of the ectopic ACTH and HCG syndromes (Orth *et al.*, 1973; Tashjian *et al.*, 1973).

II. Syndrome Resulting from Ectopic ACTH

A. Introduction

In 1966, O'Riordian *et al.* noted that somewhat more than 100 cases of the "ectopic" ACTH syndrome had by then been reported in the English literature. By 1968, a total of about 150 cases from the world literature had been recorded. Liddle *et al.* (1969) collected a group of 104 patients with tumors and a positive bioassay or radioimmunoassay for ACTH which showed the following distribution with respect to the type of tumor and the primary site: carcinoma of lung, 52; carcinoma of pancreas, 11, thymoma, 11; carcinoma of thyroid, 2; carcinoma of liver, 2; carcinoma of prostate, 2; carcinoma of ovary, 2; pheochromocytoma, 3; and benign bronchial adenoma (including carcinoid), 5. Single cases at other sites were also present, and the primary site was uncertain in seven instances.

The typical clinical features of Cushing's syndrome are not uniformly present in patients with ectopic ACTH syndrome. In contrast to the excess pituitary ACTH and primary adrenocortical tumor varieties of Cushing's syndrome, the ectopic form occurs more frequently in men than in women. The patients frequently lack centripetal obesity and cutaneous striae. Hypokalemia, weakness, and edema are more common among patients with the ectopic syndrome than among those with other varieties of Cushing's syndrome (Liddle *et al.*, 1969).

It is, of course, important clinically to differentiate the ectopic ACTH variety of Cushing's syndrome from that present in the excess pituitary ACTH variety or in primary adrenocortical tumor. A presumptive diagnosis of the ectopic syndrome can be made on the basis of corticosteroid measurements, dexamethasone suppression tests, and plasma ACTH assays. It will be recalled from Chapter 12 (Section III,D) that, in the usual dexamethasone suppression test, the oral administration of 1 mg of dexamethasone leads to a marked decrease in the plasma levels of 17-hydroxycorticosteroids (17-OHCS) in normal subjects and in diseases other than Cushing's syndrome, but not in Cushing's syndrome, as presented by bilateral adrenocortical hyperplasia, adenoma, or carcinoma.

When much larger doses of dexamethasone, namely, 8 mg/day for 2 days, are administered, a reproducible decrease of 40% or more in urinary 17-OHCS is obtained in patients with excess pituitary ACTH (Cushing's disease). Applying this procedure to 100 patients with Cushing's syndrome, Liddle *et al.* (1969) found suppression of cortisol production in 98% of patients with Cushing's syndrome resulting from excess

pituitary ACTH, but in no patients with the syndrome resulting from adrenal tumors, and in only 6% of the cases with ectopic ACTH syndrome. In this last group, the tumors in the cases that showed suppression were usually thymomas or bronchial adenomas. Thus, the suppressibility by dexamethasone distinguishes between pituitary-dependent cortisol production, on the one hand, and adrenal or ectopic tumors, on the other hand. Assay for plasma ACTH can distinguish between these latter two. Plasma ACTH is undetectable in those cases where the cause of Cushing's syndrome is an autonomous cortisol-secreting adrenal neoplasm but is detectable and frequently present in higher than normal amounts in the ectopic ACTH syndrome. Odell (1968) has also summarized these biochemical features in the 3 types of Cushing's syndrome.

B. Biochemical Aspects

Ratcliffe *et al.* (1972) have recently noted the general biochemical aspects in a series of 17 patients with the ectopic ACTH syndrome, including 4 patients with bronchial carcinoids, 1 with thymoma and carcinoid features, 1 with medullary carcinoma of the thyroid, 10 with oat cell carcinoma of the bronchus, and 1 with undifferentiated carcinoma of the bronchus. Table 16-1 shows that the mean value for the serum potassium is lowered and that for bicarbonate is elevated, findings which definitely attest to the presence of hypokalemic alkalosis. It will be re-

TABLE 16-1

Plasma Components in Ectopic ACTH Syndrome

Plasma components	Ectopic ACTH syndrome			Normal individuals	
	No. of patients	Range	Mean ± SD	Number	Mean ± SD
Potassium (mEq/liter)	17	1.4–2.9[a]	2.2 ± 0.043	107	4.52 ± 0.45[b]
Bicarbonate (mEq/liter)	15	30–49[a]	36 ± 6.6	15	28 ± 2.7[c]
Cortisol[d] (μg/100 ml)	16	40–168[a]	77 ± 43[d]	5	11 ± 4.5[e]
ACTH (μg/100 ml)[f]	13	16–800[a]	109	—	—[g]

[a] Mean and SD calculated from data of Ratcliffe *et al.* (1972).
[b] Data from Marinis *et al.* (1947).
[c] Data from Peters *et al.* (1926).
[d] Morning sample.
[e] Based on 20 observations at 8 A.M. from 5 subjects (Williams *et al.*, 1972).
[f] At time of diagnosis of tumor.
[g] Undetectable.

called that the levels of plasma cortisol depend on the episodic secretion of this steroid and that the 8 A.M. samples reflect the peak concentration in the plasma. As shown in Table 16-1, these levels are greatly increased in the ectopic ACTH syndrome.

The concentration of ACTH in plasma is also substantially elevated in patients with the ectopic ACTH syndrome (Liddle *et al.*, 1969; Ratcliffe *et al.*, 1972), as compared with its undetectability in normal plasma. Table 16-1 shows the plasma ACTH levels at the time of diagnosis of the tumor. The distribution of these and other plasma ACTH determinations in a large series of 24 patients are shown in Fig. 16-1. The concentrations ranged between 100 and 10,000 pg/ml (10–1000 ng per 100 ml) and lay between 100 and 1000 pg/ml in 83% of samples.

A radioimmunoassay was employed by Ratcliffe *et al.* (1972) in assaying plasma and tumor ACTH. Three rabbit antisera were obtained, one by using a synthetic ACTH fragment designated as the "N-terminal" fragment, a second by using a synthetic human ACTH fragment designated as the "C-terminal" fragment, and the third with natural human ACTH, designated as "mixed." The values shown in Fig. 16-1 were obtained with both the mixed and the N-terminal antiserums, since no consistent differences were obtained between them on assay of the same sample of plasma.

The source of elevation of plasma ACTH appears to be the tumor tissue. In 2 patients dying suddenly of nonmalignant disease, the concentrations of ACTH in nontumorous lung tissue were 3.6×10^{-4} μg/gm by assay with antiserum to the C fragment and 4.5×10^{-4} μg/gm by assay with antiserum to the N fragment. The concentrations in liver were 4×10^{-4} μg/gm (C) and 3×10^{-4} μg/gm (N). In the muscle tissue from a patient with malignant disease, the concentration of ACTH was 2×10^{-4} μg/gm (C). Figure 16-2 shows that the concentrations of ACTH in the tumors were much higher, ranging from about 2×10^{-3} to 1×10^{3} μg/gm of tumor tissue by radioimmunoassay with antiserum to either the C or N fragment. These increases were also demonstrable by bioassay. These results indicate very strongly that in the ectopic ACTH syndrome the tumor is the source of ACTH.

Of striking interest is the finding that considerable ACTH activity is present in tumors not apparently associated with the ectopic ACTH syndrome (Fig. 16-3). Liddle *et al.* (1969) found that 6 of 78 unselected visceral carcinomas obtained at autopsy contained appreciable quantities of ACTH and were inclined to consider that these 6 cases represented undiagnosed examples of the ectopic ACTH syndrome. A series of 14 patients with such tumors as breast, esophogeal and gastric carcinomas, lymphoepithelioma, renal carcinoma, bronchial adeno- and

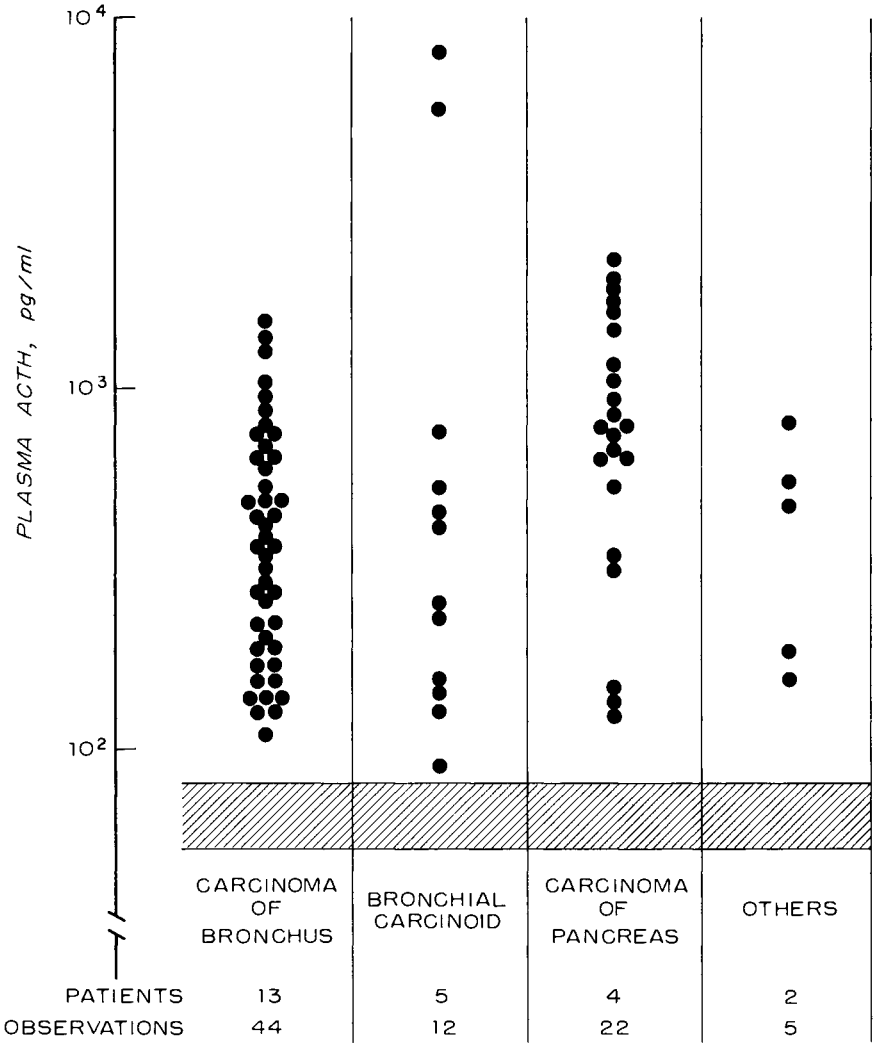

Fig. 16-1 Plasma immunoreactive ACTH concentrations related to tumor type in the ectopic ACTH syndrome. "Others" include one thymoma with carcinoid features and one medullary carcinoma of the thyroid. Hatched area indicates normal range. After Ratcliffe *et al.* (1972). Reproduced by permission of Blackwell Scientific Publications Ltd.

squamous cell carcinoma, and a pleural mesothelioma showed concentrations of ACTH ranging from about 3×10^{-4} to about 10^{-1} μg/gm tumor tissue. The values in this range overlap the lowest third of the

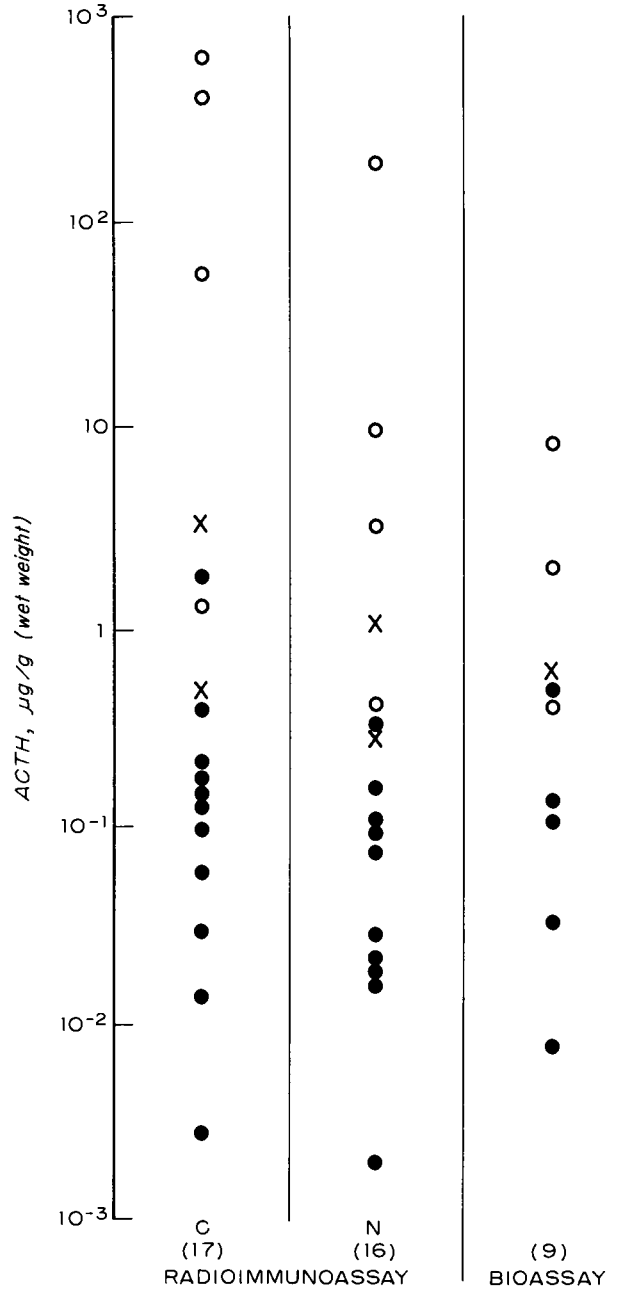

Fig. 16-2 Tumor ACTH concentrations in the ectopic ACTH syndrome: (○) bronchial carcinoids, (●) oat cell and undifferentiated carcinoma of bronchus, and (X) others (thymoma, medullary carcinoma of thyroid). C and N represent C- and N-terminal antisera used in immunoassays. After Ratcliffe *et al.* (1972). Reproduced by permission of Blackwell Scientific Publications Ltd.

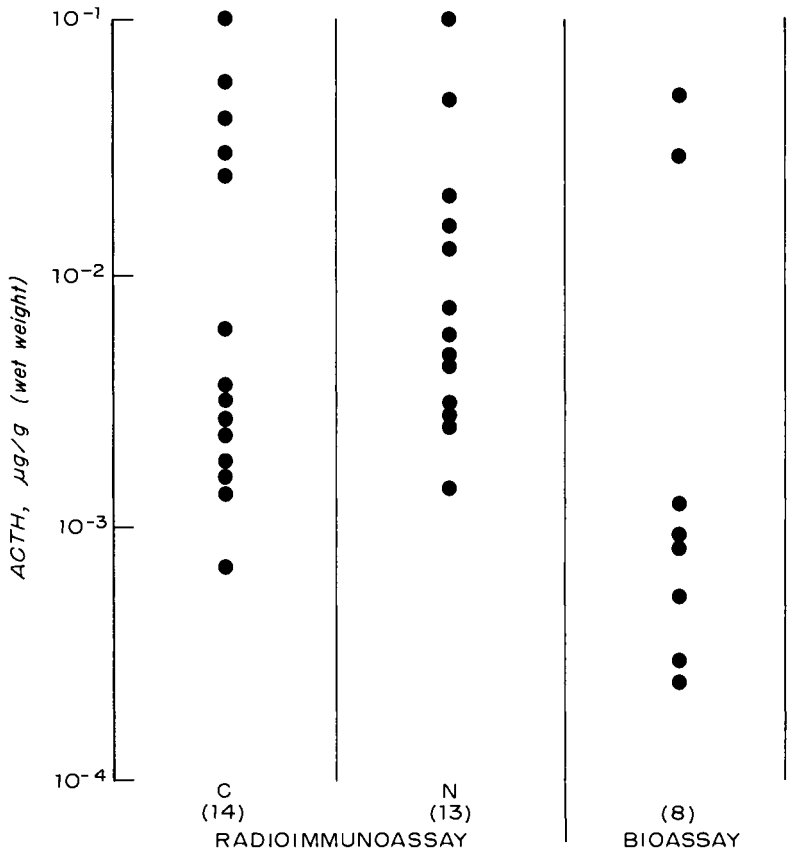

Fig. 16-3 Tumor ACTH concentrations in tumors not associated with the ectopic ACTH syndrome ("control tumors"). C and N represent C- and N-terminal antisera used in immunoassays. After Ratcliffe *et al.* (1972). Reproduced by permission of Blackwell Scientific Publications Ltd.

values in the ectopic ACTH syndrome (Fig. 16-2), and practically all of them are clearly higher than the values in nontumorous tissue. It would, therefore, appear that some tumors may contain substantial amounts of ACTH, but not to a sufficient degree to produce the ectopic ACTH syndrome (Ratcliffe *et al.*, 1972).

C. Biochemical Changes during Clinical Course of Patients with Ectopic ACTH Syndrome

The biochemical changes during the clinical course of patients with ectopic ACTH syndrome have been frequently studied (Steel *et al.*, 1967; Strott *et al.*, 1968; Jones *et al.*, 1969; Pimstone *et al.*, 1972). They

may be briefly illustrated by a case in which the ACTH was produced by a bronchial carcinoid (Jones *et al.*, 1969). A 67-year-old woman was admitted following discovery of hyperglycemia and of a lung tumor on roentgenography. During the preceding 3 months, she had gained weight and had developed typical symptoms of Cushing's syndrome. The serum sodium was slightly elevated to 149 mEq/liter; the serum potassium decreased to 2.3 mEq/liter; the chloride was slightly low, 94 mEq/liter; and the serum CO_2 content elevated to 38 mEq/liter. The blood glucose tolerance was decreased, the concentration being 430 mg per 100 ml 2 hours after administration of 75 gm glucose. The plasma 17-OHCS concentration was substantially elevated to 120 and 115 μg per 100 ml at 8 A.M. and 11 P.M., respectively. The 24-hour urinary excretions of steroids were as follows: 11-deoxycorticosteroids, 0.2 mg; total 17-OHCS, 41.5 mg; and 17-ketosteroids (17-KS), 32.0 mg. These latter two values were abnormally high.

Suitable therapy was instituted to restore her electrolyte balance, correct her hyperglycemia, and control the bronchial infection. On her ninth hospital day, administration of metyrapone in an oral dose of 250 mg every 6 hours was begun. After 5 days, metyrapone responsiveness was evident. The urinary excretions of 11-deoxycorticosteroids (11-DOCS) increased from a basal value of 0.2 to 39 mg/day, whereas the total 17-OHCS excretion increased only slightly—from about 41.5 to 49.2 mg/day. On the thirteenth hospital day, dexamethasone, 1 mg each night, was added to the metyrapone. Within 3 days, the concentrations of 11-DOCS and 17-OHCS fell to 9.5 and 15 mg/day, respectively. However, this decrease may have been more apparent than real, since metyrapone therapy was still being continued. Indeed, substantial amelioration of the patient's cushinoid features occurred during a month of combined metyrapone and dexamethasone administration. The actions of metyrapone and dexamethasone on adrenocortical steroid metabolism have been briefly discussed in Chapter 12 (Sections III,E and F). On the forty-sixth hospital day, the patient underwent surgical removal of a tumor in the left lung which, on histological examination, proved to be a bronchial adenoma of the carcinoid type.

D. Structure of Ectopic ACTH

As we have already indicated, the question arises concerning the extent to which the structure of ectopic ACTH may differ from pituitary ACTH. Certainly, there seems to be substantial evidence that in ectopic hyperparathyroidism the hormonal parathormonelike peptides are immunologi-

cally different from normal parathormone (Roof *et al.*, 1971; Riggs *et al.*, 1971). The structure of ectopic ACTH has not been fully established. Earlier studies indicated it to be essentially the same as pituitary ACTH with respect to various biological, physical, chemical, and immunological characteristics (Liddle *et al.*, 1965). Both hormones cause adrenocortical enlargement and stimulate cortisol, corticosterone, and 17-KS secretion in man. Both exert melanocyte-stimulating effect on the frog skin, *in vivo* and *in vitro*, and cause *in vitro* release of free fatty acid from adipose tissue (Liddle *et al.*, 1969). More recently, Orth *et al.* (1973) subjected extracts of tumors from 32 patients with ectopic ACTH syndrome to simultaneous bioassays and radioimmunoassays for ACTH. The three antisera used in these assays were those reacting, first, with the extreme N-terminal 1–13 amino acid sequence of ACTH, second, with the N-terminal 1–23 sequence of ACTH and, third, with the C-terminal 25–39 amino acid sequence. The results indicated that the tumors of patients with the ectopic ACTH syndrome contained an ACTH similar, if not identical, to pituitary ACTH, and, in addition, had both N-terminal and C-terminal ACTH fragments.

III. Ectopic Melanocyte-Stimulating Hormone Syndrome

The pituitary gland contains two melanocyte-stimulating hormones (MSH). The first, an α-peptide of 13 amino acids, appears to have the same sequence when isolated from pig, cow, horse, and monkey pituitaries; immunological evidence indicates that the structure is the same in humans. A second peptide, β-MSH, appears to vary in sequence and size with the species and is generally less active than α-MSH (Daughaday, 1968).

In 1963, Engel and Kahana reported the case of a patient whom they had first seen 8 years previously and who, in addition to several typical symptoms of Cushing's syndrome, also exhibited diffuse pigmentation, particularly in exposed areas of the skin. Subtotal adrenalectomy was followed by remission of Cushing's syndrome and pigmentation, but approximately 1 year later the symptoms of Cushing's syndrome reappeared, accompanied by a marked increase in pigmentation. At about this time, a large mass was removed from the mediastinum, followed by a remarkable clearing of the pigmentation. However, 3 years later, pigmentation again developed and, 1 year after that, in 1959, the patient was found to have a recurrence of the mediastinal tumor. There were few,

if any, indications of Cushing's syndrome, but there was intense pigmentation of the skin, the buccal mucous membranes, and the dorsum of the tongue. In spite of further surgical and medical measures to ameliorate her condition, she died in January 1961. Histological examination of tumor tissue from various sites showed it to be identical with that previously removed at operation from the mediastinum and similar to tumors previously diagnosed as bronchial adenoma, thymoma, and oat cell tumor. Frozen samples of plasma and tumor were saved, and several years later, Liddle and his associates (1969) showed these to contain very high concentrations of MSH. Since this first report, a number of other patients with the ectopic ACTH–MSH syndrome have been reported (Meador *et al.*, 1962; Hallwright *et al.*, 1964; Liddle *et al.*, 1965).

Evidence has accumulated that the pituitary glands, adrenocortical tumors, and tissues in ectopic ACTH syndrome secrete or contain melanotropic substances in addition to ACTH. Employing a standard preparation of α-ACTH as reference and a method involving the *in vitro* darkening of frog skin, Shimizu *et al.* (1965) performed assays of melanotropic and adrenocorticotropic activities on extracts of plasma from various groups of patients, on extracts of 3 human pituitary glands and of 15 tumors associated with the ectopic adrenocorticotropic syndrome. Table 16-2 shows that the range of the values for the melanotropic to adrenocorticotropic activity (M/A) ratio in tumor tissue from the ectopic ACTH–MSH syndrome is much higher than in the pituitary gland. Abe *et al.* (1967) found the following values for immunological β-MSH in plasma: normal humans, 0.09 ng/ml; Addison's disease, 0.5–1.1 ng/ml; Cushing's disease, 0.7–6.0 ng/ml; and ectopic ACTH syndrome, 0.9–5.1

TABLE 16-2

Ratio of M/A in Human Plasma in Various Conditions, in Pituitary Tissue, and in Tumor Tissue Associated with the Ectopic ACTH or ACTH–MSH Syndrome[a]

Nature of material from patients	No. of patients	M/A ratio	
		Mean	Range
Plasma from adrenalectomized Cushing's disease	9	25	7–78
Plasma from untreated Addison's disease	3	12	9–16
Pituitary tissue	3	14	4–23
Tumor tissue	15	202	13–960

[a] Based on data of Shimizu *et al.* (1965). Reproduced by permission of Dr. M. B. Lipsett, Editor-in-Chief, *Journal of Clinical Endocrinology and Metabolism* and The Endocrine Society.

ng/ml. The degree of clinical hyperpigmentation was well correlated with the plasma concentration of β-MSH. The concentration of β-MSH in the tumors of 11 patients with the ectopic ACTH syndrome ranged from 3 to 1600 ng and averaged 209 ng/gm of wet tumor tissue, as determined by immunoassay. These values corresponded to 25–140% of the biological MSH activity in the individual tumors. The concentrations of β-MSH in two individual pituitaries were somewhat higher, namely, 336 and 357 μg/gm of wet weight, respectively.

As is apparent from the preceding discussion, tumors that give rise to the ectopic ACTH syndrome produce not only an ACTH-like polypeptide but MSH-like polypeptides as well. Before determining the extent to which the ectopic MSH and pituitary MSH are similar, it is necessary to be able to separate MSH from ACTH. This was accomplished by Island *et al.* (1965) and Shimizu *et al.* (1965). In most tumors giving rise to the ectopic ACTH–MSH syndrome, immunoreactive β-MSH is present in sufficient amounts to account for the major portion of melanocyte-stimulating activity. Using a combination of bioassays, radioimmunoassays, and Sephadex molecular sieve fractionation, Shapiro *et al.* (1971) found three tumors in which the biological MSH activity could not be accounted for by their content of α-MSH, β-MSH, and ACTH, was physically separable from them, and showed no or incomplete cross reactivity with antibodies to them. It was concluded that the MSH in these tumors was heterogeneous and structurally different from pituitary MSH (Shapiro *et al.*, 1971).

IV. Ectopic Gonadotropic Syndrome

As the reader will recall from earlier chapters, the pituitary gland contains two hormones whose chief actions are on the gonads. The follicle-stimulating hormone (FSH) promotes follicular development in the ovary and spermatogenesis in the testis. The luteinizing hormone (LH), which is also known as the interstitial cell-stimulating hormone (ICSH), acts primarily to promote final ripening of ovarian follicles and luteinization of the ovary in the female. In the male, ICSH stimulates the Leydig cell function and the formation of testosterone. In addition, the placenta, during pregnancy and in pathological conditions such as chorionepitheliomas and hydatidiform mole (see Chapter 18), produces a gonadotropin which, in its biological actions, resembles LH (ICSH) and has little FSH-like activity. This gonadotropin is designated as human chorionic gonadotropin (HCG).

The term "ectopic gonadotropic syndrome" applies to the production of gonadotropins by neoplasms in sites other than the pituitary and trophoblastic tissues. Of interest in this connection is the occurrence in young boys of hepatic carcinomas which secrete gonadotropin and cause sexual precocity. McArthur *et al.* (1973) recently found 10 such cases in the world's literature and added 1 case of their own. Clinical evidence of sexual precocity included enlarged penis, deep voice, pubic hair, and increased skeletal musculature. In most of the patients in which the urinary 17-KS excretion was determined, this was found to be comparatively low. Biological tests in the case reported by McArthur *et al.* (1973) indicated that the ectopic gonadotropin had the properties of pituitary ICSH rather than of HCG.

Most of the cases of ectopic gonadotropic syndrome have been reported in patients with various types of lung carcinoma (Fusco and Rosen, 1966; Faiman *et al.*, 1967; Becker *et al.*, 1968; Cottrell *et al.*, 1968; Liddle *et al.*, 1969; Beck *et al.*, 1970). In practically all these instances, the ectopic gonadotropin resembled LH (ICSH) or its very similar HCG. However, Faiman *et al.* (1967) reported immunoreactive FSH in the case they studied. Beck *et al.* (1970) raised the question whether these ectopic gonadotropin-producing properties of tumors might result from trophoblastic differentiation. Accordingly, tissue from three primary and metastatic bronchial carcinomas that secreted substances with gonadotropic activity were stained by a sensitive, highly specific immunofluorescence technique capable of demonstrating human placental lactogen. However, no such lactogen was present. Since the immunofluorescence technique has demonstrated this substance to be a sensitive indicator of trophoblastic differentiation, its absence indicated that these tumors did indeed show ectopic endocrine activity.

The outstanding clinical manifestation of ectopic gonadotropinism in the adult male is gynecomastia. The clinical–biochemical correlations may be illustrated by the case reported by Becker *et al.* (1968). A 74-year-old black male was admitted with the complaints of persistent cough, loss of weight, and bilateral tenderness and enlargement of the breasts. Roentgenographic examination revealed a dense left suprahilar opacity. The sputum was positive for malignant cells. An exploratory thoracotomy showed a large nonresectable tumor involving the left hilum, descending aorta, pericardium, and left phrenic nerve. Histological examination demonstrated moderately differentiated epidermoid bronchogenic carcinoma. Postoperatively, the patient received radiation therapy and, at intervals thereafter, various forms of chemotherapy. The patient was rehospitalized about a year and a half after his initial admission and various biochemical determinations were performed at intervals

until his death in May 1966, 10 months later. The urinary excretions of estrone, estradiol, and estriol were within normal limits. The excretion of testosterone was low, particularly near the end, when it was 6.8 μg per 24 hours, as compared with a normal range of 30–200 μg per 24 hours. The excretion of 17-KS tended to be low and was definitely depressed to 3.0 mg per 24 hours several days before death. This low value was reflected in some of the 17-ketosteroids such as andosterone and etiocholanolone.

Chorionic gonadotropin is not excreted in the normal adult male, and its urinary excretion in ectopic gonadotropism is evidence of its excessive formation by the tumor. In the patient under consideration, the initial pool of urine, collected in July 1965 at the beginning of his last hospital stay contained 2300 IU of HCG per 24 hours by immunoassay and 6951 IU by bioassay. Administration of methyltestosterone, cortisone, and stilbestrol did not alter these excretions substantially. The concentration of HCG in the serum, determined at several intervals from October to December 1965, ranged from 162 to 180 IU/ml. The tumor tissue and metastases obtained at autopsy showed much higher concentrations of HCG than did the adjacent noncancerous lung. For example, the values by bioassay performed after lyophilization and ethanol extraction were, in IU/gm wet tissue: primary lung tumor, 134; metastasis, 35; and noncancerous lung, <0.5.

It has been found that FSH, LH, TSH, and HCG are heteropolymers composed of two dissimilar subunits: an alpha subunit that is nearly identical among the four hormones, and a beta subunit that confers biological specificity (Pierce, 1971). It is thus possible that there could be an unbalanced synthesis of the two subunits or faulty assembly of the subunits into the complete HCG molecule. Tashjian *et al.* (1973) have produced in culture three clones of ectopic chorionic gonadotropin-producing cells from a single human bronchogenic carcinoma. For each clone, the amount of one or the other subunit always exceeded that of the complete HCG molecule. These results reflect the variability of control of the expression of differentiated function in malignant cells and indicate the basis for the possibility that the structure of the ectopic HCG may differ from that of normal HCG.

V. Ectopic Antidiuretic Hormone Syndrome

The antidiuretic hormone (ADH) of the human posterior pituitary promotes the conservation of water by increasing the permeability to

water of the cells lining the distal convoluted tubules and collecting ducts of the nephron. Du Vigneaud (1956) identified ADH as arginine vasopressin, and elucidated the structures of this compound and the related oxytocin. Vasopressin is a nine amino acid peptide with a six-membered S—S bonded ring and a tail composed of three amino acids. Dehydration provides the usual stimulus for the release of ADH from the posterior pituitary, and the effects of ADH are decreased volume and increased concentration of urine, retention of water, expansion of total body water compartment, dilution of intracellular and extracellular electrolytes, increased glomerular filtration rate, and decreased aldosterone secretion leading to increased sodium and decreased potassium excretion (Liddle, 1968). The effects of administering ADH to normal humans on a nonrestricted fluid intake are, in general, the same and have been described in greater detail by Scheiner (1975).

The first definite report indicating the possibility of an ectopic ADH syndrome was that of Schwartz *et al.* (1957) who described 2 patients with bronchogenic carcinoma who exhibited hyponatremia, yet excreted hypertonic urine containing substantial quantities of sodium. ADH-like activity has been found in extracts of lung tumors (Amatruda *et al.*, 1963; Bartter and Schwartz, 1967), duodenal carcinoma (Lebacq and Delaere, 1965), and pancreatic carcinoma (Marks *et al.*, 1968; Vorherr *et al.*, 1968). The terms "inappropriate secretion of antidiuretic hormone" and "inappropriate ADH syndrome" have been frequently used in this connection. Bartter and Schwartz (1967) reviewed the literature of this syndrome and indicated that the ectopic or inappropriate secretion may arise not only from neoplasms but also from other diseases of the lung such as pneumonia or tuberculosis and from disorders involving the central nervous system as, for example, meningitis, head injuries, brain abscess, encephalitis, or the Guillan-Barré syndrome. However, bronchogenic carcinoma, almost always of the "oat cell" type, represents the single most common cause of the syndrome, over 50 cases having been reported by 1967.

The biochemical–clinical correlations in the ectopic ADH syndrome may be briefly illustrated in a case described by Marks *et al.* (1968). A 59-year-old white man entered the hospital with complaints of generalized weakness, low back pain, nausea, vomiting, headache, and confusion. The liver was enlarged slightly to 2 cm below the right costal margin, and chest films revealed a small area of patchy infiltration in the upper lobe of the left lung and nodular lesions in both hilar regions. The fasting blood sugar was elevated slightly to 137 mg per 100 ml. The concentrations of serum sodium and choride were low, namely, 114 and 80 mEq/liter, respectively; the serum potassium, phosphorus,

and carbon dioxide content were normal. The serum osmality was very low, 243 as compared with a normal value of about 310 milliosmoles. Removal of the primary lung tumor did not ameliorate the hyponatremia or the sodium-losing state. During the first postoperative week, glycosuria appeared and the blood sugar level rose to 236 mg per 100 ml.

An autopsy 4 months later, metastatic lung cancer was found in the liver and a primary adenocarcinoma in the pancreas. Apparently, this latter tumor was the source of ADH, for acetone extracts made from it contained very high levels of arginine vasopressin (AVP) activity, 380 mU, equivalent to 0.95 μg AVP per mg acetone powder, as compared with values of less than 0.001 μg/mg for comparable extracts from normal pancreas, the primary oat cell lung carcinoma or its metastases, normal muscle or normal lung. The normal whole pituitary contained an activity equal to 0.04 μg/mg acetone powder.

The extent to which tumors that give rise to the ectopic ADH syndrome contain AVP is illustrated by the work of Vorherr *et al.* (1968). Acetone powders, prepared from 9 bronchogenic carcinomas and 1 pancreatic adenocarcinoma in 10 patients with the classic manifestations of the ectopic ADH syndrome, were assayed. Five tumors and/or their metastases showed practically no AVP activity, that is, less than 10–20 μU/mg powder by bioassay. The other 5 had activities ranging from 47 to 763 μU/mg tumor powder. The values obtained by immunoassay were comparable. As noted above, Marks (1968) reported a value almost one-thousandfold, namely, 380 mU/mg, for the activity of the extract from a pancreatic carcinoma in a patient with the ectopic ADH syndrome.

VI. Ectopic Thyrotropin Syndrome

There have been a number of reports in the literature that patients with hydatid moles had laboratory evidence of increased thyroid function. This was first observed by Tisné *et al.* (1955) in 4 patients. Kupperman and Epstein (1958) reported the case of a patient with a hydatid mole whose increased thyroid function returned to normal upon delivery of the mole. Additional evidence of the relationship between trophoblastic tissue and hyperthyroidism is afforded by the case described in detail by Myers (1961) of a 34-year-old woman with advanced metastatic choriocarcinoma. Her 24-hour urinary excretion of chorionic gonadotropin was greatly elevated to 6–8 million IU. The following biochemical values indicated the presence of hyperthyroidism: protein-bound iodine (PBI), 15.8 and 16.7 μg per 100 ml, as compared with a normal value of 6.2 ± 2.6 μg per 100 ml; 24-hour neck uptake, as fraction of ^{131}I tracer dose, 62.5% as compared with the then normal range of 20–40%;

and serum total cholesterol, 134 mg per 100 ml, as compared with a normal range of 140–260 mg per 100 ml. Treatment of the patient with several courses of amethopterin resulted in a marked decrease of urinary chorionic gonadotropin to 60,000 IU/24 hours and to a restoration of thyroid function to normal or even subnormal levels: PBI, 3.1 μg per 100 ml; 24-hour neck uptake, 14% of the dose; and serum cholesterol, 179 mg per 100 ml.

We have so far seen that ectopic humoral syndromes may originate from a variety of neoplasms in different organs. The question arises whether the close association of thyrotropic (TSH) effects and trophoblastic neoplasms constitutes an unusual form of ectopic syndrome or whether TSH-like material is a normal product of trophoblastic tissue and is secreted in excess in trophoblastic neoplasms. Gonadotropic hormone, estrogens, and placental lactogen also appear to be elaborated in such instances (Odell *et al.*, 1967). Using chromatographic techniques, Hennen (1965) was able to purify from human placenta a chorionic-thyroid-stimulating hormone (HCTSH). Immunological studies disclosed a rather close but not complete relationship between human pituitary TSH and HCTSH.

In view of the preceding considerations, it appears preferable to avoid the use of the term "ectopic TSH syndrome" and discuss instead the effects of TSH in trophoblastic neoplasms. In 1967, Odell and his associates summarized the evidence for these in a series of 11 patients, consisting of 8 women with choriocarcinoma, 2 with hydatid moles, and 1 male with testicular teratoma having trophoblastic elements. In general, these patients showed few or no clinical symptoms of hyperthyroidism. Tachycardia was present in all, but anemia and other factors may have accounted for this. A widened pulse pressure and minor skin changes were present in about half of the patients. Other signs were present in one or two patients each. However, the laboratory evidence was much more definite. Table 16-3 shows that the 24-hour gonatotropin excretion and the PBI were elevated, some to very high levels, in each instance. The radioiodine uptake was increased, and the basal metabolism rate (BMR) was substantially elevated in all except 1 patient. Plasma TSH levels were elevated by bioassay but not by immunoassay. This may be accounted for by the assumption that the trophoblastic cell elaborates a material with TSH-like activity which differs immunologically from pituitary TSH. In 2 patients, extracts of the tumor showed substantial TSH-like activity, namely, 40 and 300 mIU per 100 gm wet weight. Large amounts of chorionic gonadotropin from normal trophoblastic tissue showed no intrinsic TSH-like activity, even though bioassayed by three different methods (Odell *et al.*, 1967).

VII. Ectopic Syndromes Resulting from Human Chorionic Somatomammotropin, Human Placental Lactogen, and Human Growth Hormone

In 1962, Josimovich and MacLaren discovered that the peripheral and retroplacental sera of pregnant women at term and their placentas contained a substance which was not only closely related immunochemically to human pituitary growth hormone but was also highly lactogenic as determined both in pigeon crop assays and in milk-promoting activity in the pseudopregnant rabbit. It was designated as human placental lactogen (HPL), but because of its growth promoting qualities, it was also called chorionic growth hormone-prolactin (Grumbach *et al.*, 1968) and human chorionic somatomammotropin (HCS) (Li *et al.*, 1968). This hormone was subsequently obtained in a highly purified form; it was shown to be a protein of a molecular weight of about 20,000 with a single polypeptide chain and to have certain amino acid sequences similar to that of human pituitary growth hormone (Li *et al.*, 1968). In Chapter 17, we shall consider whether this preparation is a single hormone possessing both growth and lactogenic properties or whether it

TABLE 16-3

Evidence for Production of TSH Activity in Patients with Trophoblastic Tumors[a]

Laboratory parameter	Values in patients with tumors	Normal values
Gonadotropin excretion (IU/24 hours)[b]	2–10 × 10^5	<20
PBI (μg/ml)	9.2–20.0	4.0–8.0
24-Hour thyroidal radioiodine uptake (%)	46–88	15–45[c]
BMR	11–75	−10 to +10
Plasma TSH bioassay (IU/100 ml)[b]	1.0–2.2[d]	0.3
Tumor TSH bioassay (IU/100 gm wet weight)[b]	4.0–30.0	—
Plasma TSH immunoassay (pg/ml)	<3.0[e]	<3.0

[a] From Odell *et al.* (1967) with modifications. Reproduced by permission of Hoeber Medical Division (Harper and Row).

[b] These values have been converted from mouse uterine units (MUU) to international units on the basis that 10 MUU equals 1 IU.

[c] These are values given by Odell *et al.* (1967). As was pointed out in Table 13-1, more recent values for normal uptake are lower.

[d] Four patients.

[e] Three patients.

is a mixture of two hormones, each having its own characteristic properties.

Human growth hormone (HGH), either of pituitary or chorionic origin, is not included among the list of ectopic hormones produced by neoplasms (Lipsett *et al.*, 1964; Liddle *et al.*, 1969; Greenberg *et al.*, 1964). However, during the past 20 years, there have appeared reports on the occasional association of bronchial carcinoma with acromegaly and osteoarthropathy. A number of investigators have also commented on the dramatic relief from arthralgia and tissue changes, such as finger clubbing and periosteal proliferation, following removal of a pulmonary tumor (Steiner *et al.*, 1968). In 1964, Bariéty *et al.* reported that, in a series of 250 cases of bronchial cancer, 45 had an "osteoarticular syndrome"; 34 cases had finger clubbing only, 9 had painful arthropathy, and 4 had complete osteoarthropathy.

Impressed by the clinical similarity between acromegaly and generalized osteoarthropathy, Steiner *et al.* (1968) determined the plasma growth hormone in a series of 12 patients: 8 with tumors, some of them with finger clubbing or osteoarthropathy; 3 without cancer but with polyarthritis or related conditions; and 1 with acromegaly. All except 2 patients were within the normal range, namely, 3.7 ± 1.7 (SD) ng/ml for men and 5.09 ± 3.9 (SD) ng/ml in females. As might be expected, the patient with acromegaly had a very high plasma level of HGH, more than 100 ng/ml. One of these patients was a 57-year-old man with a pulmonary neoplasm in the upper left lobe, severe skeletal pains, definite acromegaloid traits, and significant clubbing of the fingers and toes. The plasmsa HGH levels were elevated preoperatively to 38 and 23 ng/ml in two separate determinations. After removal of the lung tumor, the subjective joint and leg pains and the stiffening and swelling of the fingers disappeared. The plasma HGH level was now 3.5 ng/ml.

As might be anticipated, the production of HCS and its levels in the plasma would be increased in trophoblastic tumors such as choriocarcinomas of the testis or ovary. Of interest are the findings in patients with tissue-proved malignancies not originating in trophoblastic or gonadal tissue. Weintraub and Rosen (1971) surveyed 128 patients falling into this class. The radioimmunoassay for HCS showed no cross reaction with HGH and minimal cross reaction with human chorionic gonadotropic (HCG) hormone; HCS was found in the plasma from 11 patients and in tumor from 4 of these. When unconcentrated HCS was used in the assay, the plasma levels, expressed as ng/ml, were as follows: normal <1.0; two undifferentiated lung tumors, 14 and 9; epidermoid lung tumor, 2.0; hepatoma, 1.9; hepatoblastoma, 2.8; lymphoma, 1.9; and pheochromocytoma, 1.5. In 4 patients with lung tumors, the use of concentrated HCS in the assay gave plasma values ranging

from 0.007 to 0.250 ng/ml. The normal level was <0.002 ng/ml. Human chorionic somatomammotropin was detected in tumor extracts from 4 cases with undifferentiated large cell carcinomas of the lung, and these patients also had evidence for ectopic HCG production.

VIII. Ectopic Gastrin (Zollinger-Ellison) Syndrome

In 1955, Zollinger and Ellison described a syndrome in which gastric hypersecretion and peptic ulceration were associated with a noninsulin-secreting pancreatic adenoma of islet-cell type. Two-hundred and sixty cases of this syndrome had been reported by 1964 (Ellison and Wilson, 1964) and over 600 by 1969 (Thompson *et al.*, 1972). In our discussion of neoplasms of the gastrointestinal tract, liver, and pancreas (Chapter 8), we considered some of the aspects of the Zollinger-Ellison syndrome (Section V,C,4). It is appropriate to extend this discussion in the present context.

Zollinger and Ellison (1955) had suggested that the tumor secreted a stimulant of gastric secretion into the circulation. Several years later, R. A. Gregory *et al.* (1960) showed that the pancreatic tumor of a patient with the Zollinger-Ellison syndrome did indeed contain a gastrin-like substance, as determined by bioassay. In 1964, R. A. Gregory and Tracy culminated many years' work on gastrin by isolating from hog antral mucosa two heptadecapeptides which were highly potent stimulants of gastric acid secretion. These were of identical amino acid constitution, except that gastrin II had a sulfate ester group on a tyrosyl residue and a terminal amide group (H. Gregory *et al.*, 1964). A pair of peptides of closely similar constitution and identical physiological properties was soon isolated from human antral mucosa (R. A. Gregory *et al.*, 1966). Structural and synthetic studies revealed that these differed from the peptides isolated from hog mucosa only in that the methionine residue in position 5 was replaced by leucine (Bentley *et al.*, 1966). Gastrin II was the tyrosine sulfate ester of gastrin I. That these gastrins could be produced by the pancreatic tumor in the Zollinger-Ellison syndrome was demonstrated by R. A. Gregory and his associates (1967) who isolated a mixture of two closely related substances that stimulated gastric acid secretion and had the same amino acid composition as human gastric gastrin. Employing a sensitive radioimmunoassay for gastrin which they developed, McGuigan and Trudeau (1968) showed that 4 patients with the Zollinger-Ellison syndrome contained markedly elevated levels of serum gastrin, tenfold or greater than the normal level of 425 ± 136 (SD) pg/ml. Gastrin was identified immunochemically in the extract of a pancreatic islet-cell tumor from 1 patient at a level of 1.05 μg/gm of tumor tissue.

Liddle *et al.* (1969) have noted that the ectopic gastrin syndrome appears to be the only type of ectopic syndrome in which the neoplasm is resident in only one organ, namely, the islets of Langerhans. It is thus possible that the gastrin is produced by a rare type of islet cell which is the prototype of the gastrin-secreting neoplasms of the patients with the Zollinger-Ellison syndrome.

The mortality in patients with the Zollinger-Ellison syndrome who have been treated without surgery is 73%, as compared with 47% in patients treated by subtotal gastric resection, and only 13% in those subjected to primary total gastrectomy (Wilson and Ellison, 1966). The problem of early diagnosis is, therefore, an important one, and the development and use of a sensitive radioimmunoassay for gastrin has permitted definitive diagnoses to be made much earlier. For example, during a period of 4 years, Thompson *et al.* (1972) studied the serum of 508 patients, 92 of whom were originally suspected of having the Zollinger-Ellison syndrome. Of 15 who were definitely proved to have it, 10 had serum gastrin values higher than 1000 pg/ml; 2 had values between 500 and 1000 pg/ml; 1 had a value between 250 pg/ml, the upper limit of normal, and 500 pg/ml; 1 had levels that were occasionally below 250 pg/ml; and the remaining patient had values consistently less than 250 pg/ml.

As with other serum biochemical parameters in disease, their use as indicators of specific disease processes must take into account the possibility of complicating factors. Korman *et al.* (1972) have pointed out that patients with renal failure may show high levels of serum gastrin. Thus, in 29 patients with normal renal function and a serum creatinine less than 1.2 mg per 100 ml, the serum gastrin was 38 ± 8.9 (SE) pg/ml. In 34 patients with mild renal impairment and serum creatine values ranging from 1.2 to 3.0 mg per 100 ml, the serum gastrin was 51 ± 9.1 (SE) pg/ml. In a group of patients with severe renal impairment and serum creatinine concentrations greater than 3.0 mg per 100 ml, the serum gastrin was 220 ± 80 (SE) pg/ml. Indeed, 2 of the patients in this last group had very severe renal impairment and serum gastrin levels greater than 1500 pg/ml. Incidentally, it may be noted that the normal values and range for serum gastrin obtained by Korman *et al.* (1972) were lower than those employed by Thompson *et al.* (1972). The occurrence of elevated levels of serum gastrin in renal impairment indicates that the kidney may play a metabolic and excretory function in the disposal of gastrin.

High serum gastrin levels are also found in pernicious anemia. The mean fasting value in a series of 21 patients was 1036 ± 215 (SE) pg/ml, as compared with a range of 0–120 pg/ml in 60 normal subjects (Hansky

et al., 1971). Three factors have been considered as possibly accounting for such elevations: overproduction of gastrin by enterochromaffin cells in the gastric mucosa of patients with pernicious anemia, decreased utilization of gastrin associated with the loss of parietal cells, and lack of inhibition of gastrin release.

IX. Ectopic Erythropoietin Syndrome

As has been appreciated for many years, erythropoietin is a substance which is present in the plasma and is involved in the formation, maturation, and release of blood cells into the blood stream. It appears to be a mucoprotein and to have a molecular weight of approximately 28,000. It exists in a free and a bound form, with about one-fourth being in the free form, and the remainder being bound to various proteins. Most of the available evidence indicates that erythropoietin is usually formed in the kidney, but that, under some conditions, may be formed elsewhere (Wintrobe, 1969; Miale, 1972). Although its chief action is considered to be on the stem cell, it has been suggested that erythropoietin may also produce an increase in the absolute number of normoblasts in the bone marrow, may stimulate hemoglobin synthesis in existing normoblasts, or may cause the release of cells from the marrow.

The most recent and comprehensive literature survey of the ectopic erythropoietic syndrome is that of Thorling (1972). The various types of tumors and the number of each were listed as follows: renal carcinoma, 118; miscellaneous renal tumors, 12; hepatocellular carcinoma including one harmatoma, 64; cerebellar vascular tumor, 50; and uterine fibroma, 23. A few cases of each of the following neoplasms have also been recorded: prostatic carcinoma, gastric cancer, pheochromocytoma, ovarian carcinoma, and bronchogenic carcinoma. It may be recognized that other reviewers of the literature have used alternate criteria and obtained different distributions of the incidence of the various types of the erythropoietic syndrome. Thorling's views (1972) are indicated in a quotation from Herodotus in the introduction to his monograph: "I am obliged to report what I have heard, but I don't have to believe it."

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17

Neoplasms of the Breast

I. Introduction*

Carcinoma of the breast is the most common form of cancer occurring in white women over 40 years of age and, indeed, accounts for about one-fifth of all cancers in women (Ackerman and del Regato, 1970). The number of deaths from this tumor in white women in the United States in 1967 was 25,589, or 21.0% of all white female deaths from all types of cancer. The corresponding number in nonwhite women was 2,193, or 16% of all nonwhite female deaths from all types of cancer (Segi and Kurihara, 1972). It has been estimated that, in 1973, the incidence of breast cancer in the United States would be 73,600, or about 11.7% of the incidence of cancer at all sites, and that the number of deaths would be 32,650, or 9.4% of deaths from all types of cancer in both sexes (Silverberg and Holleb, 1973). It should be noted that breast cancer also occurs in males, albeit very rarely. In 1967, 209 white and 32 nonwhite males died from this cause in the United States. The death rates for carcinoma of the breast vary greatly with geography. Thus, in the United States, the rate in 1966–1967 was 28.71 per 100,000 white

* The following abbreviations are used most commonly in the present chapter: ACTH = adrenocorticotropic hormone; DHEA = dehydroepiandrosterone; HGH = human growth hormone; HPL = human placental lactogen; HPRL = human prolactin; MMT = mouse mammary tumor; MMTV = mouse mammary tumor virus; 17-OHCS = 17-hydroxycorticosteroids; PIF = prolactin-inhibiting factor; PRF = prolactin-releasing factor; TRH = thyrotropin-releasing hormone. The systematic names of the various steroids referred to in this chapter are listed in Chapter 12, Table 12–1.

women, similar to the rates in the countries of Western Europe. In contrast, the rate in Japan was 4.16 (Segi and Kurihara, 1972).

Although the normal biochemistry of the breast has received little attention in the usual texts on biochemistry, the magnitude and importance of the associated oncological problems have stimulated many biochemical approaches. We shall discuss these presently. Neoplasms of the breast have been classified in several ways as, for example, morphologically (McDivitt *et al.*, 1968) and by extent and frequency of metastases (Ackerman and del Regato, 1970). The degree to which the various types play a role in the biochemical aspects of breast cancer will also be considered presently.

II. Steroid Metabolism

A. Estrogen Metabolism

The distribution and metabolism of estrogen in the patient with breast neoplasm has been of interest since the early studies of Twombly *et al.* (1948) and of Albert *et al.* (1949). The tissue distribution of radioactive estrogen and metabolites in postmenopausal women with breast neoplasms may be illustrated by the study of Demetriou *et al.* (1964). Twenty minutes after the end of a 10-minute intravenous infusion of 680 μg [4-^{14}C]estradiol-17β, the mean values for radioactive estrogen in the tissues of 24 women with breast carcinoma and 6 with benign disease of the breast, expressed as nanograms per gram of tissue, were as follows: normal mammary tissue, 7.4; body fat, 6.8; malignant tumor, 13.3; benign tumor, 4.5; muscle, 6.0; and epidermis, 9.0. The concentration in the malignant tumor was significantly higher than that in mammary tissue or body fat. The relative concentrations of radioactivity of estradiol, estrone, and E_p, an uncharacterized fraction, varied from tissue to tissue and for a single tissue, from patient to patient but, on the average, expressed as percent of total radioactivity, were 76%, 13%, and 13%, respectively.

Some aspects of the dynamics of metabolism and tissue uptake of estrogen in women with advanced cancer were explored by Pearlman *et al.* (1969). Nine patients were studied. [6,7-^{3}H]Estrone in nine experiments and [6,7-^{3}H]estradiol-17β in one experiment were infused intravenously at a constant rate. Clearance of estrogen from plasma was much slower in 2 patients who had extensive metastases to the liver. In these patients, the uptake in the noncancerous breast tissue and adipose tissues did not differ significantly from that in cancerous breast tissue. In another

patient, the uptakes in the carcinoma and in a metastatic lymph node were clearly much higher than those in other tissues. The experiments as a whole were too few in number to yield any definite answer about the relative uptakes of estrogen in the normal and carcinomatous breast tissue.

More extensive data concerning this problem, particularly with respect to growth rate or hormonal dependence of the tumor, have been presented by Braunsberg *et al.* (1971). Tritium-labeled estradiol was infused for a period of 3–6 hours just before and during surgery. The tissue–plasma ratio of free steroid radioactivity in 21 patients with carcinoma of the breast ranged from about 1 to 10, with 14 of the carcinomas showing levels greater than 2. In contrast, primary carcinomas of the colon, rectum, stomach, bladder, and ovary as well as normal muscle, breast, and other tissues had a ratio of about 1. Adipose tissue had ratios ranging between 1 and 2. One specimen of normal liver had a high value of 10. In most of the tumors analyzed for free extractable radioactivity, the proportion of the estradiol moiety was predominant, and the estrone fraction was less than 10%. There was no significant correlation between the tissue–plasma ratio of free steroid radioactivity and either DNA or hydroxyproline, indicating that there was no dependence on the number of cells present or the proportion of connective tissue.

It is naturally relevant to determine whether the patient with breast cancer metabolizes estrogen in a different manner from that of the normal woman. Hellman *et al.* (1971), among others, have addressed themselves to this question. A group of 23 women with breast cancer, of varying age, menstrual status, and presence or absence of metastases, were each given an intravenous tracer dose of [^{3}H]estradiol, and the urine was collected for 3 days. The fraction of administered radioactivity that was conjugated and hence present in the glucosiduronate extract was 41% in the normal controls and essentially the same as the fraction, 43%, in the patient group.

The extent of formation of estriol from the administered [^{3}H]estradiol, expressed as a fraction of the glucosiduronate extract in the female patients with breast cancer, was 20%, significantly higher ($p < 0.001$), than the fraction, 12%, in the group of 12 control patients. Eleven of the 23 cancer patients had values greater than the upper 95% confidence limit of normal. The extent of formation in 6 men with breast cancer averaged 37% (Zumoff *et al.*, 1966). The mean value for endogenous estriol glucosiduronate excretion in 10 female breast cancer patients was 5.3 μg/day and was more than twice the mean value, 2.5 μg/day, in 5 normal control subjects. The difference between the groups was not statistically different. Nor was there any significant difference between

the mean value, 9.6 μg/day, for the endogenous estrone glucosiduronate urinary excretion in the breast cancer patients and that, 6.3 μg/day, for this excretion in the normal group. However, occurrence of several values for both the estrone and estriol glucosiduronate excretions in the cancer patients that were much higher than the upper limit of normal indicates that some patients with breast cancer may form significantly greater than normal quantities of estriol and estrone from radioactive estradiol tracers or from endogenous precursors.

The adrenocorticotropin (ACTH)-stimulated estradiol production rate in a series of 8 premenopausal women with breast cancer ranged from 107 to 350 μg/day (Barlow *et al.*, 1969) and was reduced to 10–49% of these levels after ovariectomy. In postmenopausal women with breast cancer, the ACTH-stimulated estradiol production rate was somewhat lower, ranging from 47 to 122 μg/day, and there was no consistent reduction after ovariectomy (Barlow *et al.*, 1969). These results indicate that, in women with breast cancer, postmenopausal ovaries make no contribution to estrogen production. This consideration, as well as a review of the clinical literature, raises the question whether therapeutic oopherectomy without adrenalectomy is of benefit in postmenopausal women with breast cancer.

That the urinary excretion of total and individual estrogens may increase significantly in a group of ovariectomized breast cancer patients about 8–10 days postoperatively in response to the intramuscular injection of 50 mg testosterone propionate was demonstrated by Sharoukhova *et al.* (1972). The injection of larger doses, 500–3000 mg, of testosterone propionate led to still further increases in the total and individual estrogens. The ratio of estriol, E_3, to the sum of estrone, E_1, and estradiol, E_2, decreased from a preinjection value of 1.4 to one of 1.1 after 500 mg testosterone propionate, to 0.75 after 1000–3000 mg, and to 0.45 after doses greater than 3000 mg.

B. Testosterone and Other Steroid Metabolism

1. Sulfurylation of Dehydroepiandrosterone

The conjugation of steroids with sulfate is an important metabolic step and is mediated by sulfate-activating enzymes and steroid sulfokinases (or sulfotransferases). Sulfokinase, which mediates the formation of ester-sulfate derivatives of steroids, occurs in normal human liver and adrenal gland tissue and in extracts of human primary and secondary breast carcinomas (Adams, 1964). In contrast, the normal breast tissue surrounding the tumors reveals no activity or, in some instances, only

very weak activity. Normal ovarian tissue, obtained from cancer patients undergoing oophorectomy, did not generally possess this enzyme activity, although trace levels were detected in about 20% of the specimens examined (Adams, 1964). Adams and Wong (1968) undertook to explore some metabolic steroid reactions in carcinomatous breast tissue. Incubation of labeled dehydroepiandrosterone (DHEA) (5-androstene-3β-ol-17-one) with minced specimens from several cases of breast carcinoma resulted in the formation of about 0.7–1.3% androstenedione (4-androsten-3,17-dione) and indicated the presence of a Δ^5-3β-hydroxysteroid dehydrogenase -isomerase system in breast carcinoma tissue, a system which effects the oxidation of the OH group at C-3, and the shift of the double bond from C-5 to C-6 to the position at C-4 to C-5. The same system was indicated when incubation with pregnenolone (4-pregnen-3β-ol-20-one) yielded progesterone (4-pregnen-3,20-dione). An "α-triol," formed to the extent of 1.2–6.8% from the incubation with DHEA, was identified as androst-5-ene-3β,16α,17β-triol which may be an intermediate in the "direct" pathway to estriol.

The finding that steroid sulfatase was present in homogenates of breast carcinoma tissues, together with the previously demonstrated occurrence of sulfokinases in this tissue (Adams, 1964), indicated that sulfated forms of steroids may act as intermediates in some of the conversions that we have described above. A summary of these, as formulated by Adams and Wong (1968), is shown in Fig. 17-1.

These observations have been confirmed and extended by Dao and Libby (1968). Aliquots of supernatants, obtained by centrifuging a 0.25

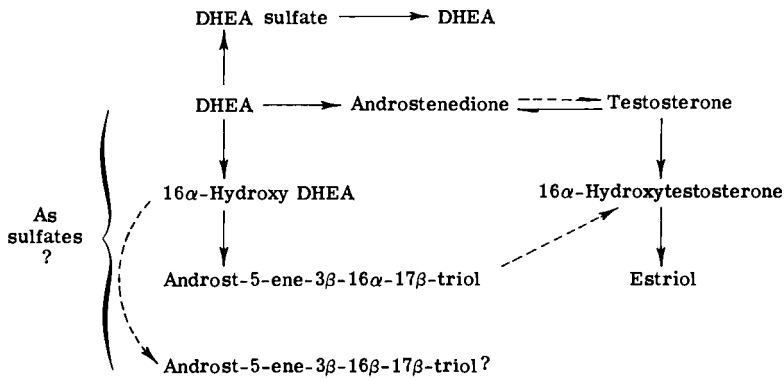

Fig. 17-1 Summary of steroid conversions demonstrated *in vitro* with breast carcinoma tissues. Solid lines indicate reactions which have been proved and broken lines indicate possible reactions. From Adams and Wong (1968). Reproduced by permission of the Cambridge University Press.

M sucrose tissue homogenate at 12,000 *g* for 15 minutes, were incubated for 1 hour at 37°C in a mixture containing Tris buffer, pH 7.4, ATP, $MgCl_2$ labeled $Na_2{}^{35}SO_4$, and steroid. Sulfokinase activity was expressed as picamoles of steroid sulfate formed per milligram protein per hour. Dehydroepiandrosterone, pregnenolone, deoxycorticosterone, corticosterone, 19-nortestosterone, and estradiol-17β were used as substrates. In mammary cancer tissues from 11 patients who had no evidence of distant metastases and underwent mastectomy, the activities varied widely. For example, the rates of synthesis of DHEA sulfate ranged from zero in 3 cases to 28.6 pmoles/mg protein per hour in the most active case. These 3 cases also failed to show sulfokinase activity with the other 5 steroid substrates, but the remaining 8 cases had activities that, in general, were of the same order of magnitude as that obtained with DHEA. In biopsy specimens of normal mammary gland tissue obtained from 4 patients, the sulfokinase activity for all 6 steroids was not measurable in 3 patients and very low in the other. This finding confirms and extends the earlier observation by Adams (1964). As was to be expected from an organ that possesses much conjugating activity for many compounds, the sulfokinse activity of normal liver was considerable for all 6 steroids tested. For example, with DHEA as substrate, the activity in specimens from 13 subjects ranged from 25 to 384 and averaged 168 pmoles/mg protein per hour. It was of interest that, in 3 cases, metastases to the liver from carcinoma of the breast showed rates of sulfate synthesis of the same or even higher order of magnitude than normal liver, rather than resembling the lower rates of carcinomatous breast tissue.

The following sequence of sulfate-activating enzymes is necessary for the formation of steroid sulfates: (a) the interaction of ATP and inorganic sulfate, mediated by sulfate adenyltransferase, to form 3-phosphoadenyl sulfate (APS) and inorganic pyrophosphate; (b) the interaction of APS and ATP, mediated by the enzyme adenylsulfate kinase, to form adenosine 3′-phosphate 5′-sulfatophosphate (PAPS) and ADP (White *et al.*, 1973; Libby and Dao, 1970). The 3′-phosphate 5′-sulfatophosphate, which may be considered as "active sulfate," then interacts with the steroid to form the steroid sulfate; this step is mediated by a sulfokinase (or sulfotransferase). Lack of any one of these three enzymes would result in the failure of steroid sulfate formation. We have already noted that a small proportion of breast carcinoma preparations were inactive and such preparations were indeed found to be lacking the two enzymes, sulfate adenyltransferase and adenylsulfate kinase, though having measurable amounts of 3β-hydroxysteroid sulfotransferase and estrone sulfotransferase (Libby and Dao, 1970).

The capacity of breast carcinomatous tissue to sulfurylate steroids has been proposed by Dao and Libby (1971) as a criterion for predicting whether patients with metastatic breast carcinoma will respond successfully to bilateral adrenalectomy. In a series of 79 postmenopausal women with disseminated breast cancer, 27 had no sulfokinase activity in the neoplastic breast tissue. All 27 were stated to have failed to respond to adrenalectomy. In those patients with sulfokinase activity present in the neoplastic breast tissue, the incidence of favorable response (objective remission) appeared to depend on the ratio of the rate of synthesis of DHEA sulfate to that of estradiol-17β sulfate (Table 17-1). Of the 25 patients whose breast tumors were enzymically active and showed ratios of DHEA sulfate to estradiol sulfate synthesis equal to or greater than unity, 18, or 72%, were alive at periods ranging from about 7 to 20 months postoperatively. Of 54 patients whose tumors either lacked enzyme activity or sulfurylated estradiol more efficiently than DHEA, only 8, or 12%, were alive at periods ranging from about 7 to 19 months postoperatively.

In another group of 92 patients undergoing mastectomy for primary carcinoma of the breast without distant metastases, studies of the steroid-sulfurylating activity and of the ratios of rates of DHEA sulfate to estradiol sulfate synthesis in breast carcinoma tissue also suggested that such assays might have a prognostic significance.

2. Blood Plasma Levels of Dehydroepiandrosterone and Androsterone Sulfates

Employing a method involving gas–liquid chromatography, Wang *et al.* (1968) determined concentrations of these steroids in the plasma

TABLE 17-1

Rate of Synthesis of Steroid Sulfates by Carcinomatous Breast Tissue and Its Relationship to Response of Patient to Bilaterial Adrenalectomy[a]

No. of patients	Sulfokinase activity in tumor	Ratio of rates of synthesis of sulfates of DHEA:estradiol-17β	Response to adrenalectomy	
			Remission	Failure
27	None	—	0	27
21	+	>1	17	4
4	+	1	1	3
27	+	<1	3	24

[a] From Dao and Libby (1971).

of normal men and women. Very little DHEA sulfate was found to be present in the plasma before the age of 7 in either sex. At about this age, there was a marked increase in the plasma level, with a peak of about 100–200 μg per 100 ml for men and women being attained at about the third decade, and a decline setting in thereafter to a level of about 50 μg per 100 ml. Wang *et al.* (1968) found it possible to fit polynomial equations to these data and linear regression equations to the portion above the age of 20. It should be noted that these concentrations of plasma DHEA sulfate are much higher than those of *free* plasma DHEA. It will be recalled (Chapter 12, Section II,E,3) that the latter is secreted episodically and may range from a maximal concentration of 0.6 μg per 100 ml at 7 A.M. to a nadir of about 0.2 μg per 100 ml at approximately 7:30 P.M.

A similar method of determination for plasma androsterone sulfate was developed by Wang *et al.* (1968). The semilogarithmic plots of concentration against age were similar in form to those obtained for plasma DHEA. A peak value of about 50 μg per 100 ml was attained at about the third decade.

It has been suggested that DHEA sulfate is the principal C_{19} steroid synthesized by the adrenal cortex (Wieland *et al.*, 1963). Brownsey *et al.* (1972) have recently obtained the following mean values, expressed as the logarithms of the concentration in micrograms per 100 ml plasma, in tumors of the breast: 2.04 ± 0.22 in 34 women with benign neoplasms; 1.92 ± 0.28 in 23 women with primary carcinoma; and 1.77 ± 0.41 in 26 women with advanced, metastatic carcinoma. The mean age was about 50 years in each of these groups. There were no statistically significant differences between the benign and the primary groups or between the primary and advanced groups. However, the difference between the benign and advanced groups was significant. When the logarithmic values were converted into the usual terms of micrograms per 100 ml plasma, the mean values for DHEA sulfate in the 3 groups were: benign, 110; primary, 83 and advanced, 59.

C. Urinary Excretion of Steriods

1. Development of a Discriminant Function in Metastatic Carcinoma of the Breast

About 1960, Bulbrook, Hayward, and their associates inaugurated a series of studies on the urinary excretion of steroids in patients with metastatic carcinoma of the breast with a view to determining whether the urinary excretion of various steroids could serve as a guide to the

effects of ablative hormonal therapy such as hypophysectomy, oophorectomy, or adrenalectomy (Bulbrook *et al.*, 1960, 1962a,b; Hayward *et al.*, 1961; Atkins *et al.*, 1968). The response to treatment was measured by a method devised by Walpole and Paterson (1949) and consisted in determining and averaging growth or regression of the malignant lesions at various intervals after operation. Those patients showing growth of lesions were considered to be therapeutic "failures."

In an earlier study, Hayward *et al.* (1961) found no significant differences between the "regression" and the "failure" groups with respect to the urinary daily excretion of any of the following: total 17-ketosteroids, total estrogen, estrone, estriol, DHEA, androsterone, 11-oxy-17-ketosteroids, pregnanediol, and gonadotropin. In contrast, the mean daily excretion of 17-hydroxycorticosteroids (17-OHCS) in the regression group was 7.1 mg, significantly less ($p < 0.05$) than the mean value, 11.0 mg, for the excretion in the failure group. Similarly, the mean daily excretion of etiocholanolone in the regression group was 805 μg, significantly higher than the mean excretion, 543 μg, in the failure group. In order to utilize more incisively the differences between the excretions of 17-OHCS and etiocholanolone in patients of the 2 groups, the use of a discriminant function was invoked (Bulbrook *et al.*, 1960). The equation was

$$80 - 80\,(\text{daily excretion of 17-OHCS in mg}) + \text{daily excretion of etiocholanolone in } \mu\text{g}$$

Because of the various complications in some cases such as the presence of diabetes insipidus, metastases to the pituitary, or other conditions, they were excluded from calculation. Figure 17-2 shows the results of calculating the values of the discriminant for each of 59 patients and correlating these values with the clinical outcome after adrenalectomy or hypophysectomy. Later studies by Bulbrook *et al.* (1962b) considered the correlation between the value of the discriminant and age in normal women and in women with breast carcinoma.

In 1968, Atkins *et al.* reviewed their 10 years' experience with the use of the discriminant in 206 patients with metastatic breast cancer. This group included a subgroup in which the decision to perform an adrenalectomy or hypophysectomy was made randomly, another subgroup of patients with a positive discriminant who were assigned to hypophysectomy, those with a negative discriminant to adrenalectomy, and other groups in which the reverse allocation was made.

Table 17-2 shows a total of 206 patients, and the correlation of the nature of the discriminant to the clinical response following adrenalectomy or hypophysectomy. Of the patients with positive discriminants who were treated with hypophysectomy, 47% had successful outcomes,

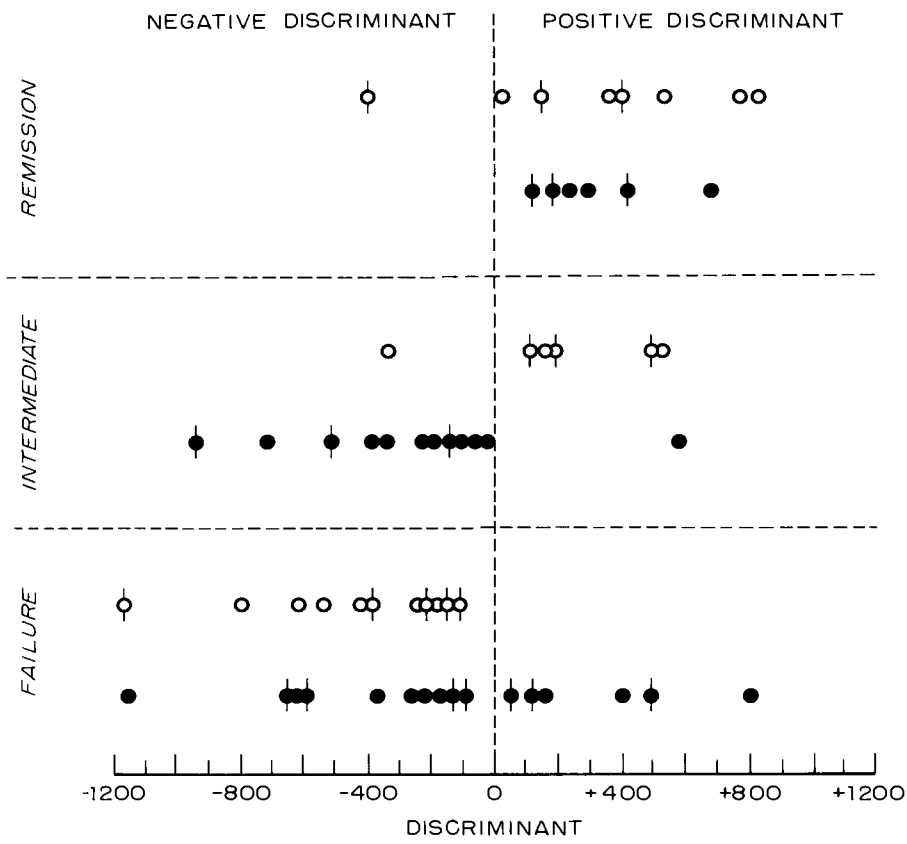

Fig. 17-2 Correlation between the discriminant (based on 17-OHCS and etiocholanolone levels) and the actual clinical results of adrenalectomy or hypophysectomy. (○) Hypophysectomy and (●) adrenalectomy. The discriminant was calculated from data on the cases plotted without vertical lines through the points. An additional series (see text) is shown as circles with vertical lines through them. From Bulbrook *et al.* (1960). Reproduced by permission of The Lancet Limited.

whereas 23% were failures. Conversely, of those with negative discriminants, only 11% were successes and 67% were failures. The differences were highly significant. In the patients who were subjected to adrenalectomy, differences existed between the percentage of responses in those with positive and negative values for the discriminant, but none of these differences was statistically significant.

Several other factors appeared to affect the relationship between the positivity or negativity of the discriminant and the response to operative endocrine ablation. These included previous mastectomy; the period

TABLE 17-2

Response of Patients with Positive or Negative Discriminants to Adrenalectomy or Hypophysectomy[a]

Group	No.	Success (Remission) No.	Success (Remission) Percent	Intermediate No.	Intermediate Percent	Failure No.	Failure Percent
A. Response to hypophysectomy							
Positive discriminants	47	22	47	14	30	11	23
Negative discriminants	46	5	11	10	22	31	67
B. Response to adrenalectomy							
Positive discriminants	57	18	32	11	19	28	49
Negative discriminants	56	7	12.5	18	32	31	55

[a] Based on data of Atkins *et al.* (1968).

between the time of mastectomy and the first recurrence of disease, usually designated as the "free period"; and the length of the postmenopausal period.

The review of Atkins *et al.* (1968) indicates that the Bulbrook discriminant can predict with some degree of probability the success or failure of endocrine surgery in a group of patients. However, it is not so exclusive as to be able to prognosticate in any definite way the result for an individual patient. As was noted, such clinical factors as the free period and the duration of the postmenopausal state may also play a role in the success or failure or endocrine surgery. Armitage *et al.* (1969) examined the role of other variables by means of a computer program. In the following sections, we shall consider the application of the Bulbrook discriminant in early cancer of the breast and in prospective studies.

2. The Discriminant in Early Cancer of the Breast

It would obviously be of value to elicit, in patients with early breast cancer, that is, with operable lesions confined to the breast and axilla, some guide to the desirability of performing ablative endocrine surgery subsequent to mastectomy. Bulbrook *et al.* (1962b) reported that over half of a group of 40 women with early breast cancer had negative discriminants, and the remainder had positive discriminants. In a total of 71 mastectomized patients, by the end of 8 years, nearly 3 times more patients had died in the negative discriminant group than in the positive

(Hayward and Bulbrook, 1968). Wade *et al.* (1969) found that patients under the age of 55 with early breast cancer tend to have discriminants that are lower than in normal women but that in older women the reverse tended to occur. However, these differences were not statistically significant. A control group of 33 women who had surgical operations for conditions other than cancer of the breast or for benign mammary dysplasia gave mean values at any given age which did not differ significantly from those of normal women or of women with early breast cancer. In general, the standard errors of the estimates of the mean discriminants in the 3 groups studied by Wade *et al.* (1969), namely, healthy controls, operation controls, and early breast cancer, were quite large. No support was obtained for the view that the discriminant was specifically affected in early breast cancer and could, therefore, have any predictive value.

3. Prospective Studies of Breast Cancer

In 1961, a prospective study was set up in Guernsey, one of the Channel Islands off England, to determine whether the measurement of urinary androgen and corticosteroid metabolites, including 17-OHCS and etiocholanolone, routinely performed in a population of approximately 5000 healthy women volunteers, aged 35–55 years, might reveal abnormal steroid excretions and thus herald the appearance of breast cancer (Bulbrook and Hayward, 1967; Bulbrook, 1970). When a diagnosis of breast cancer was made, up to 10 normal women, matched for age, weight, menopausal status, etc., were selected as a control group. A random serial number was taken from the population of volunteers, and the normal woman so identified was termed a "pseudocancer" patient. A group of controls was also selected for this patient.

The method of analysis was based on the deviations of 17-OHCS and etiocholanolone values from their respective group means. This method may be illustrated by Case 2 of Bulbrook and Hayward (1967). The mean value and standard deviations of the excretions of 17-OHCS and etiocholanolone in 6 women, including the cancer patient and her 5 controls, were 7.24 ± 2.57 (SD) and 1.30 ± 0.462 (SD) mg per 24 hours, respectively. The weighted deviation, d_x, for 17-OHCS, which is represented in the formula $d_x = (x - \bar{x})/S_x$ (the difference between the mean and the individual values in milligrams divided by the standard deviation in milligrams), was calculated for each control and the cancer patient. It had a value of -1.17 without dimensions for the cancer patient. A similar calculation yielded a value of -1.55 for etiocholanolone in the cancer patient.

The results obtained in these studies (Bulbrook and Hayward, 1967; Bulbrook, 1970) could be depicted in any of several ways. For example, to illustrate one of these, the weighted deviations of the individual 17-OHCS and etiocholanolone were plotted as coordinates. This gave a scatter of points about the axes in which most of the control values tended to occupy the central areas, and in which the majority of the values for the cancer patients tended to occupy the periphery. In other words, the majority of the women who subsequently developed manifest cancer of the breast had excreted amounts of 17-OHCS and etiocholanolone that differed significantly from those found in the urine of the majority of controls.

By the end of 1970, the Guernsey prospective study had yielded 27 cases of breast cancer (Bulbrook *et al.*, 1971). Of these, 21 had shown urinary excretion values for etiocholanolone below the median value of 1.2 mg per 24 hours in the matched controls. The urinary excretions of 17-OHCS were distributed more evenly throughout the normal range. Table 17-3 shows the mean values for these steroid excretions, expressed as log micrograms per 24 hours, as well as for androsterone. The mean value for the excretion of dehydroepiandrosterone (DHEA) for the precancer cases did not differ significantly from those of the controls or

TABLE 17-3

Urinary Excretion of Steroids in Women in Prospective Study Who Subsequently Developed Cancer (Precancer Cases), in Matched Controls and in Normal Population[a]

Group	No.	Mean and standard deviation, expressed as log μg/24 hours, of urinary excretion of steroids[b]		
		17-OHCS	Etiocholanolone	Androsterone
Precancer cases	27	1.82 ± 0.16	2.91 ± 0.28[c,d]	2.95 ± 0.25[e,d]
Matched controls	187	1.84 ± 0.15	3.05 ± 0.24	3.09 ± 0.28
Normal population	1506	1.81 ± 0.17[f]	3.12 ± 0.26	3.15 ± 0.27

[a] Data from Bulbrook *et al.* (1971). Reproduced by permission of The Lancet Limited.

[b] Logarithms of values for excretions were used since these gave a normal distribution.

[c] Significantly lower than matched controls at $p = <0.01$.

[d] Significantly lower than normal population at $p < 0.001$.

[e] Significantly lower than matched controls at $p = <0.05$.

[f] This value was based on 4941 cases.

normal population. In contrast, the mean values for urinary excretions of androsterone and of etiocholanolone were significantly lower in the precancer cases than in the matched controls or in the general population.

It should be noted particularly that the subnormal excretions of etiocholanolone occurred, in general, long before the diagnosis of breast cancer was evident. Indeed, the mean time between the collection of the urine specimen and diagnosis was 44 months; the shortest time was 5 months and the longest almost 9 years. Eight of the 21 women developed cancer of the breast 5–9 years after urine analysis, and 7 of these had exhibited low etiocholanolone excretions at that time.

4. The Incremental Ratio Discriminant

Moore and his associates (1968) have used a discriminant based on adrenal function during ACTH secretion. Two days of resting secretion were compared with 2 days under stimulation by 40 units of ACTH given intravenously per day over 6–8 hours. The increments in urinary excretions of 17-OHCS and 17-ketosteroids evoked by ACTH were then expressed as fractions of the resting values and were incorporated into the discriminant, D, in the following expression:

$$D = -4.5 + 0.44\ (\text{17-OH IR}) + 0.73\ (\text{17-K IR}) + 0.037\ \text{FI}$$

where 17-OH IR is equal to the incremental ratio for urinary 17-OHCS, corrected for creatinine, based on the excretions during 48-hour resting and 48-hour stimulated periods; 17-K IR is the value obtained in the same manner for urinary 17-ketosteroids; and FI is the free interval, that is, the interval in months between treatment for primary breast carcinoma and the local recurrence of tumor or the appearance of metastases. A positive value for D is correlated with a favorable response and a negative value with palliative failure. In one series, Moore *et al.* (1968) found that the discriminant identified 12 out of 15, or 80%, of the responders, and 12 out of 16, or 75%, of the nonresponders.

5. Urinary Estrogens in Breast Cancer

Adlercreutz *et al.* (1967) reported that in 3 normal women, followed throughout the course of the menstrual cycle, a good correlation existed between the urinary excretion of estrogen and that of androgen metabolites. The possibility has been raised by Bulbrook (1970) that low androgen excretions in patients with cancer of the breast might also accompany low urinary excretions of estrogens. In general, the data concerning the excretion of estrogens are conflicting. For example, Persson and Risholm

(1964) reported that patients with metastatic breast carcinoma excreted significantly larger quantities of estriol than healthy women of comparable age, whether pre- or postmenopausal, but that the excretion of estrone and estradiol was essentially the same in the cancer patients as in healthy women. On the other hand, Marmorston *et al.* (1965) found that the mean excretion of estradiol by patients with breast cancer was significantly lower than the level excreted by well, control subjects, whereas differences in the excretion of "estriol" and "total estrogen" were not significant. Lemon *et al.* (1966) observed that estriol excretion is generally reduced. Using an expression which they termed the estrogen quotient (Eq), namely,

$$\frac{\text{estriol}}{\text{estrone} + \text{estradiol}} = \text{Eq}$$

all expressed as micrograms excreted in 24 hours, these investigators found that 21% of 34 women without cancer and, in contrast, 62% of 26 women with cancer, had values lower than that of the median estrogen quotient.

D. Receptors in Carcinoma of the Breast

1. Introduction

During the past several decades, a substantial body of knowledge has developed to the effect that various groups of substances such as drugs, chemical neurotransmittors, peptide and steroid hormones act by virtue of combining in very small amounts with certain receptive sites on protein molecules in the target organs and thereby initiating a series of cellular events characteristic of these substances (Hammes *et al.*, 1973). Many hormones combine with receptors associated with membranes, and the interactions with these have been studied recently (Tanford, 1973; Pardee, 1973; Rodbell, 1973). The binding and fate of sex steroids have claimed particular attention during the past decade (E. V. Jensen and Jacobsen, 1962; Baulieu *et al.*, 1971). In general, available evidence indicates that, in target tissues, steroid hormones are bound by cytoplastmic receptors of high affinity and specificity and that "the steroid receptor complex moves to the nucleus where physiological changes are initiated via a modification of genetic expression" (Thomas, 1973).

The principal characteristics of the interaction of estrogenic hormones with responsive tissues *in vivo* were elucidated chiefly by studies on the incorporation and retention of radioactive steroid, mainly in the

uterus, after the administration of physiological amounts of various steroids to rats (E. V. Jensen *et al.*, 1968). Although we shall discuss this aspect more fully in Chapter 18, it may be noted here briefly that, in the rat, the uterus shows a striking affinity for estradiol, 17α-methyl estradiol, 17α-ethinyl estradiol and hexestrol, and a transient affinity for estriol. Estrone and mestranol [1,3,5(10)-estratrien-17α-ethinyl-3, 17β-diol 3-methylether] do not bind to target tissue, but they probably exert an estrogenic effect because of their metabolic conversion to estradiol and 17α-ethinyl estradiol and the accumulation of the latter in the target organs (Jensen *et al.*, 1971).

2. *In Vivo* Interaction of Estradiol and Human Breast Tissue

Since 1964, a number of investigators have shown that human breast tumors can accumulate higher concentrations of intravenously injected labeled estradiol-17β than surrounding normal tissues (Demetriou *et al.*, 1964; Deshpande *et al.*, 1967). Deshpande *et al.* (1967) studied a group of 23 women with breast cancer, injecting tritiated estradiol at various times, 9–595 minutes, preceding mastectomy. Both the neoplastic breast tissue and the surrounding normal breast tissue accumulated and retained estradiol for up to 5 hours. Estradiol accounted for about 70–100% of the total radioactivity in both the normal and neoplastic breast tissue. The concentration of labeled estradiol accumulated in the carcinomatous breast tissue was about 3 times that present in the normal breast tissue. Dromastanolone (2α-methyl-5α-androstan-17β-ol-3-one) propionate, which is closely related to testosterone (4-androsten-17β-ol--3-one), inhibited the uptake of estradiol by the tumor, but had no apparent effect on the uptake by normal breast tissue.

3. *In Vitro* Assays for Estradiol Receptors in Carcinoma of the Breast

The uptake of estradiol by breast tissue and, more broadly, of estrogens by other target tissues such as the uterus bespeaks the possibility of demonstrating this interaction *in vitro*. Indeed, a number of such studies, utilizing different procedures, are now available (Braunsberg and James, 1967; Sander, 1968; Johansson *et al.*, 1970; Feherty *et al.*, 1971; E. V. Jensen *et al.*, 1971; Wittliff *et al.*, 1971).

A few of these may be illustrated briefly. Tumor, freed of surrounding tissue and fatty, necrotic and hemorrhagic areas, was sliced and 3 groups were incubated for 1 hour at 37°C in Krebs-Ringer phosphate buffer

containing bovine albumin and glucose with various combinations of radioactive estradiol-17β and unlabeled estradiol-17β (Johansson *et al.*, 1970). The incubation medium for the first group of slices contained only 2×10^{-9} *M* [^{3}H]estradiol-17β; the medium for the second contained this concentration and 2×10^{-7} *M* of the enantiomer of the unlabeled estradiol-17β. The medium for the third group of slices contained the low concentration of the labeled and the high concentration, 2×10^{-7} *M*, of unlabeled estradiol-17β. The enantiomer of estradiol-17β is a considerably less potent estrogen and also has a very low affinity for estrogen receptors. The binding of estradiol-17β, which was suppressed by the nonlabeled carrier but not by the nonlabeled enantiomer, was considered specific. After incubation, the tritium content of each tissue slice was measured individually, and was expressed as dpm per mg wet weight. The mean $\pm$ SE was calculated for each group of slices from a particular tumor.

For any given tumor, the difference in concentrations between groups I and III was considered as the most reliable measure of the receptor-bound estradiol in the tumor. The difference between groups II and III was an indicator of the stereospecificity of the estradiol-17β binding, and followed the value of the difference, I to III. Upon statistical treatment of the data, Johansson *et al.* (1970) found that 14 of 31 cancers of the breast, or 35%, and 2 of 26 benign tumor showed significant binding.

Another procedure involved the homogenization of carefully trimmed tissue, centrifugation at 1000 *g* for 15 minutes and incubation of this supernatant with varying concentration of [^{3}H]estradiol under conditions yielding maximum formation of bound estradiol (Feherty *et al.*, 1971). Dextran-charcoal suspension was then added to each sample to absorb the free estradiol, and the incubation was continued for an additional 10 minutes at 30°C. The reacting mixture was collected, centrifuged, and an aliquot of the resulting supernatant was counted.

Using this procedure, Feherty *et al.* (1971) found that 37 of 53 breast carcinoma biopsies, or 70%, contained high affinity estradiol receptors, ranging in concentration from 0.3 to 22.6×10^{-15} mole/mg of tissue. The magnitude of these bindings could not be related to any histological feature or to cellularity of the biopsies. Only 3 of 41 benign breast biopsies contained any high-affinity estradiol receptor; the concentrations were low, ranging from 0.3 to 0.6×10^{-15} mole/mg of tissue. Normal breast tissue did not contain any detectable estradiol receptors.

E. V. Jensen and his associates (1971) incubated trimmed normal and neoplastic breast tissue slices in Krebs- Ringer-Hensleit glucose buffer with [6,7-^{3}H]estradiol and an estrogen antagonist such as

nafoxidine. Slices were removed from the incubation at various times and combusted to convert the tritiated hormone to tritiated water, which was counted.

The results on incubation of the slices from primary breast cancer revealed two patterns: (a) those showing a low uptake, about 1000 dpm/mg dry tissue, which was not depressed in the presence of nafoxidine or other inhibitor; (b) those showing a substantially larger incorporation, about 3000 dpm/mg dry tissue, which was reduced by the inhibitor. These were termed "negative" and "positive" patterns, respectively. Metastatic carcinomas of the breast revealed similar positive and negative patterns. Of 51 patients with primary carcinoma of the breast, 24, or 47%, had positive patterns; 25 had negative patterns; and 2 had borderline values.

A cytosol prepared by freezing, shattering, and pulverizing tissue in liquid nitrogen, homogenizing the resultant powder in Tris-HCl buffer, and centrifuging the homogenate at 105,000 *g* for 30 minutes was employed by Wittliff *et al.* (1971). Aliquots of tissue cytosols were incubated with Tris-HCl buffer or with buffer containing inhibitor and then added to a reaction vial containing tritiated estradiol-17β. A suitable portion of this reaction mixture was layered onto a cold, linear gradient of sucrose (10–30%) that also contained buffer and was centrifuged for 12 hours at 308,000 *g*. Each gradient was fractionated by the usual procedure of puncturing the bottom of the tube and collecting a suitable number of drops into a scintillation vial. Since each tumor cytosol was measured in the presence and absence of the specific competitive inhibitor, CN-55,945-27, the specific binding was considered to be the difference in radioactivity bound only in the 9S region of these gradients, and was expressed as femtomoles (fmoles or 10^{-15} mole) estradiol-17β bound per milligram protein.

Of 75 malignant breast tissues, 29, or 39%, were positive, with an average binding capacity of 43.0 ± 5.3 (SE) fmoles/mg protein and a range of 10.3–137.6 fmoles/mg protein. An additional 10 carcinomas were considered borderline, with an average binding capacity of 5.6 ± 0.4 (SE) fmoles/mg protein and a range of 3.3–7.4 fmoles/mg protein. Thirty-six, or 49%, of the 75 malignant tissues were negative, having binding capacities less than 2 fmoles/mg protein. In contrast, of 22 nonmalignant and normal samples of breast, only one showed a low but positive binding, namely, 9.4 fmoles/mg protein. The remainder were all negative.

The preceding reports indicate that a substantial proportion of human mammary carcinomas, 35–70%, depending on the method used and the particular group studied, contain specific high-affinity estradiol receptors

which are not detected in normal breast tissue or in the large majority of benign lesions. The variability of estrogen receptor concentration in the malignant tumors may result from the effect of endogenous hormone and receptor sites that are already occupied or possibly from the histological character of the tissue (Feherty *et al.*, 1971).

4. Estrogen Binding as an Aid in Prognosis of Operative or Other Therapeutic Procedures in Cancer of the Breast

As we have seen in an earlier section, substantial efforts have been made to utilize the excretion of various steroids, notably 17-OHCS and etiocholanolone, as a prognosticator of the probable success or failure of operative procedures, such as adrenalectomy or hypophysectomy, in affecting the clinical course of patients with metastatic breast cancer.

Only a few studies have so far been done to correlate the results of *in vitro* estrogen binding with the subsequent course of disease. E. V. Jensen *et al.* (1971) reported a series of 33 patients with metastatic carcinoma of the breast, 28 of whom were subjected to adrenalectomy, with 7 also having oophorectomy at the same time, and 1 having subsequent hypophysectomy. Three of the 33 patients had hypophysectomy, 1 had radiation castration, and 1 was treated with depo-testosterone. Of the 19 patients in this group who had a negative receptor pattern, 17 failed to respond, 1 died in the immediate postoperative period, and 1 had a remission. Of 11 patients with positive patterns, 5 had significant remissions, 2 were failures, and 4 did not survive the immediate postoperative period. Of 3 patients with borderline patterns, 2 were failures and 1 was a surgical casualty.

The preceding results indicate that some correlation may exist between a positive pattern (presence of specific estrogen receptors) and favorable clinical response followng ablative endocrine therapy. Conversely, a negative pattern may be correlated with a failure to respond. Savlov *et al.* (1974) have recently presented some preliminary data also supporting this view. Of 5 patients undergoing adrenalectomy, 2 without cytoplasmic 8S receptors failed to respond. Three patients with a positive pattern had objective and subjective responses of more than 6 months. Of 5 patients receiving additive endocrine therapy, 4 with tumor lacking cytoplasmic 8S estrogen receptors failed to respond. The remaining patient, whose tumor contained the receptor, responded.

Obviously, there is a paucity of data in this area, and additional cases are being collected in order to determine more precisely the extent to which the presence of estrogen receptors will indicate a favorable re-

sponse to ablative hormonal therapy in patients with metastatic carcinoma of the breast (Savlov *et al.*, 1974).

E. In Vitro Sensitivity of Breast Cancer Tissue to Hormones

Earlier in this chapter, we discussed the urinary excretion of steroids, particularly the combination of 17-OHCS and etiocholanolone in a discriminant function, as a means of predicting the response of patients with advanced breast cancer to hormonal ablation. We noted in the summary study of Atkins *et al.* (1968) on 206 patients that this approach provided only approximate predictions. For example, 47% of patients with positive discriminants responded favorably to hypophysectomy, while 30% had an intermediate or equivocal response, and 23% were associated with failure.

Within the past few years, Hobbs and his associates (Salih *et al.*, 1972a,b; Flax *et al.*, 1973) have explored the *in vitro* sensitivity of breast cancer tissue slices to various hormones as a possible screening method for predicting the response to ablative hormonal therapy. The procedure may be briefly described (Salih *et al.*, 1972a). Thin slices were obtained aseptically at operation. Some were chilled in *n*-hexane at —70°C, and others were maintained for 24 hours in Trowell's T_8 medium alone or in the medium containing various concentrations of estradiol-17β or other hormone. After the maintenance period, the tissues were chilled quickly in *n*-hexane at —70°C, and 8 μm sections were cut from cultured and uncultured specimens in a cryostat. The sections were stained with hematoxylin and eosin for histological study, and duplicates were treated for determination of the total dehydrogenase activity of the pentose-phosphate shunt by the reduction of neotetrazolium chloride to highly colored formazan.

Histological examination of breast cancer tissue showed that maintenance of the specimen in only control medium for 24 hours led to substantial deterioration of the tissue, as compared with the tissue taken at operation. The presence of 10^{-6} *M* estradiol-17β in the medium prevented this deterioration to some degree, and a concentration of 10^{-5} *M* was still more effective. The histochemical determination for dehydrogenase activity yielded parallel results. Moderate activity was evident in the tissue taken at operation. Maintenance in the medium for 24 hours without hormone led to a substantial decrease in this activity. Incubation in the presence of 10^{-6} *M* estradiol-17β prevented the decrease to some extent. In the presence of 10^{-5} *M* estradiol-17β, very high dehydrogenase activity was elicited. In general, a tumor was considered to be dependent

only when the total dehydrogenase activity of the pentose shunt pathway was greater than in both the fresh-frozen uncultured biopsy specimen and the biopsy specimen cultured in medium alone without added hormones (Flax *et al.*, 1973).

Potentially, this *in vitro* technique provides a very direct method for determining hormone dependency. For example, biopsy tissues from 7 cases of carcinoma of the breast maintained in T_S medium containing 10^{-5} *M* estradiol-17β showed high dehydrogenase activity and an improved histology when compared with the uncultured tissues. When the estrogen was absent from the medium, 5 of these tissues failed to survive and 2 survived poorly, as determined by the histological and histochemical criteria described above. The 7 tumors from which these tissues came could be described as estrogen-dependent. In contrast, tissues from 7 other tumors showed estrogen-independency. When the slices were maintained in the absence of estrogen, 4 of the 7 showed deterioration in their histological appearance, 2 showed the same appearance, and 1 revealed improvement. The dehydrogenase activity did not alter in 6 of the cases, and one actually showed an improvement. When the slices were incubated in the presence of estrogen, neither the histological appearance nor the dehydrogenase activity improved as compared to the controls. Indeed, there was a worsening of both these parameters (Salih *et al.*, 1972a).

This type of analysis has been extended to determine the range of dependency of carcinoma of the breast on other hormones (Salih *et al.*, 1972b; Hobbs *et al.*, 1973; Flax *et al.*, 1973). Table 17-4 summarizes the results on 130 patients who had biopsies of their primary or meta-

TABLE 17-4

***In Vitro* Hormonal Dependence of 130 Patients with Breast Cancer**[a]

Group	No. of patients	Percent
Independent	62	48
Dependent	68	52
Prolactin only	19	15
Estradiol only	14	11
Testosterone only	14	11
Prolactin and estradiol	13	10
Prolactin and testosterone	8	6

[a] After Flax *et al.* (1973). Reproduced by permission of The Lancet Limited.

static tumors tested in this manner. The proportion of hormone-dependent tumors was 52%, and the various types of hormonal dependency are also listed. The series has been enlarged to 192 patients, and a recent report (J. R. Hobbs, personal communication, 1973) has yielded essentially the same results, 43% independent and 57% dependent. Of the 57% dependent, the distribution was as follows: prolactin only, 17%; estradiol only, 8%; testosterone only, 9%; prolactin and estradiol, 13%; and prolactin and testosterone, 10%.

The extent of correlation between *in vitro* steroid dependence and clinical progress on subsequent corrective therapy has been actively pursued. Of the 14 cases reported by Salih *et al.* (1972a), 7 showed *in vitro* estrogen dependence. Five of these 7 received antiestrogen therapy consisting either of drugs, radiation, or hormonal ablation, and only 2 or possibly 3 of these showed remission. Two of the 7 patients whose tissues showed *in vitro* estrogen-dependence did well without any treatment. Of the 7 cases whose *in vitro* test showed estrogen-independence and who received either no therapy, radiotherapy, or chemotherapy, 5 did well. Both Stoll (1972b) and Brewin (1972) have pointed out that, in the entire series of 14, the clinical progress was followed in only 8 patients who had received endocrine therapy. Stoll (1972b) noted that there was evidence of clinical remission in 2 of the 5 cases where the tumor showed estrogen-dependence and in 1 of the 3 cases where the tumors did not show any estrogen-dependence. Both Stoll (1972b) and Brewin (1972) considered the number of cases too few and the modalities of endocrine manipulation too diverse to substantiate the suggestion by Salih *et al.* (1972a) that the results available up to that time warranted a correlation between *in vitro* dependency tests and clinical results.

We have already noted (Table 17-4) that, of 130 patients with breast carcinoma, *in vitro* studies of their carcinomatous breast tissue showed that 14 were testosterone-dependent. Antiandrogen measures were successful in 6 of 8 patients. However, these results were reported within several months after institution of therapy (Flax *et al.*, 1973). A more extensive correlation has been recently described by J. R. Hobbs (personal communication, 1973). Where appropriate therapy was given as indicated by *in vitro* dependency to estrogen, androgen, or prolactin, an objective response was obtained in 20 of 22 patients. When these groups of patients were given the hormones to which biopsy had shown dependency as, for example, estrogen administered to patients with tumor *in vitro* dependency, the clinical condition worsened. Indeed, the *in vitro* test correctly predicted this outcome in 27 of 29 patients.

As we have seen, the *in vitro* determination of hormonal dependency

is the most recent of several procedures designed to predict the response of patients with breast carcinoma to hormonal manipulation, either by endocrine ablation or by the administration of antihormonal compounds. The conditions for determining successful response have not been rigidly defined and, because of the novelty of the procedure in several cases, the period of observation following the institution of therapy has been too short. The concept underlying this procedure is a rational one, but obviously more cases and longer periods of observation are necessary to determine whether it is superior to other measures of prediction we described above, namely, the discriminant based on urinary steroid excretion, the assay for sulfotransferase activity of carcinomatous breast tissue, or the determination of estrogen binding.

III. Prolactin and Carcinoma of the Breast

A. Introduction

As has been previously noted (Chapter 11, Section I), human prolactin (HPRL), also known as the lactogenic hormone, is one of the hormones found in the human pituitary gland and has an important role in the regulation of lactation in mammals. Until recently, it was also considered to possess the properties of promoting growth, but this appears largely to result from the difficulties encountered in separating it from human growth hormone (HGH). Human prolactin has now been isolated as a separate molecule (Lewis *et al.*, 1971; Hwang *et al.*, 1972). It may be of interest to note the reasons for the difficulties involved in this isolation. First, dried human pituitary contained an overwhelming excess of HGH, 5–10% of the dry weight, as compared with only 0.1% for HPRL. Second, HGH, unlike growth hormone from other species, has 10–20% of the prolactin activity of the best sheep prolactin standards.

The molecular weight of HPRL is about 22,000, and there are some clear differences between the NH_2-terminal residues of this hormone and HGH. In HPRL, the N-terminal amino acid is leucine, as contrasted with phenylalanine in HGH. There is extensive homology between sheep and human prolactins and between HGH and human placental lactogen (HPL). There is very little homology between HPL, which we discussed in Chapter 16, and human prolactin HPRL, at least insofar as the first 24 N-terminal residues are concerned (Niall, 1972; Friesen and Hwang, 1973).

Most of the concepts concerning the control of prolactin secretion have been derived from work done chiefly in rats (Meites, 1972a), and

we may assume that the regulation in man is very similar. The secretion of prolactin is inhibited by a hypothalamic substance designated as prolactin-inhibiting factor (PIF). There is also some evidence for a prolactin-releasing factor (PRF). Both these factors, PIF and PRF, like other hypothalamic-releasing factors, are synthesized in the hypothalamus, harbored in the median eminence, and transported in the portal venous system which passes down the pituitary stalk to the anterior lobe of the pituitary (Friesen and Hwang, 1973).

As we shall note presently, several agents have become important in the study of mammary cancer; their role may be briefly reviewed here. The secretion of PIF is controlled by catecholamines by means of dopaminergic fibers. Accordingly, administration of levodopa in man leads to an increase in catecholamines which, in turn, increases PIF secretion and inhibits prolactin secretion (Malarkey *et al.*, 1971; Friesen *et al.*, 1972; Friesen and Hwang, 1973). Ergot drugs appear to have a dual effect, acting directly on the pituitary prolactin cells to inhibit prolactin secretion and acting on the hypothalamus to produce a marked change in its catecholamine levels (Lu *et al.*, 1971; Hökfelt and Fuxe, 1972). Prolactin secretion is increased by psychotropic agents such as phenothiazines and by thyrotropin-releasing hormone, acting at the level of the hypothalamus and pituitary gland, respectively. For example, the phenothiazine, chlorpromazine, depletes the effective catecholamine levels in the hypothalamus, leading to a decrease of PIF and, hence, to an increase of prolactin secretion (Kleinberg *et al.*, 1971). The thyroid-releasing hormone (TRH) is a very potent releaser of prolactin (Friesen and Hwang, 1973).

B. Prolactin in Human Serum

A variety of bioassays have been used for prolactin. These include the pigeon crop assay, the rabbit intraductal mammary gland assay, induction of histological changes in midpregnant mouse mammary glands, stimulation of the induction of [^{32}P]casein synthesis, and the induction of *N*-acetyl-lactosamine synthetase in midpregnant mouse breast tissue. In general, these assays lack specificity and sensitivity, particularly with regard to determination of HPRL in serum (Friesen and Hwang, 1973). Radioimmunoassays have largely overcome these drawbacks.

Using a radioimmunoassay method, Hwang *et al.* (1971) found the following concentrations of serum prolactin in various groups: 42 males, aged 16–84, 0–28 ng/ml, with 16% having concentrations higher than 15 ng/ml; 47 females, aged 16–85, 0–30 ng/ml, with 30% having concentrations higher than 15 ng/ml; and in 9 menstruating females, daily samples

during the month ranged from about 5 to 25 ng/ml, with no ovulatory peak and no increase in the luteal phase. Serum prolactin levels in the postmenopausal period were of the same order of magnitude. These and other data on the serum prolactin levels in the newborn and in pregnant women are shown in Table 17-5.

C. Prolactin and Cancer of the Breast

1. Introduction

Considerable experimental work in mice and rats indicate that prolactin is an important hormone in the genesis of mammary cancer, acting most probably as a promoter or cocarcinogen (Pearson *et al.*, 1969; Meites, 1972b). In rats with mammary tumors induced by dimethylbenzanthracene (DMBA), the administration of prolactin decreases the latent period of the appearance of tumors and increases the incidence and growth rate of the tumors. The administration of prolactin also leads to the recurrence of hormone-responsive tumors that have regressed

TABLE 17-5

Serum Prolactin Concentration in Normals under Different Conditions[a]

Group	Mean (ng/ml)	Range (ng/ml)
Children	10.8	7–17
Adult males	7	0–28
Adult females		
Follicular phase	10	4–20
Luteal phase	11	5–42
Pregnancy		
First trimester	30	7–70
Second trimester	60	20–450
Third trimester	120	36–300
Term	200	50–600
Postpartum		
Day 1	150	50–300
Day 7	40	7–60
Newborn		
Cord blood	200	100–500
1 Week	75	20–150

[a] Based on data of Friesen and Hwang (1973). Reproduced, in part, by permission of Annual Reviews, Inc.

as the result of oophorectomy, adrenalectomy, or hypophysectomy. When endogenous prolactin levels are increased as the result of administering chlorpromazine, the incidence and growth of mammary tumors are increased. Conversely, when pituitary prolactin secretion is decreased with ergot drugs, the incidence of DMBA-induced tumors and the incidence of spontaneous mammary tumors are also decreased (Yanai and Nagasawa, 1971).

2. Effect of Levodopa in Human Breast Cancer

There is much active work going on at present to explore the role of prolactin in human breast cancer (Friesen and Hwang, 1973). Hwang *et al.* (1971) reported a case in which the level of serum prolactin was 10 ng/ml, well within the normal limit, and decreased to less than 2.5 ng/ml after hypophysectomy. On the basis of evidence that the sensitivity of the hypothalamic centers tends to decrease with increasing age or in the presence of breast or endometrial cancer, Stoll (1972a) suggested that the concentration of dopamine might be depleted in the median eminence and that the administration of levodopa would stimulate therapeutic activity.

Stoll (1972a) accordingly administered levodopa to a group of patients with breast carcinoma and soft-tissue recurrences or metastases. Since no patient showed any definite evidence of tumor response after levodopa alone for 2 months, estrogen therapy, which had been ineffective previously, was added. Of 7 patients in this trial, 3 showed a 50% or greater decrease in the bulk of measurable tumor.

However, Stoll (1972a) did not submit any proof of prolactin suppression. This was supplied in 1 case by Minton and Dickey (1972) who treated a 44-year-old premenopausal woman who developed constant pain from rib metastases 18 months after a radical mastectomy. Levodopa was administered in a dose of 250 mg every 4 hours for 48 hours without evidence of clinical improvement. The serum prolactin decreased from a pretreatment level of 37.0 ng/ml to one of 22.0 ng/ml. The follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels showed a transitory rise on the first day of treatment, but were essentially the same as the pretreatment levels on the second day. The dose of levodopa was then increased to 500 mg every 4 hours for 48 hours. Bone pain disappeared, but reappeared after the cessation of the therapy. The level of serum prolactin continued to decrease slightly and attained a level of 17.8 ng/ml. The LH and FSH serum levels did not alter much. After 12 hours without therapy, the prolactin rose again to 43.2 ng/ml. Oophorectomy was then performed and, during the next 4 days,

the serum prolactin decreased, oscillating between 20.5 and 16.0 ng/ml. The serum FSH rose gradually to 15.0 mIU/ml, about threefold the pretreatment level. The serum LH decreased during the withholding of levodopa and the first 2 days' postoophorectomy, but then rose toward the pretreatment level.

The studies of Stoll (1972a) and of Minton and Dickey (1972) provide only preliminary data that levodopa may be of use in the treatment of metastatic breast cancer. However, the responsiveness of serum prolactin to the administration of this compound as well as to other influences suggests the possibility that the determination of serum prolactin may be of value in providing an objective criterion for following the clinical status of breast cancer patients.

3. Effect of Ergot Drugs on Human Breast Cancer

We noted earlier in this section that, in rats, ergot drugs can decrease pituitary prolactin release, and can counteract the stimulatory effect of estrogen on prolactin release (Lu *et al.*, 1971). 2-Br-α-ergocryptine (CB 154) has been found to inhibit growth of the DMBA-induced mammary carcinoma in the rat (Heuson *et al.*, 1970). Nineteen patients with advanced breast cancer were treated with this drug according to a definite dosage schedule. No objective remission was observed in any of these patients (European Breast Cancer Group, 1972). No information was presented as to whether or not the tumors in this series of patients were prolactin-dependent. Salih *et al.* (1972b) reported that prolactin dependence was present in 32% of 50 human breast cancers.

IV. RNA-Dependent DNA Polymerase and RNA Homologies in Carcinoma of the Breast

A. Introduction

In Chapter 9, we considered RNA-dependent DNA polymerase (reverse-transcriptase) and RNA homologies in human leukemia. Spiegelman and his associates (Spiegelman *et al.*, 1970; Schlom *et al.*, 1971; Axel *et al.*, 1972a,b) have explored the possibility that human cancers might contain RNA molecules or portions of RNA molecules that were homologous to various tumor virus RNA's in animals. The role of viruses in animal cancers can be determined by inoculation of virus into the animals and observing whether or not cancer develops. Obviously, this procedure is not permissible in man. The establishment of homologies

between the RNA molecules of certain tumors of man and animals does not necessarily signify viral etiology in man, but does provide evidence for the involvement of virus-related information in human tumors.

As we pointed out in Chapter 9, the extent of structural similarity between RNA molecules from two different sources can be determined by preparing a DNA homologous to RNA, utilizing the "reverse-transcriptase" method, then hybridizing the resulting DNA with the second type of RNA molecule. At the end of the 18-hour annealing period, the reaction mixture was added to an EDTA-CS_2SO_4 mixture and centrifuged at 44,000 rpm for 60 hours. Fractions were collected and assayed for trichloroacetic acid precipitable radioactivity. The formation of DNA–RNA hybrid complexes during the annealing period was manifested by movement of the radioactive DNA toward the RNA region of the density gradient. The nuclear and polysomal fractions of the mammary tumor tissue contained RNA molecules complementary to [^{3}H]DNA from mouse mammary tumor virus, as revealed by a shift of 30–35% of the [^{3}H]DNA to the regions of the gradient corresponding to RNA and hybrid density. In contrast, the gradient exhibited no shift after annealing of the viral [^{3}H]DNA with polysomal RNA from mouse liver (Axel *et al.*, 1972b). Examples of these are shown in Fig. 17-3. This technology and its further implications have recently been reviewed by Spiegelman *et al.* (1974).

B. Homologies between Human Breast Cancer and Mouse Mammary Tumor Virus

This technique was now applied to human neoplasms and, more specifically, to a consideration of the homology between human breast cancer RNA and mouse mammary tumor virus (MMTV) RNA. The criteria for determining a positive reaction, namely, that a hybridization peak actually exists, are indicated in the legend to Fig. 17-3. Of 29 human malignant breast cancers tested, 19, or 66%, were positive, that is, they contained RNA that could hybridize with DNA complementary to the RNA of MMTV. None of the other types of breast tissues or of other human neoplastic tissues yielded any positive tests.

Several implications arise from the results that have been presented here concerning the homologies in carcinoma of the breast and, in Chapter 9, concerning the homologies in human leukemic cells. Axel *et al.* (1972a) noted that their experiments provided no measure of the extent of the homology existent between the RNA found in human breast tumor and the genome of the MMTV. To obtain such information, it would be necessary to have MMTV-DNA in amounts sufficient to saturate the relevant RNA strands in tumor pRNA. In the opinion of these investi-

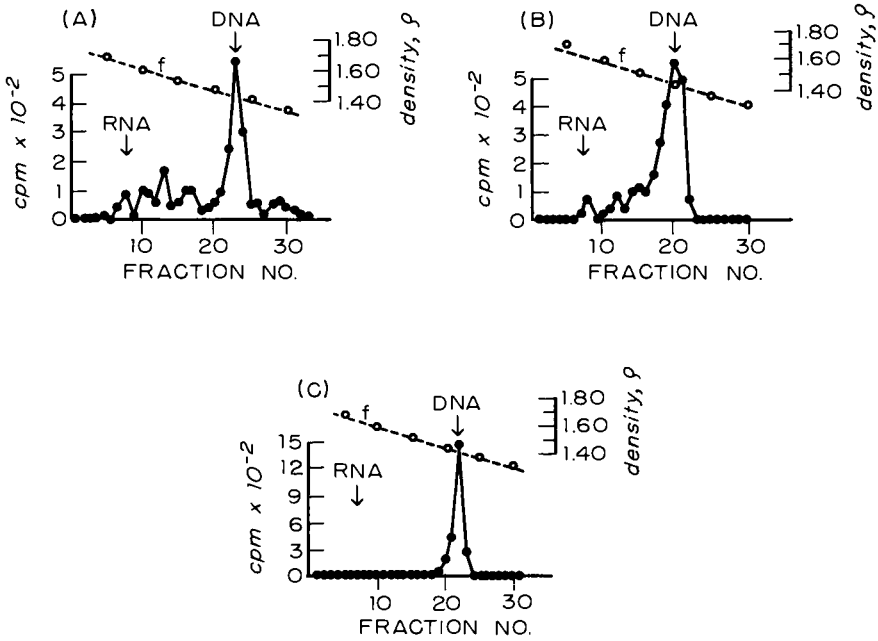

Fig. 17-3 Cs_2SO_4 equilibrium centrifugation of viral [^{3}H] DNA after annealing to RNA from following sources: (A) polysomal RNA from mouse mammary tumor, (B) nuclear RNA from mouse mammary tumor, and (C) polysomal RNA from normal mouse liver. In each case, 250 μg of RNA was annealed to viral [^{3}H]DNA at 37°C for 18 hours. From Axel *et al.* (1972b).

gators, the result obtained with the various types of human neoplasms did not constitute definite proof of a viral etiology but rather the involvement of virus-related information.

C. Search for a Human Breast Cancer Virus

Nonetheless, there are intimations that breast cancer has a viral etiology. Moore and his colleagues (1971a) found that some human milks contain particles physically identical to the mouse mammary tumor (MMT) virus which is transmitted in milk and can cause mammary carcinomas (Bittner, 1936). Moore *et al.* (1971a) fractionated and examined by electron microscopy milk from 212 women. They found characteristic type B particles resembling the MMTV in the milks of 7, or 5%, of 156 women in the Philadelphia area with no history of breast cancer in their immediate families; of 6, or 60%, in the milks of 10 American women with a history of breast cancer in their immediate families, and in 18, or 39%, of the milks of 46 women from the Parsi

community of Bombay. The structure of the intact particle shows, on negative staining, a head of about 100 μm in diameter, a long curved tail, and knobbed surface protrusions. In an editorial in *Nature*, Spiegelman (1972) was quoted as stating that, in his opinion and personal feelings, if a woman had a familial history of breast cancer and a Bittner-like virus in her milk, it would interdict the nursing of a child.

The Parsi community is of interest because of intermarriage within this community for religious reasons. The incidence of mammary cancer in this community is not significantly different from that in the United States, i.e., 50–55 per 100,000 population. However, the relative frequency, as a fraction of all cancer, is about 3 times as high as in the rest of the population of Bombay, namely, 49.4% versus 16.1% (Paymaster and Gangadharan, 1970; Moore *et al.*, 1971b).

In addition to morphological characteristics, several other similarities have been demonstrated between the MMT virus and the virus obtained from human milk samples. Schlom *et al.* (1971) examined 13 human milks for type B particles by electron microscopy and for RNA-dependent DNA polymerase activity of the material isolated from the 1.16 to 1.19 gm/ml density region of the gradient. Nine milks showed no particles, and in none of them was polymerase activity elicitable. In contrast, 4 revealed viruslike particles by electron microscopy, and all of them had RNA-dependent DNA polymerase (reverse transcriptase) activity. The existence of 60–70S RNA template was also demonstrable in both the human milk virus and the MMT virus (Schlom *et al.*, 1972; Schlom and Spiegelman, 1971).

The question arises whether the virions found in human milk are causally related to human breast cancer, as is the case with breast cancer in the mouse. It has already been pointed out that it is obviously impossible to attempt the direct introduction of virus in man. Other ways of demonstrating a causal relationship have received considerable attention (Moore *et al.*, 1971b; Sarkar and Moore, 1972). Epidemiological surveys have failed to establish an etiological role (Sarkar and Moore, 1972). Several attempts have been made to establish stable virus-producing cell lines from breast tumor tissues or from pleural effusion tumor cells. In general, these have failed, but recently Keydar *et al.* (1973) have been able to obtain human cell lines which produce and secrete particles with the biochemical and biophysical properties of oncornaviruses for as long as 11 months of continuous passage. These lines were obtained from human embryonic cells infected with milk from human breast cancer patients, or from co-cultivation of human embryonic cells with human breast tumor cells. Hence, the oncornaviruses that were produced may be involved in the etiology of human breast cancer. However, as Keydar *et al.* (1973) pointed out, it is necessary to consider and evaluate other

factors such as the possible contamination of the cultures with some nonhuman oncornavirus, derepression of a viral genome in the biopsy cells, or infection of the cultures with a ubiquitous passenger oncornavirus in the human milk. The literature on these and other factors in humans has been recently reviewed (Marx, 1974).

V. Other Biochemical Aspects of Breast Cancer

A. Introduction

In previous chapters, we discussed several biochemical aspects of human breast cancer in connection with other topics. For example, in Chapter 2 (Section II,D), we noted the patterns of tissue lactate dehydrogenase isoenzymes and of serum glycolytic enzyme elevations in patients with carcinoma of the breast, and in Chapter 3 (Section III,C,2), we discussed the significance of alterations of the serum alkaline phosphatase activity in this disease. In Chapter 10 (Section VII,B), we considered the calcium and phosphorus metabolism in skeletal metastatic carcinoma of the breast. In the present section, we shall describe those studies which cannot be readily classified among the topics we have mentioned.

B. Biochemical Components in Normal and Carcinomatous Breast Tissue

Biochemical analyses of normal and neoplastic human breast tissues have been carried out in order to determine whether characteristic differences exist. Obviously, were such differences to be found, they might be made the basis for prognostic tests, similar to those we discussed earlier in this chapter, in order to determine which patients might benefit from hormonal therapy or endocrine ablation. Smith *et al.* (1966) compared results of biochemical determinations on samples of normal and of neoplastic human breast tissues from each of 15–20 females. When the weights or activities were expressed per mg DNA, the mean values for total protein and RNA were significantly lower, and the activities of lactate dehydrogenase, 6-phosphogluconate dehydrogenase, and isocitrate dehydrogenase were significantly higher in the tumor tissue. No such differences were obtained for acidic nuclear protein, glucose-6-phosphate dehydrogenase, phosphohexose isomerase, or free and total β-glucuronidase. However, when these activities or weights were expressed per 100 mg protein, the differences were all significant, except for β-glucuronidase.

The increases in the activity of glycolytic enzymes in the breast

tumor tissues are an affirmation of the old Warburg concept and of repeated observations since then (see Chapter 2, Section II,B) that tumor possesses increased glycolytic activity. Increases in the activities of enzymes of the hexose monophosphate shunt in cancer have also been previously noted (Aisenberg, 1961).

Several other changes in the composition of carcinomatous breast tissue have been described. Employing histochemical methods, Csuka and Sugár (1971) reported that in benign conditions of the breast such as fibrocystic mastopathy or papilloma of the ducts, the surfaces of both myoepithelial and epithelial cells displayed adenosinetriphosphatase (ATPase) and uridinetriphosphatase (UTPase) activity. In carcinomas of the breast, UTPase activity remained unchanged, whereas the membrane-bound ATPase activity was decreased. In another histochemical study, H. Jensen and Schiødt (1971) have reported that no or only slight alkaline phosphatase activity was present in the cells of breast carcinoma. However, the surrounding zone showed "reaction zones" formed by proliferating fibroblasts with high phosphatase activity. This stromal response occurred in 65% of the biopsy specimens of 40 carcinomas and was doubtfully positive in another 7.5%. In 32 cases of fibroadenomatosis, only 19% of the biopsies revealed reaction zones with phosphatase activity.

The concept that malignant cells possess decreased adhesiveness stimulated a number of studies indicating that the calcium content of malignant tissues was decreased (Coman, 1944; Brunschwig *et al.*, 1946; deLong *et al.*, 1950). Employing homogenates in 0.9% NaCl and absorption spectrometry, Seltzer *et al.* (1970), however, found the opposite to prevail, reporting a mean value of 1.36 ± 0.41 (SD) mg per 100 ml per gm wet weight in a series of nine malignant breast tumors. This was significantly higher than the content, 0.93 ± 0.37 (SD) mg per 100 ml per gm wet weight, of specimens removed from the same breast at a site distant from the primary tumor. Eight of the serum calcium values were within normal limits, and the ninth slightly elevated to 11.2 mg per 100 ml. No correlation was found between the serum and tumor levels of calcium.

C. Other Metabolic Aspects of Patients with Breast Cancer

1. Tryptophan Metabolism

We have already considered the metabolism of tryptophan in cancer of the bladder (Chapter 6) and in other malignant neoplastic disease

including carcinoma of the breast (Chapter 6, Section II,C). It will be recalled that the mean values for the excretions of each of the three metabolites, 3-hydroxykynurenine, xanthurenic acid, and 3-hydroxyanthranilic acid, were higher in a group of mastectomized patients than in a group of controls. However, there was a considerable overlap of the ranges in the 2 groups. The possibility was raised that abnormal tryptophan metabolism in several types of cancer as well as non-neoplastic disease was the result of a stress-induced increase in tryptophan pyrrolase in all these diseases (Altman and Greengard, 1966). Rose and Randall (1972) have challenged this view. In their studies, 36% of 36 patients with "early" cancer, that is, patients with localized disease, and 29% of 24 patients with recurrent cancer had abnormal excretions of tryptophan metabolites. Moreover, all patients with metastases to the bone had normal excretion of tryptophan metabolites, although some were subjected to the stress of bone pain. Only patients with advanced breast cancer have an increased rate of cortisol production and elevation in the unbound plasma cortisol (V. Jensen *et al.*, 1968). In the opinion of Rose and Randall (1972), these considerations militate against considering increased excretion of tryptophan metabolites in breast cancer as solely resulting from the "stress" of illness.

Indeed, Davis *et al.* (1973) have sought to determine whether a correlation exists between abnormal tryptophan metabolism and altered urinary steroid excretion in patients with breast cancer. Subjects were classified as having abnormal tryptophan metabolism if, after the administration of 2.0 gm of L-tryptophan, the 24-hour urinary excretions of two or more of the following metabolites, kynurenine, acetylkynurenine, *ortho*-aminohippuric acid, anthranilic acid glucuronide, 3-hydroxykynurenine, kynurenic acid, and xanthurenic acids, were at least 2 standard deviations above the mean value for controls. Twelve of the 25 patients with carcinoma of the breast were designated as having abnormal tryptophan metabolism and, in this group, the excretion of etiocholanolone was 0.86 ± 0.52 (SD) mg per 24 hours, significantly lower than the excretion, 1.59 ± 0.64 (SD) mg per 24 hours, in a group of 12 normal control women, or than the excretion, 1.80 ± 1.33 (SD) mg per 24 hours, in a group of 13 breast cancer patients with normal tryptophan metabolism. There were no significant differences in the excretion of 17-hydroxycorticosterone or androsterone.

2. Serum Fucose

We have previously discussed the nature of glycoproteins in human plasma and their elevation in cancer and in other diseases (Chapter

1, Section III,C,3). The reader will recall that the methylpentose, fucose, is a component of these glycoproteins. Utilizing the method of Dische and Shettles (1948), Rosato *et al.* (1971) explored the possibility that this pentose would be elevated in patients with carcinoma of the breast. In initial studies, Rosato (1967) found that 13, or 87%, of 15 patients with breast masses, subsequently demonstrated to be malignant, had serum fucose levels of 12 mg per 100 ml or higher, whereas only 6, or 13%, of 46 patients with benign breast masses had serum levels higher than this concentration. In two subsequent studies of 90 and 150 patients, respectively, the serum fucose was expressed as micrograms per milligram protein. Combination of the results of these studies showed that 56, or 73%, of 77 patients with cancer had concentrations greater than 3.35 μg per 100 ml, whereas 47, or 23%, of 163 patients with benign disease had serum concentrations higher than this value. On the whole, therefore, this procedure has only a very limited utility. The levels in the serum of patients with neoplasms of other organs or in nonneoplastic disease were not studied. In all probability, the level of serum fucose merely reflects that of glycoproteins and has all the limitations which that procedure has as a test for the presence of breast cancer or, more generally, of any type of cancer (see Chapter 1, Section III).

3. Tissue and Serum Lipids in Patients with Carcinoma of the Breast

The subject of lipid metabolism in cancer was considered generally in Chapter 1 (Section IV). Specifically with regard to carcinoma of the breast, it was noted that the concentrations of total cholesterol, esterified cholesterol, and free fatty acids were higher, and the concentration of triglycerides was lower than in normal breast tissue (Hilf *et al.*, 1970).

Utilizing a combination of the ethanol-zinc precipitate procedure of Lever *et al.* (1951) and of the ultracentrifugal technique of Gofman and his associates (1949; De Lalla and Gofman, 1954), Barclay and her co-workers (1955) found that in a group of 20 patients with carcinoma of the breast, the mean value for the concentration of plasma α-lipoprotein was significantly less than in a group of 10 normal women of the same age range. The differences held whether the fractions were assayed in terms of cholesterol or phospholipid. The concentration of plasma β-lipoprotein was essentially the same in both groups.

In subsequent studies (Barclay *et al.*, 1957), it was shown that oophorectomy for palliation of advanced mammary carcinoma resulted in statistically significant increases for the group as a whole with respect to

the following plasma parameters: total cholesterol, total phospholipid, α-lipoprotein cholesterol, α-lipoprotein phospholipid, total β-lipoprotein cholesterol, total β-lipoprotein phospholipid, low density β-lipoprotein cholesterol, and low density β-lipoprotein cholesterol. These parameters were also analyzed with respect to each of 2 subgroups, those with skeletal and those with osseous metastases, each of which could be subdivided further into those who had and those who had not responded favorably to the operative procedure. Further studies by the Barclay group have been concerned with the effect of the extent of breast disease on plasma lipoproteins (Barclay *et al.*, 1959).

Changes in the serum lipoprotein pattern of patients with carcinoma of the breast have been generally, but not completely, confirmed by other workers. Using filter paper electrophoresis, Miller and Erf (1956) reported that the concentration of α-lipoprotein was decreased but, in contrast to the findings of Barclay *et al.* (1955), that of β-lipoprotein was increased in patients with advanced carcinoma of the breast. Significant increases in the β-lipoprotein fraction have also been demonstrated by means of a simpler turbidimetric procedure (Kellen, 1968).

Table 17-6 shows the results of a recent comprehensive survey of lipoproteins and other plasma lipids in women with metastatic carcinoma

TABLE 17-6

Blood Plasma Lipids and Lipoproteins in Women with Metastatic Breast Carcinoma[a]

Lipids and lipoproteins	Breast cancer patients		Healthy women	
	Number	Mean ± SE	Number	Mean ± SE
Cholesterol[b]	211	236 ± 5.0[d]	53	274 + 6.8
Triglycerides[b]	135	104 ± 5.5	53	101 ± 6.0
Phospholipids[b]	100	238 ± 12.7	10	240 ± 13
Free fatty acids[c]	108	824 ± 31.9[d]	23	596 ± 50.2
α-Lipoproteins[e]	162	28.5 ± 1.54	44	25 ± 1.2
β-Lipoproteins[e]	109	65.7 ± 1.24	—	66.8 ± 1.30
Origin lipoprotein[e]	109	10.0 ± 0.74	—	7.7 ± 0.67
α-Lipoprotein[f]	50	57.2 ± 2.57	—	65
β + pre-β-lipoprotein as cholesterol[b,f]	50	161 ± 7.99[d]	11	192 ± 11.2

[a] Based on study of Feldman and Carter (1971).
[b] Expressed as mg per 100 ml.
[c] Expressed as μEq per liter.
[d] Significantly different from values in control group.
[e] Performed by paper electrophoresis and expressed as percent of total.
[f] Performed by preparative ultracentrifugation.

of the breast who were postmenopausal or had undergone radiation or surgical castration (Feldman and Carter, 1971). It may be seen that the concentrations of plasma free fatty acids were significantly higher, and the concentrations of cholesterol significantly lower than in a control group of healthy postmenopausal women. In contrast to the results of Barclay *et al.* (1955) and of Miller and Erf (1956), there was no alteration in the levels of α-lipoprotein.

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18

Hydatidiform Mole, Choriocarcinoma, and Neoplasms of the Uterus

I. Introduction*

The uterus is a hollow, pear-shaped muscular organ, situated between the bladder and the rectum, in the midline of the female pelvis. It is about 3 in. or 7.5 cm in length, and about 2 in. or 5 cm wide. It consists of a fundus or superior portion, a corpus or intermediate section, and a cervix or lower portion. The endometrium, which lines the cavity of the uterus, is a mucous membrane of simple columnar epithelium containing many glands that extend into the muscular layer of the uterus.

In 1967 in the United States, 5,834 white women died from cancer of the cervix, and 4,908 white women from cancer of other regions of the uterus. The corresponding figures for nonwhite women were 1,684 and 822, respectively. The rates per 100,000 population for all parts

* The following abbreviations are used most commonly in the present chapter: FIGLU = formiminoglutamic acid; FSH = follicle-stimulating hormone; HCG = human chorionic gonadotropin; HCS = human chorionic somatomammotropin; HGH = human growth hormone; HPL = human placental lactogen; HPRL = human pituitary prolactin; KA = King-Armstrong (units); 17-KS = 17-ketosteroids; LDH = lactate dehydrogenase; LH = luteinizing hormone; MUU = mouse uterine units; 17-OHCS = 17-hydroxycorticosteroids.

of the uterus were 12.25 for whites and 20.03 for nonwhites (Segi and Kurihara, 1972). According to the 1947 cancer survey of the United States, the sex-age adjusted incidence was 23.6 per 100,000 population (Ackerman and del Regato, 1970). The death rates vary considerably with the geographical areas, with a low rate of 5.17 in Israel and a high rate of 29.16 in Austria for the years 1966–1967 (Segi and Kurihara, 1972). The predicted incidence in the United States for the year 1973 was 46,000 out of a total of 665,000 cases of cancer, and the predicted number of deaths was 11,800 (Silverberg and Holleb, 1973). Malignant neoplasms of the uterus are thus the second most common form of cancer in women in the United States—breast cancer, of course, being first.

The age distribution for cancer of the cervix is 30–39 years, 17%; 40–49 years, 24%; 50–59 years, 23%; and 60–69 years, 18% (Cutler, 1968). The peak incidence occurs at a later age in cancer of the body of the uterus: 30–39 years, 3%; 40–49 years, 13%; 50–59 years, 28%; 60–69 years, 31% and 70–79 years, 17% (Cutler, 1968).

Before turning our attention to the biochemical aspects of neoplasms of the body of the uterus and of the cervix, we shall first consider the hydatidiform mole and choriocarcinoma, which are present in the uterus and attached to its wall, but do not arise from any of its histological elements. The origin of these two tumors is the trophoblast which is a layer of extra-embryonic ectodermal tissue on the outside of the blastodermic vesicle.

II. Hydatidiform Mole

A. Introduction

From a broad point of view, this condition is only part of the spectrum of chorionic tumors that may arise from the germinal cells of the gonads of either sex or from the placenta in which the fertilized ovum is embedded (Li, 1971). In addition, trophoblastic tumors may arise, albeit very rarely, from misplaced primordial cells in the pineal body, mediastinum, and retroperitoneal area (Fine *et al.*, 1962).

B. The Normal Trophoblast

Fertilization of the ovum usually occurs in the midportion of the Fallopian tube. At this time, the diploid number of chromosomes is established, the sex is determined, and the fertilized ovum begins to cleave (Hellman *et al.*, 1971). It reaches the uterine cavity between

72 and 96 hours after fertilization. It now consists of about 16 cells, with the peripheral cells due to form the trophoblast and the inner cells to differentiate into the embryo and amnion. In the later stages of development, at approximately the 58 or 104 cell stage, the cells become grouped into a central or inner mass and a peripheral layer, known as the trophoblast. By the accumulation of fluid between the central mass and the peripheral layer, a blastodermic vesicle or blastocyst is formed. At about the eight to tenth day, the fertilized ovum or embryo becomes embedded in the decidua of the uterine wall (Percival, 1969).

After embedding, the trophoblast begins to differentiate into two parts, namely, the syncytiotrophoblast, which consists of nucleated protoplasmic buds, bands, and reticula but has no distinct cell outlines, and the cytotrophoblast, which consists of definite cells. These two layers, known as the chorion, constitute the outermost envelope of the growing fertilized ovum. At about this stage, the endometrium shows advanced gestational hyperplasia, with prominent, dilated and rapidly growing capillaries that appear to leak and contribute to the massive stromal edema (Hertig, 1968; Bagshawe, 1968; Percival, 1969; Hellman *et al.*, 1971). At about the thirteenth day after fertilization, the chorion around the surface of the ovum begins to form villi. This primitive villous period lasts approximately 2 days. The chorionic villi then begin to branch and, during the next 5 weeks, are gradually transformed into the definitive organ, the placenta. At this point, the chorion has reached a maximum diameter of about 80 mm. During this period, too, the embryo develops rapidly and, at the end of 5 weeks, is a well-formed fetus, 30 mm in length (Percival, 1969).

C. Hydatidiform Mole

1. Origin

At some time after fertilization, the conceptus may develop gross cyst-like swelling of its chorionic villi as a result of accumulation of fluid within the mesenchymal core. The blood vessels in the core begin to disintegrate and then disappear, whereas the chorionic epithelium continues to proliferate. Hertig (1968) noted that the average age after onset of amenorrhea in large series of blighted ova with hydropic swelling was about 10 weeks; of the transitional mole, about 16 weeks; and of the true mole, 20 weeks. Edmonds (1959) reported that the mean age of abortuses with hydatidiform degeneration was 10.2 weeks, and that of transitional moles was 16.6 weeks. The average age of typical hydatidiform moles has been variously given as 17.4 weeks (Edmonds,

1959), and of 14.4 weeks with a range of 7–32 weeks (Marquez-Monter *et al.*, 1963).

2. Incidence

The incidence of hydatidiform mole as a fraction of known pregnancies varies very widely with geographical location. In several series from the United States, the incidence has ranged from 1:1000 to 1:2500 (Bagshawe, 1969). Some of the incidences reported for various European countries are France, 1:500; Great Britian (Belfast), 1:1190; and Russia, 1:333. Those reported from Asian countries appear particularly high: Japan, 1:232; Taiwan, 1:120; Philippines, 1:173; and India, 1:160 to 1:400. The incidence appears to be highest among women less than 20 years of age and over 40 years of age (Douglas, 1959; Edmonds, 1959; Bagshawe, 1969). In their series of 104 cases, seen in 1961 at the General Hospital of Mexico City, Marquez-Monter *et al.* (1963) found 30% in the 11–20-year decade, and the peak incidence, 41%, in the 21–30-year decade.

3. Human Chorionic Gonadotropin Production and Levels in Urine and Blood

The production of chorionic gonadotropin, estrogen, progesterone, and of other steroids and their levels in blood and urine during the normal menstrual cycle was considered in some detail in Chapter 15. Of interest here is the production of these hormones during pregnancy and the degree to which the occurrence of a mole alters this production. Figure 18-1 shows the mean serum and urine concentrations of human chorionic gonadotropin (HCG) of 600 Asian women during the course of normal pregnancy (Teoh, 1967). There was considerable variability in these values from person to person, but the general trend was essentially similar to that obtained with the same laboratory procedure (the hemagglutination–inhibition test) for Irish (McCarthy *et al.*, 1964) and Swedish (Mishell *et al.*, 1963) populations.

Ascheim and Zondek (1927) were the first to show that excretion of HCG tended to be higher in molar pregnancies than in normal ones at corresponding periods after onset of amenorrhea, and this has been amply confirmed since then. In 16 cases of hydatiform mole, Hamburger (1944) found the excretion of HCG ranging from 6 IU to 20×10^3 IU/ml of morning urine (6×10^3 to 20×10^6 IU/liter) with 80% of the cases having an output equal to or greater than 300 IU/ml. Among 71 cases of normal pregnancy, only 6% showed excretions greater

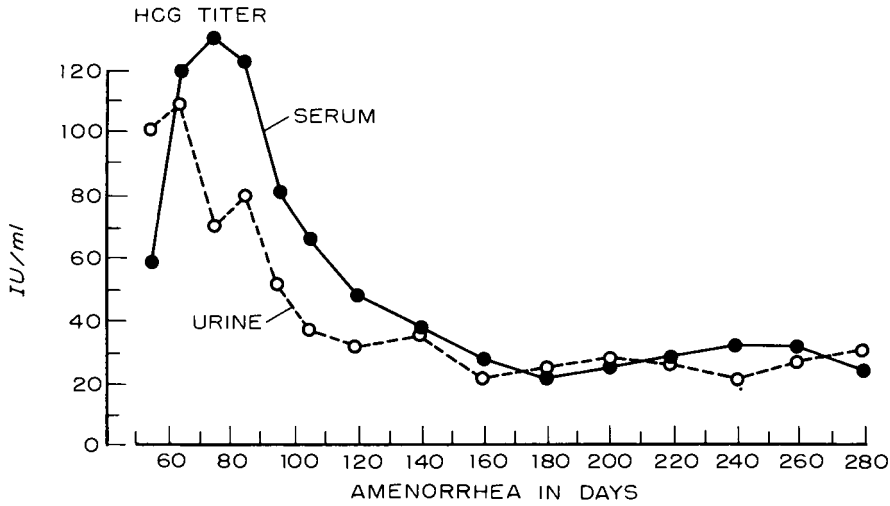


Fig. 18-1 Mean serum and urine HCG concentrations of 600 Asian women with normal pregnancy. From Teoh (1967). Reproduced by permission of the Royal College of Obstetricians and Gynaecologists.

than this value, and the highest excretion was 900 IU/ml. In another series of 45 women with moles, 49% had excretions equal to or greater than 30 IU/ml, and 13% equal to or greater than 600×10^3 IU/ml, as contrasted with only 6 and 0.5%, respectively of 205 women with normal pregnancies (Hobson, 1958).

Hobson (1958) observed that the total HCG contents of 8 moles weighing from 143 to 990 gm ranged from 12×10^3 to 1.8×10^6 IU. There was no relationship between weight and total content, but those instances in which the gestation period was 21–25 weeks showed distinctly lower concentrations and total contents than those with gestation periods of 12–17 weeks. This indicates the possibility that damaged or dead moles may produce much less HCG and that the urinary excretions in patients with such moles may be relatively low. The excretion of HCG decreases sharply after the complete removal of a hydatidiform mole (Hamburger 1944, Hobson, 1958). For example, 4 patients studied by Hobson (1958) had preoperative urinary excretions ranging from approximately 100 to about 2000 IU/ml, and showed decreases to 1 IU/ml or less, 12–14 days after operation.

The serum levels of HCG also tend to be elevated in patients with hydatidiform mole. As may be seen from Fig. 18-1, employing a hemagglutination–inhibition procedure in an extensive study of 600 normally pregnant Asian women, Teoh (1967) observed that the mean value

for serum HCG rose from a level of 60 IU/ml at about 55 days past the last menstrual period to a peak of approximately 130 IU/ml at 80 days. It then declined gradually to reach a level of 20–30 IU/ml at about 160 days of the amenorrheic period. In 21 cases with intact hydatidiform mole, ranging in age from 70 to 280 days, the serum HCG levels ranged from 60 to 1920 IU/ml and averaged 833 IU/ml. Indeed, all values were higher than the normal mean values for the corresponding stage of pregnancy, and 6 patients had values higher than 1280 IU/ml serum (Teoh *et al.*, 1972).

4. Estrogen and Progesterone Production and Levels in Urine and Blood

Prior to 1960, there were occasional and sparse reports of alterations in the urinary excretion of estrogen and progesterone metabolites in patients with hydatidiform mole. More definite data have since been forthcoming. As a basis for evaluating urinary excretions of estrogens in patients with mole, Frandsen and Lundwall (1966) studied these excretions in 242 daily specimens from 16 women throughout normal pregnancies. The estrone was reduced to estradiol in the procedure used so that these two estrogens were determined as one moiety. The excretion of estrone-estradiol increased from a mean value of about 100 μg per 24 hours in the tenth week of pregnancy to approximately 2000 μg per 24 hours at term. The mean value for estriol excretion similarly increased from about 0.5 mg per 24 hours to about 25 mg per 24 hours at term. The 95% confidence limits embraced a range of approximately 50–100% about the mean.

Several studies have shown that the urinary excretion of estrogens is generally decreased in patients with hydatidiform mole (Bonanno *et al.*, 1963; Frandsen and Stakemann, 1964; Johnson-Brinck *et al.*, 1970). For example, a 56-year-old woman who had irregular menstrual periods for 2 years and none for about 25 weeks prior to admission and the diagnosis of a mole excreted 21 μg estrone and 12 μg estradiol per 24 hours. The sum, 33 μg per 24 hours, was far below the lower value, 499 μg per 24 hours, for the normal range for that period of gestation (Frandsen and Lundwall, 1966; Frandsen and Stakemann, 1964). Figure 18-2 shows instances of the urinary decrease of estriol in patients with hydatidiform mole. The decrease in estriol excretion is relatively more pronounced, as is evident from the value of the ratio of estriol to that of the sum of estrone and estradiol. Whereas the mean value for this ratio in normal pregnancy rises from approximately 3.6 at 10–13 weeks to 10 at 25–26 weeks and about 15 at 39–41 weeks (Frandsen and Lundwall, 1966),

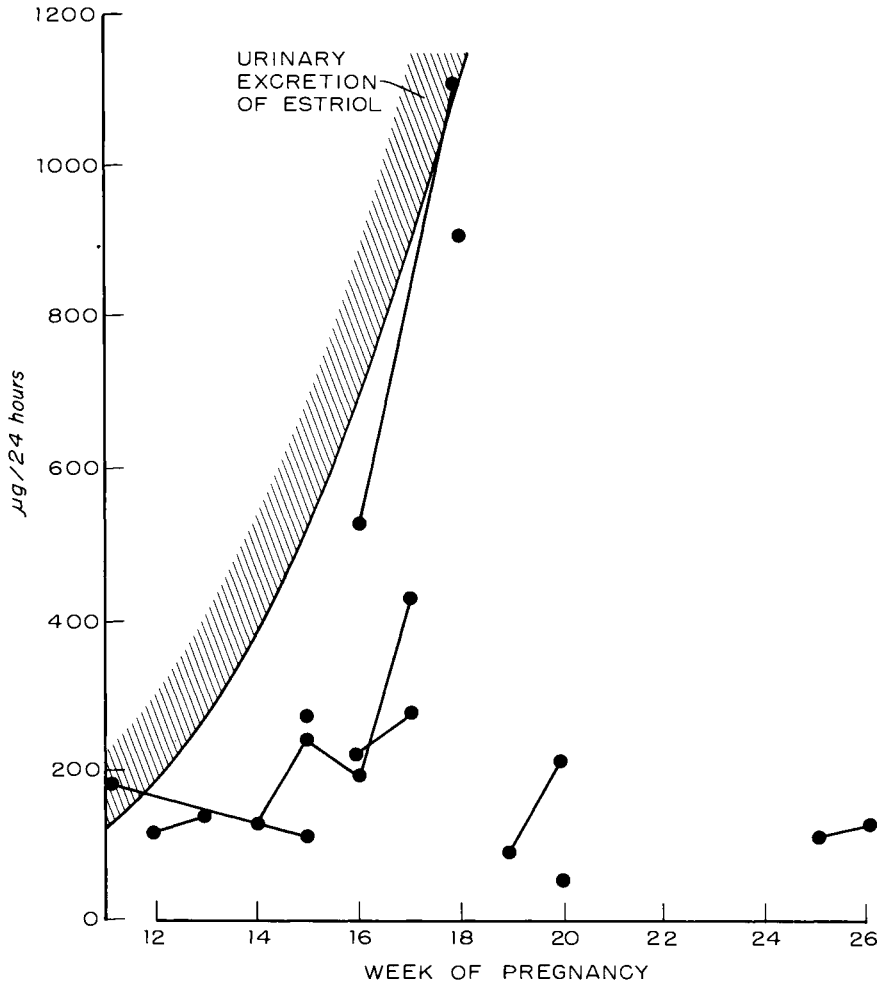


Fig. 18-2 Excretion of estriol in patients with hydatidiform mole. Hatched area represents urinary excretion of estriol at various periods of normal gestation; (●) excretion determined at one or more periods during the course of gestation in patients with mole. From Frandsen and Stakemann (1964). Reproduced by permission of Periodica, Copenhagen.

the ratios in patients with mole were less than 2.0 up to 16 weeks of amenorrhea. The highest ratio observed was only 3.1 in a patient with 26 weeks of amenorrhea (Frandsen and Stakemann, 1964).

It will be recalled (Chapter 15, Section II,C,3) that the concentration of progesterone in the blood serum and excretion of its metabolite, preg-

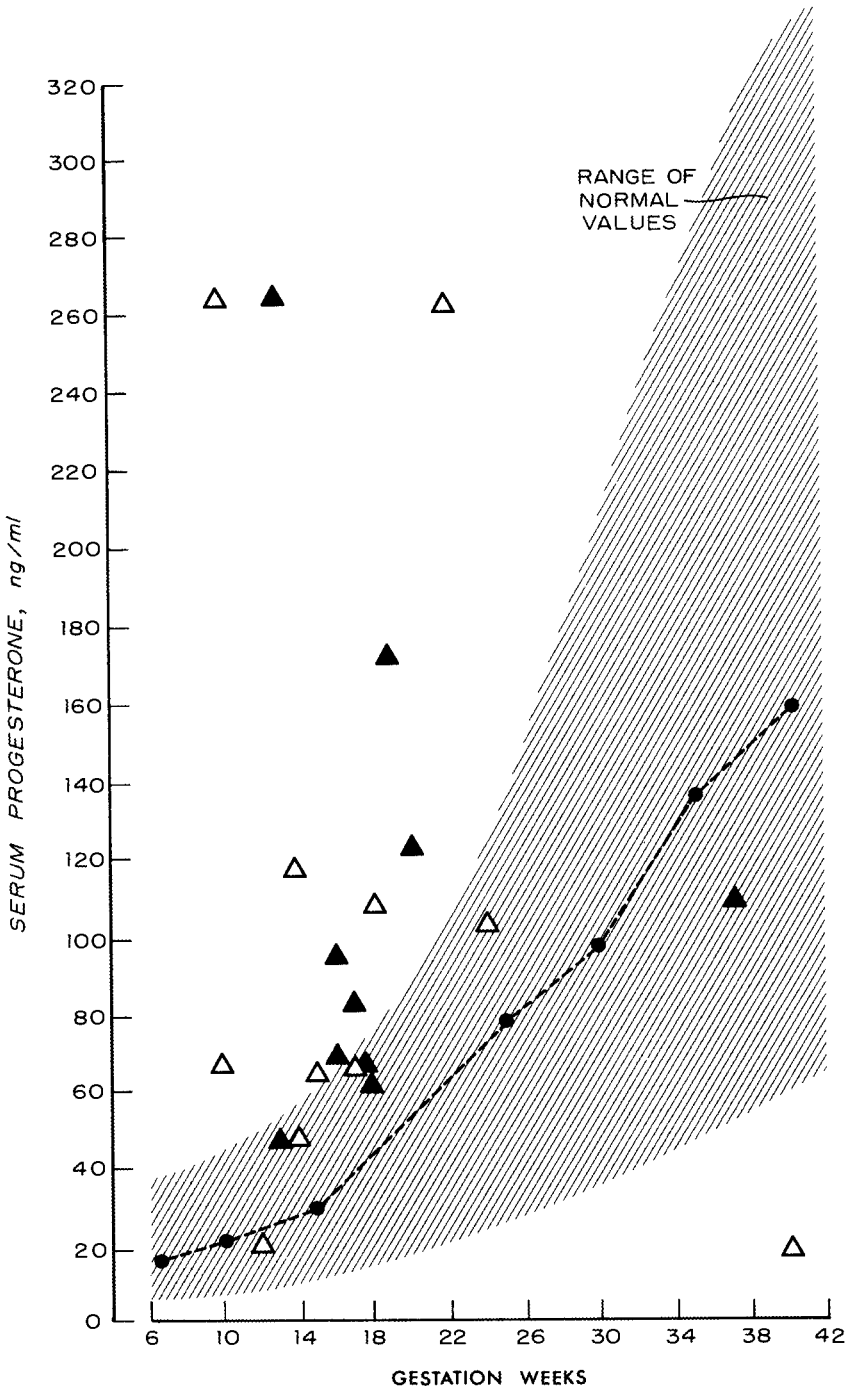
nanediol, in the urine fluctuate during the menstrual cycle, beginning to increase immediately following the luteinizing hormone (LH) peak at midcycle, reaching a maximum several days after this peak and returning to normal by the onset of menses. Upon reference to Chapter 15, the reader will note that the serum progesterone level is about 0.5 ng/ml in the first part of the menstrual cycle, then begins to rise, reaching a maximum of about 15 ng/ml about 7 days after the midcycle and decreases again as the onset of menses approaches. During normal pregnancy, there is, despite considerable variability at each stage, a steady sigmoid-shaped increase in the mean concentration of serum progesterone, begining with approximately 15 ng/ml at 6 weeks and reaching a mean value of about 160 ng/ml at term (Teoh *et al.*, 1972). Figure 18-3 shows that, despite the broad range of normal levels at various stages during pregnancy, the serum progesterone levels in the patients with mole were elevated above the upper limit in 12 patients, within the normal pregnancy range in 8 patients and in the nonpregnant range in 1 patient.

The normal urinary excretion of progesterone is relatively low, amounting to about 15–20 μg per 24 hours at the peak, but this steroid gives rise to metabolites that are excreted in much greater amounts. The best known of these is pregnanediol, the reduction product of progesterone, which is excreted throughout the menstrual cycle, but reaches a maximum amount, approximately 3–5 mg per 24 hours, about 5–8 days after the midpoint, or LH peak, of the menstrual cycle (Chapter 15). During pregnancy, pregnanediol excretion increases continuously until, at term, it reaches levels which range, according to various investigators, from 25 to 90 mg per 24 hours (Bodansky and Bodansky, 1952).

In general, the urinary excretion of pregnanediol in patients with hydatidiform mole is of the same order of magnitude as the maximum excretion in normal menstruating women, but occasionally there are values that are more characteristic of pregnancy. Thus, the average excretions in 5 women with mole ranged from 1.9 to 7.7 mg per 24 hours and averaged 4.5 mg per 24 hours (Frandsen and Stakemann, 1964). On the other hand, MacNaughton (1965) reported 5 cases, 2 of whom showed substantially elevated levels of urinary pregnanediol, namely, 31.5 and 24.5 mg per 24 hours at 17–19 weeks after cessation of amenorrhea.

5. Serum Placental Lactogen

In 1962, Josimovich and MacLaren found that the human placenta and the sera of pregnant women at term contained substantial quantities of a protein that was highly lactogenic, readily promoting crop sac



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growth in the pigeon and milk production in the pseudopregnant rabbit. In these respects, this substance, which was termed "human placental lactogen" (HPL), resembled human pituitary prolactin (HPRL). However, these differed structurally in several respects.

As we observed in Chapter 17 (Section III,B), there is very little homology between HPRL and human growth hormone (HGH) with respect to the sequence of the first 24 NH_2-terminal residues (Niall, 1972; Friesen and Hwang, 1973), only 4 amino acids being similarly positioned in the sequence. In contrast, the homology between HPL and HGH is more extensive; of the first 17 amino acids in the amino terminal sequence, 11 were found to be identical between the two hormones. It follows from the preceding that there is very little homology between HPL and HPRL; 2 amino acids occupied the same position in the 24 amino acid terminal sequence of the two hormones.

Using a radioimmunoassay procedure, Samaan *et al.* (1966) found that in normal pregnancies the serum HPL rose from zero levels during the first few weeks to mean values of about 1 μg/ml at about 25 weeks, about 2.5 μg/ml at 32 weeks, and to a maximum of about 3.5 μg/ml from 35 weeks on to term. Within 1 hour after parturition, the values decreased to approximately 0.4 μg/ml. Using a different HPL preparation for iodination and standard, Josimovich *et al.* (1971) obtained somewhat higher mean values, about 3 μg/ml at 25 weeks of pregnancy, 5 μg/ml at 32 weeks, and a maximum of about 9 μg/ml near term. It is apparent, therefore, that alterations in trophoblastic disease must be judged, taking into account the particular HPL preparation used as standard in the immunoassay.

The serum HPL activity is decreased markedly in patients with hydatidiform mole, indeed, to levels as low as 1–10% of the values expected in normal pregnancy at the corresponding gestational ages. For example, Samaan *et al.* (1966) found the serum HPL values in 4 patients to range from 0.02 to 0.16 μg/ml, values characteristic of the very early weeks of normal pregnancy. With the HPL preparation employed as standard in these studies, a mean maximum value of about 3.0 μg/ml was obtained at term of normal pregnancy. Table 18-1 again demonstrates the decreased serum HPL levels in patients just before they were operated on and the presence of molar pregnancy proved. In a study of 94 cases of hydatidiform mole, Goldstein (1971) obtained find-

Fig. 18-3 Serum progesterone in normal pregnancy and hydatidiform mole. Hatched area encloses range of normal values. Broken line represents mean serum progesterone in normal pregnancy. (△) Hydatidiform mole without lutein cyst and (▲) hydatidiform mole with theca lutein cyst. From Teoh *et al.* (1972). Reproduced by permission of Periodica, Copenhagen.

TABLE 18-1

Decreased Serum HPL Levels in Patients with Undelivered Molar Pregnancy[a]

Patient No.	Gestational age (weeks)	Serum HPL	
		In molar pregnancy (μg/ml)	Expected in normal pregnancy (μg/ml)
1	24	0.05	8.0
2	19	0.22	4.0
3	17	0.026	2.0
4	14	0.230	1.5
5	13	0.014	1.0
6	14	0.36	4.0
7	16	0.24	2.0

[a] From data of Saxena *et al.* (1968). Reproduced by permission of C. V. Mosby Company.

ings indicating that the level of serum HPL activity was not so much an evidence of the hormonal activity of the trophoblastic tissue as reflected by the urinary excretion of HCG, but rather of the length of gestation and the absence or presence of possible malignancy, as determined by the histological criteria of Hertig and Sheldon (1947).

D. Invasive Mole

1. Introduction

Invasive mole has also been termed malignant hydatidiform mole, penetrative mole, and destructive mole (chorioadenoma destruens). This tumor is characterized by excessive trophoblastic overgrowth and, as the name indicates, by penetration of the trophoblastic elements, including whole villi, into the myometrium and sometimes even further into the adjacent parametrium or vaginal vault (Hellman *et al.*, 1971). Although it has been suggested that, in some cases, diagnosis can be made on the basis of the occurrence of intra-abdominal hemorrhage or by findings on palpation, Hertig (1968) has stated that the presence of an invasive mole cannot be foretold solely by the size of the uterus, the amount of bleeding, the urinary HCG titer or the immediate postmolar course. The existence of an invasive mole is usually recognized by the occurrence of vaginal bleeding and abdominal pain or tenderness

several weeks or months following the evacuation of a mole. According to Bagshawe's review of the literature (1969), this occurs in about 20–25% of cases of hydatidiform mole. Infrequently, molar villi can be transported to the brain or to the lung (Hertig, 1968; Bagshawe, 1969); at the latter site, they grow and may perforate the pleura.

2. Biochemical Aspects

There are relatively few data available concerning hormonal production in invasive mole. Bagshawe (1969) listed the range of HCG excretion as 400–100,000 IU per 24 hours. These would correspond approximately to a range of 0.3–65 IU/ml. In view of the fact that the gestational age was not stated, it is difficult to state that these values were distinctively different from those of normal pregnancy. Utilizing a biological method (B) of assay for urinary HCG that depended on change in weight of rat seminal vesicle and an immunological method (I) utilizing the hemagglutination inhibition, Hobson and Wide (1968) observed that the ratio (B/I) of results obtained by these two methods was 1.34 for a group of 7 women with invasive mole, and significantly higher than the ratio, 0.634, for 10 patients with choriocarcinoma. From a diagnostic point of view, a rapidly rising HCG excretion at any time after the evacuation of a mole should be considered as an indication of rapid proliferation of tissue from an invasive mole.

The serum HPL activity is also profoundly decreased in patients with invasive mole, as compared with the values expected in normal pregnancy at the corresponding gestational stages. However, it does not appear as markedly decreased as in ordinary molar pregnancy. For example, Goldstein (1971) obtained a mean serum HPL level of 0.34 ± 0.08 (SD) μg/ml for a series of 11 patients with invasive mole, whereas Saxena *et al.* (1968) had previously obtained serum HPL values ranging from 0.05 to 0.36 and averaging 0.16 ± 0.13 (SD) μg/ml for a series of 7 patients with ordinary hydatidiform mole. The same HPL standard had been used in these assays, and it would appear that the difference between the mean values for the 2 groups is significant.

III. Choriocarcinoma

A. Introduction

Choriocarcinoma is a malignant epithelial tumor composed of pure trophoblast (Hertig, 1968) which shows a greatly exaggerated tendency

to invasive growth and erosion of blood vessels. The gross picture is that of a rapidly growing, dark red, ragged mass that may involve the endometrium, invade the myometrium, and penetrate the peritoneum. Microscopically, columns and sheets of trophoblast penetrate the blood vessels, either in a plexiform arrangement or in a completely disorganized form (Hellman *et al.*, 1971). The absence of a villus pattern is characteristic. Choriocarcinoma may metastasize to the lung and from there to any other organ. Although choriocarcinoma may also arise from germinal cells or from misplaced primordial cells, we shall first consider the much more frequent type that originates from the fertilized ovum.

The incidence of this type of choriocarcinoma varies widely. Bagshawe (1969) quoted several estimates of the incidence in pregnancies: 1:13,850 in a 1937 report from Philadelphia; 3:50,000 in 1959 from New York City; and 1:40,000 in 1968 from Rhode Island. The incidence in Asian hospital reports appears to be much higher, ranging from 1:250 to 1:3,708 deliveries, and even these incidences may be lower than the proportion of deaths from choriocarcinoma to deaths from other causes as determined at autopsy, such as 1:114 for Hong Kong, 1:128 for Tokyo, and 1:150 for Manila (Bagshawe, 1969). The incidences for various types of trophoblastic disease seeen in the University Hospital at Taipei, Taiwan from 1950 to 1960 were hydatidiform mole, 1:125 deliveries; invasive mole, 1:561; and choriocarcinoma, 1:496 (Wei and Ouyang, 1963).

The type of pregnancy that precedes choriocarcinoma is of interest. A general estimate is that 40% of cases occur after hydatidiform mole, 40% after abortions, and 20% after normal pregnancies (Hellman *et al.*, 1971). Hertig (1968) has given the following figures for his series: 50.0% after mole, 25.0% after abortions, and 2.5% after normal pregnancies.

B. Biochemical Aspects

1. Introduction

Our discussion of the biochemical aspects of hydatidiform mole has formed the basis for a consideration of these aspects in choriocarcinoma, for the differences are essentially a matter of degree.

2. Chorionic Gonadotropic Hormone

Earlier in this chapter (Section II,C,3), we referred to Hamburger's (1944) study on the increased excretion of HCG in 16 patients with

molar pregnancy before removal of the mole. Eight of these and 41 other patients who had an uncomplicated course after operation showed a dramatic drop immediately after the removal of the mole, and then a slower decrease. However, in 23 other cases of mole, the course was complicated, and it was later found that 4 of these had molar remnants, 13 had choriocarcinoma, and, in 6, the diagnosis was uncertain. In these 23 cases, the HCG excretion tended to rise after operation, with the occurrence of urinary excretions amounting to from 30,000 to 300,000 IU/liter for at least 1 month after the removal of the mole. Similar observations have been made by other investigators (Frandsen and Stakemann, 1964). Bagshawe (1969) found that the distribution of elevated values of urinary HCG in a series of 92 cases of choriocarcinoma was about the same as in patients with hydatidiform mole (Table 18-2) but, of course, was much higher than the excretion in normal pregnancy.

There appear to be very few values for the HCG content of choriocarcinoma, but these are much lower than that of normal placental tissues

TABLE 18-2

Initial HCG Excretion in 92 Patients with Choriocarcinoma before Chemotherapy[a]

No. of cases	Urinary excretion of HCG IU/24 hours[b]
4	$<10^3$
19	10^3 to 10^4
27	10^4 to 10^5
26	10^5 to 10^6
16	$>10^6$

[a] From Bagshawe (1969). Reproduced by permission of Edward Arnold (Publishers) Ltd., London.

[b] In accordance with most investigators, we have been expressing the excretion of HCG as IU per milliliter. The daily volume of urine in an adult varies greatly, depending primarily upon the intake and the body surface, but may be said to average about 1500 ml. Division of the value in the table by 1500 yields a value in terms of HCG per millimeter.

at corresponding stages of gestation. For example, of 2 cases of choriocarcinoma reported by Allison (1961), one showed an HCG content of 30 IU/gm of tumor obtained from the uterus, and the other a content of 35 IU/gm of secondary tumor in the lung. In contrast, a mean value of 93 IU and a range of 10–445 IU/gm of placenta were obtained in 7 cases that were therapeutically aborted at 6–17 weeks of gestation. In 11 women who had curettage for incomplete abortion at 6–12 weeks of gestation, the curetting had a mean value of 220 IU HCG per gm tissue, with a range of 20–800 IU/gm.

3. Estrogen and Progesterone Production and Levels in Urine and Blood of Patients with Choriocarcinoma

We have previously noted that the excretions of estrogens in patients with hydatidiform mole are usually much lower than those in normal pregnancies of a corresponding gestational period. The excretions in patients with choriocarcinoma are decreased, in general, to a similar extent, although occasionally still lower to the levels present in nonpregnant women (Frandsen and Stakemann, 1964; Johnson-Brinck *et al.*, 1970; Zondek and Finkelstein, 1967). For example, Frandsen and Stakemann (1964) studied a 33-year-old woman who had a hydatidiform mole removed with accompanying decrease in HCG excretion. One year later, elevated amounts began to be excreted once more. The uterus and tubes were removed at operation and were found free of abnormal tissue. During the next year, she was discovered to have tumor in the lung which, on operation and subsequent examination, proved to be choriocarcinoma. She died 6 months later. In this patient, the initial estrogen excretions, expressed as micrograms per 24 hours, were estrone, 13; estradiol, 9; and estriol, 44. These were essentially within the nonpregnant range. They increased terminally to 68, 30, and 185 μg, respectively, but were still below the range found in pregnancy (Hellman *et al.*, 1971). In several other cases of choriocarcinoma reported in the literature, the excretions of estrogens were even lower than those in nonpregnant, menstruating women at midcycle (Ross *et al.*, 1970; Johnson-Brinck *et al.*, 1970).

In a series of 10 patients with choriocarcinoma, the serum progesterone levels ranged from 1.3 to 61 ng/ml (Teoh *et al.*, 1972). Six of these had concentrations that were within the limits observed for the luteal phase of the normal menstrual cycle (Fig. 15-6); 2 patients had slightly elevated serum progesterone levels, 24 and 61 ng/ml, comparable with

the values in early pregnancy (Fig. 18-3); and two had very low values, namely, 1.3 ng/ml. These findings have been taken to indicate that there is little *in vivo* secretion of progesterone by the tumor cells. The excretion of pregnanediol, progesterone's chief metabolite, in patients with choriocarcinoma appears to be of the same order of magnitude as in patients with hydatidiform mole or nonpregnant women. In the patient studied by Frandsen and Stakemann (1964), the excretion ranged from 0.9 to 4.6 mg per 24 hours throughout the course of her illness.

4. Serum Placental Lactogen

The levels of serum placental lactogen (HPL) are decreased markedly in patients with choriocarcinoma as they are in those with hydatidiform mole. For example, in 2 cases reported by Samaan *et al.* (1966), the concentrations were 0.1 and 0.025 μg/ml, as compared with values ranging from about 0.6 μg/ml at 18 weeks of normal pregnancy to about 3–5 μg/ml at term. Table 18-3 summarizes other data in the literature and indicates that the serum HPL level does not differentiate between noninvasive hydatidiform mole and choriocarcinoma nor, with respect to the latter, between metastatic and nonmetastatic disease. However, as we have noted earlier in this chapter, the serum HPL level is higher in patients with invasive mole.

Low as the level of serum HPL is in hydatidiform mole or choriocarcinoma, it may decrease still further as a result of therapeutic intervention. Figure 18-4 illustrates such a case. The patient was a 24-year-old woman who, after evacuation of a molar pregnancy, developed choriocarcinoma metastatic to the lungs. For about 16 days following curettage, the serum HPL remained at levels of about 0.02 μg/ml. With the institu-

TABLE 18-3

Serum HPL in Patients with Trophoblastic Disease

Disease	No. of patients	Mean ± SD (μg/ml)	Range (μg/ml)
Hydatidiform mole[a]	7	0.16 ± 0.13	0.014–0.36
Invasive mole[b]	11	0.34 ± 0.08	0.02–0.72
Nonmetastatic choriocarcinoma[b]	3	0.092 ± 0.03	0.01–0.27
Metastatic choriocarcinoma[b]	9	0.086 ± 0.04	0.01–0.34

[a] Data from Saxena *et al.* (1968).
[b] Data from Goldstein (1971).

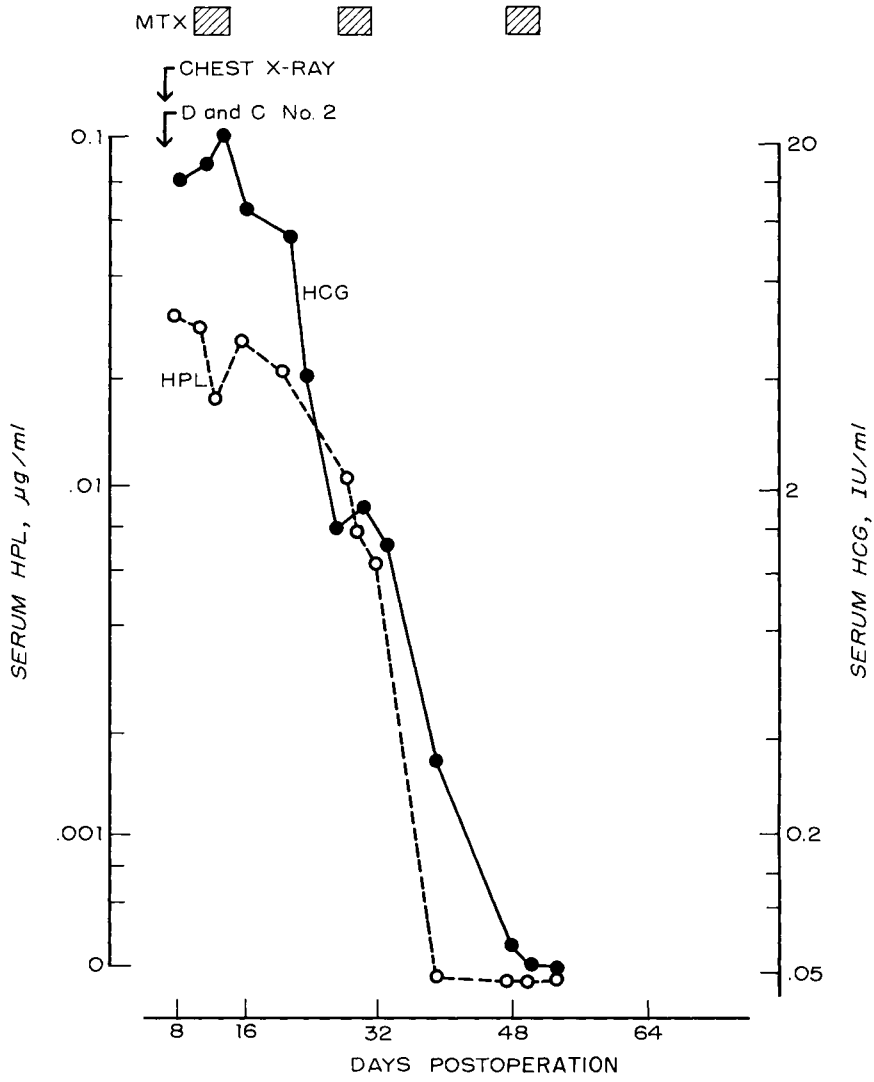

Fig. 18-4 Decrease of serum HPL and HCG levels in a patient with choriocarcinoma metastatic to the lungs, following institution of methotrexate (MTX) therapy. See text for further details. From Saxena *et al.* (1968). Reproduced by permission of C. V. Mosby Company.

tion of methotrexate therapy for several days at intervals of about 12–13 days, the serum HPL began to decrease markedly, reaching undetectable levels 38 days following curettage and beginning of therapy.

5. Serum Levels and Urinary Excretion of HCG in Treatment of Patients with Choriocarcinoma

In the case we have just described, the serum chorionic gonadotropin (HCG) also decreased following therapy, but this decrease was less marked, and elevated levels were still present when serum HPL could not be detected. Saxena *et al.* (1968) have reported several other cases of patients with hydatidiform or invasive mole who, after curettage or evacuation and institution of methotrexate therapy, exhibited the same phenomenon, namely, decreases in serum HCG that were less marked than the decreases in HPL.

In 1956, Li *et al.* observed that the administration of methotrexate to patients with metastatic chorionic carcinoma altered dramatically what, until then, had been a progressively worsening course in such patients. Li (1971) recently listed a total of 608 cases of trophoblastic disease, consisting of series reported from various parts of the world. A sustained complete remission, lasting from 3 to 10 years at the time of the report, had been obtained in 48–88% of the various series of treated cases.

Measurement of the urinary excretion of HCG has proved of special usefulness in following the course of trophoblastic disease, particularly since this condition is now so amenable to chemotherapeutic treatment. Li (1961) described the case of a patient who had severe vaginal bleeding caused by an overwhelming choriocarcinoma of the uterus, with extension to the vagina. The initial urinary HCG excretion was about 420,000 IU per 24 hours. She responded well to treatment with methotrexate, and the HCG excretion dropped to very low levels. Approximately 7 months later, the urinary excretion of HCG began to rise again, reaching the very high level of 2,500,000 IU per 24 hours. However, this was the herald, not of recurrence of the tumor, as was first suspected and for which she was treated, but, fortunately, of pregnancy which eventuated in the delivery of twins (Li, 1961, 1971). The effect of drug combination therapy is illustrated by a case of choriocarcinoma with pulmonary metastases in which methotrexate was of no avail clinically and in which the 24-hour urinary excretion of HCG did not decrease, oscillating between about 10×10^3 to 20×10^3 IU. After about 80 days of this therapy, a combination of actinomycin D and chlorambucil was administered for various periods during the next 60 days. After an initial rise in urinary HCG, the clinical response was very dramatic; the pulmonary lesion disappeared and the daily HCG excretion dropped from a maximum of 160×10^3 IU to very low levels.

Methotrexate is the 4-amino-N^{10}-methyl derivative of folic acid. The

question has arisen whether patients with trophoblastic disease metabolize methotrexate differently from those with other types of cancer or other diseases. It has been reported that patients with cancer frequently have low serum folate levels (Rama Rao *et al.*, 1965), but this situation also holds for hospital patients with nonmalignant, grave disease (Spector *et al.*, 1966) and may indeed be correlated with poor nutritional status (Hellman *et al.*, 1964).

In a group of 34 patients with trophoblastic tumors comprising adenocarcinoma, invasive mole, malignant teratoma, and other invasive trophoblastic neoplasms, the serum folate levels were related to the excretion of HCG and also to the interval between the end of the antecendent pregnancy and the time of the study. The correlation coefficients were 0.51 and 0.52, respectively (Hughes and Bagshawe, 1968). Serum folate levels were lower than 5.0 ng/ml, the lower limit of normal, in 19 of the 34 patients. Initial urinary formiminoglutamic acid (FIGLU) excretion, determined for an 8-hour period starting 1 hour after the ingestion of L-histidine, was found to be above normal, that is, greater than 20 mg per 8 hours, in 12 of the 16 subjects studied.

To orient the reader, we may note that FIGLU is a metabolic derivative of histidine in the following sequence: histidine → urocanic acid → imidazolonepropionic acid → formiminoglutamic acid. The formimino group can then be transferred by a specific transferase to the N^5 position of tetrahydrofolic acid which hydrolyzes and cyclizes to the $N^{5,10}$-methenyl derivative (White *et al.*, 1973). Animals or human beings deficient in folate have an excessive excretion of FIGLU. However, no conclusive relationship between the excretion of FIGLU and serum folate levels was obtained in the study by Hughes and Bagshawe (1968). Nor were the data adequate enough to determine whether the excretion of FIGLU was dependent on the type of trophoblastic disease or upon the particular therapeutic regimen employed. In general, although the serum folate activities were very low in some patients with trophoblastic disease, and these decreases appeared to depend on the extent and duration of the disease, they were of the same magnitude as had been reported in patients with other forms of malignant disease.

IV. Nongestational Choriocarcinoma

A. Gonadal Choriocarcinoma

As we indicated at the beginning of this chapter, choriocarcinoma may also arise from the germinal cells of the gonads of either sex or, much more rarely, from misplaced primordial cells in the pineal body, mediastinum and retroperitoneal area. Athough it is recognized that they

are not related to tumors of the uterus, we may briefly discuss them at this point. The incidence of gonadal cell tumors in males is about 1:50,000. The proportion of these that are choriocarcinomas varies from 0.4 to 16% in various reports (Friedman, 1959; Li, 1971).

The clinical–biochemical findings in gonadal, nongestational choriocarcinoma may be briefly illustrated. A 28-year-old man who noted swelling of his left testis was subjected to orchiectomy 3 months later, and the diagnosis of choriocarcinoma was established (Li *et al.*, 1960). After operation, repeated chest roentgenograms revealed multiple large metastases to both lungs, and intravenous pyelography suggested retroperitoneal lymph node enlargement. The urinary HCG excretion ranged from 300,000 to 640,000 IU per 24 hours. He was placed on triple chemotherapy of actinomycin D, methotrexate, and chlorambucil; the HCG excretion dropped to very low and then undetectable levels after the third 5-day course of therapy, or approximatey 35–40 days after instituting therapy, and remained at this level during the next 18 months prior to the report. All evidence of disease, as determined by intravenous pyelography and chest roentgenograms, had disappeared by the eighth month following institution of therapy (Li *et al.*, 1960).

Wider *et al.* (1969) have reported a series of 4 patients with primary ovarian cancer containing choriocarcinoma. The patients were treated, as above, with combined methotrexate, actinomycin D, and chlorambucil. The urinary HCG excretions were determined by the mouse uterine-weight assay method, and were expressed in mouse uterine units (MUU) where 1 such unit is equal to 0.1 IU. The admission urinary HCG ranged from a level of between 20 and 50 IU (200–500 MUU) per 24 hours in 1 patient to higher levels in the others, up to 200,000 IU per 24 hours, as compared with a normal level of less than 20 IU per 24 hours in females with functioning ovaries. After institution of therapy, the HCG levels decreased in 3 of these 4 patients to the normal range and, at the time of the report, they were alive and well at periods ranging from 14 months to 4 years and 7 months after the time of diagnosis. Previous experience with such tumors had shown that incomplete surgical removal of the presence of metastatic disease was associated with death 8–12 months from the time of diagnosis.

The fourth patient in the series was an 8-year-old girl who had had evidence of sexual precocity, abdominal pain, and a large abdominal mass. Excision of the left ovary revealed teratocarcinoma with choriocarcinoma. One month later, pulmonary metastases appeared and triple therapy was instituted. The HCG excretion at this time was between 2,000 and 5,000 IU per 24 hours, rose to between 20,000 and 50,000 IU, and then decreased to the normal range. There appeared to be some regression of the pulmonary metastases. In spite of continued therapy,

the HGC excretion rose and the pulmonary lesions enlarged. She died 1 year and 1 month after diagnosis, and autopsy revealed extensive metastases of choriocarcinoma to the lungs and many other organs.

More extensive biochemical studies have been carried out by Greenwood *et al.* (1971) in a 27-year-old man complaining of back pain and gynecomastia who died 9 days after initiation of triple chemotherapy. Roentgenograms of the chest had shown multiple, bilateral, dense infiltrates, and intravenous pyelography had shown displacement and external obstruction of the ureter with hydronephrosis. Autopsy revealed extensive metastases to the lungs, kidney, breast, adrenal glands, liver, dura, and tetroperitoneum. Although the testes appeared grossly free of neoplasm, detailed sectioning revealed a small scar which was considered to be the primary site of the choriocarcinoma.

Values for serum triiodothyronine resin uptake, serum concentrations of testosterone and androstenedione, and urinary excretion of 17-ketosteroids at this point were normal. The following serum components were slightly elevated: protein-bound iodine, 9.1 μg per 100 ml, as compared with a normal range of 4–8 μg per 100 ml; estrone, 15.2 ng per 100 ml, as compared with a normal range of 3–10 ng per 100 ml; and estradiol, 7.8 ng per 100 ml, as compared with a normal range of 2–6 ng per 100 ml. Of striking interest were the marked elevations in the following: serum HCG, 500,000 μg (of the standard LER 907 preparation) per 100 ml, as compared to a normal level of less than 10 μg per 100 ml; chorionic somatomammotropin (HCS), 50 ng/ml, as compared with a normal level of less than 1 ng/ml.

The reader will recall that we have previously discussed somatomammotropin (Chapter 16, Section VII). It is a hormone which has both lactogenic and growth-promoting properties and is produced by the normal placenta. In the particular case under study, Greenwood *et al.* (1971) found that HCS was localized by immunofluorescence in the malignant trophoblast cells. Histochemical methods showed that steroid sulfatase and 3β-hydroxysteroid dehydrogenase activities were also localized in the neoplastic cells. However, no evidence was obtained in the neoplastic cells for enzyme activities which convert androgens to estrogens. Greenwood *et al.* (1971) suggested that, in the patient they studied, the neoplasm may have synthesized HCS and contributed to the elevated serum estrogen levels by the production of precursors, and formed some of the hormones which led to gynecomastia.

B. Extragenital Choriocarcinomas

We have already indicated the probability that choriocarcinomas may occur at sites other than the uterus or gonads, presumably from mis-

placed primordial cells (Fine *et al.*, 1962; Li, 1971). Fine and his associates (1962) reported the case of a 49-year-old white male with complaints of waning potency and gynecomastia, and the finding of increased excretion of urinary HCG. At autopsy, a tumor was present in the mediastinum which showed 2 types of tumor cells—one with abundant eosinophilic, finely granular and reticulated cytoplasm, and the second with abundant cytoplasm and multiple hyperchromatic clumped nuclei near the periphery of the cell. Similar nodules were found in other organs, but not in the testes. Fine *et al.* (1962) collected a total of 109 cases from the literature, 19 of which they considered as acceptable cases of extragenital choriocarcinoma since no scars, cysts, or benign tumors were found in sections from the serially blocked testes.

The incidences of primary sites in the 18 acceptable cases were as follows: mediastinum, 8; retroperitoneum, 4; abdominal viscera, 2; pelvic viscera, 2; and intracranium, 1. The pregnancy test, indicative of elevated HCG excretion, had been performed in 11 of the 18 cases, and was positive in all. Gross and microscopic evidence of gynecomastia was present in 6 cases.

Ozaki *et al.* (1971) reported a case of primary choriocarcinoma of the stomach and, in reviewing the 20 cases that had been reported from the English, German, and Japanese literature until then, decided that only 5 of these had met reliable histological and biological criteria. Of the 18 cases listed by Ozaki *et al.* (1971) as having been reported prior to 1962, 13 had been included in the review by Fine *et al.* (1962). As has been noted before, few biochemical data are available. Goldstein (1971) submitted values, ranging from 0.04 to 0.23 μg/ml, for the initial serum HPL level in 5 cases of nongestational choriocarcinoma, but some of these were gonadal, and it is not clear how many were extragenital. In any case, this range was essentially the same as that for nonmetastatic choriocarcinoma or metastatic choriocarcinoma. Another case had a value of 0.87 μg/ml, closer to that of the range of invasive mole.

V. Enzymes in Uterine Neoplasms

A. Introduction

In Chapters 2, 3, and 4, we considered the enzyme patterns in human cancer tissue, the individual enzymes in serum and tissues of patients with cancer, the isoenzyme patterns in neoplastic tissues and their normal counterparts, and certain groups of enzymes whose activities in serum were useful in the diagnosis and management of patients with various types of neoplasms. Only passing reference was made there to enzyme

activities in uterine carcinoma. We shall now consider this topic in greater detail.

B. Lactate and Other Dehydrogenases

The distribution of the five isoenzymes of lactate dehydrogenase in various normal tissues, with the exception of the uterus, and the change in their proportion in neoplasms of these tissues were considered in Chapter 2 (Section II,C,5). It was pointed out that neoplasia was accompanied by a shift in the isoenzyme pattern, so that the proportion of the most cathodic or, in terms of the International Union of Biochemistry agreement, the LDH-4 and -5 components, was increased. Okabe *et al.* (1968), using the reverse American nomenclature for the isoenzymes, that is, LDH-I for the most cathodic and LDH-V for the most anionic isoenzyme, showed that the same relationship holds for tumors of the human uterus, namely, an increase in the proportion of the cathodic components and decreases in that of the anionic components.

Okabe *et al.* (1968) achieved 16- to 87-fold degrees of purification for the various isoenzymes from normal uterus and 4- to 34-fold degrees of purification for the isoenzymes from cervical cancer. Employing these purified preparations, Okabe *et al.* (1968) discovered several differences between the lactate dehydrogenase isoenzymes from normal uterine tissue and those of neoplasms (Table 18-4). Thus, the rates of reduction of NAD analogs by the isoenzymes from cervical cancer were generally higher than those by the isoenzymes from normal uterus and its myoma.

TABLE 18-4

Percentages of the Isoenzymes of Human Lactate Dehydrogenase in Water Extracts of Normal Uterus and Its Neoplasms[a]

Uterine tissue	Isoenzyme				
	Most cathodic I	II	III	IV	Most anionic V
Normal	4	15	45	30	6
Leiomyoma	13	32	35	16	4
Leiomyosarcoma	38	44	20	5	4
Cervical cancer	42	29	17	9	3

[a] From Okabe *et al.* (1968). Reproduced by permission of the American Chemical Society.

Again, the isoenzymes from cervical cancer were inhibited to a greater extent by oxalate and α-ketoadipate than were the isoenzymes from normal uterus and uterine myoma.

Some attempts have been made to utilize lactate dehydrogenase (LDH), malate dehydrogenase, and several other enzymes diagnostically in neoplasms of the human uterus, either by studying the levels of these enzymes in the serum or in the neoplastic tissue. Schlegel and Canzler (1972) were unable to obtain any significant differences between the serum LDH isoenzyme pattern of normal individuals and that of patients with cancer of the uterus. Nor was there any consistent relationship between the level of isoenzyme activity in the tumor and that in the serum. Kidess *et al.* (1972b) studied the activities of lactate and malate dehydrogenases as well as of aldolase in histologically differentiable areas of untreated carcinoma of the endometrium. The only statistically significant alterations were an increase of the activity of aldolase in exophytic growing carcinoma and a decrease of malate dehydrogenase activity in necrotic tissue.

C. *Miscellaneous Enzymes*

1. Introduction

A large number of enzymes have been studied with regard to their alteration in neoplastic uterine tissue or in the serum of patients with tumors of the uterus. For example, leucine aminopeptidase activity is significantly higher in infiltrating carcinomas of the endometrium, but alkaline phosphatase shows no significant difference in several histologically different carcinomas of the uterus (Kidess *et al.*, 1972a). In the following sections, we shall consider alterations in various serum enzyme activities.

2. Serum Alkaline Phosphatase Activity

The mean serum alkaline phosphatase activity in 75 patients with endometrial carcinoma was 6.36 King-Armstrong (KA) units and not significantly different from those in 2 control groups, namely, 6.36 KA units in those with glandular cystic hyperplasia of the endometrium or 6.98 KA units in a "normal" group exhibiting no hyperplasia of the endometrium. However, use of the χ^2 test revealed statistically significant differences in the frequency of elevated activities of serum alkaline phosphatase. The proportion of patients with endometrial carcinoma or with glandular cystic hyperplasia of endometrium having decreased values was significantly greater than in the control group (Laurová *et al.*, 1971). The

mechanism of these changes is not clear. Levine (1964) had shown that serum alkaline phosphatase activity was lower in 50–60-year-old women with abnormal uterine bleeding than in normal menopausal women of this age, and had suggested that the most likely candidates for endometrial disease were to be found in the former group.

3. Fibrinolytic Activity

Extractable fibrinolytic activity is greater in invasive carcinoma of the endometrium and uterine cervix than in normal uterine tissue. Such fibrinolytic activity may result from new vessel formation as well as from the neoplastic cells per se (Todd, 1964). It will be recalled that, in general, such activity is exercised by an enzyme, plasmin (fibrinolysin), which exists in plasma and in tissues in an inactive form, plasminogen, and which must be activated. Employing histochemical methods, Zeller *et al.* (1970) found that fibrinolytic activity requiring plasminogen activator was absent, and nonspecific protease fibrinolytic activity was present in endometrial carcinoma. In invasive cervical carcinoma, plasminogen activator fibrinolytic activity was present not only in the neoplastic cells but also in the vascular components that accompanied the invading cells.

4. Hexokinase Isoenzymes

The existence of hexokinase in multiple molecular forms in rat liver was demonstrated both by column chromatography and by electrophoresis (González *et al.*, 1964; Katzen and Schimke, 1965). These isoenzymes were designated as types I, II, III, and IV in terms of increasing electrophoretic mobility, and their proportions were shown to alter in rat liver tumors. For example, as compared with normal rat liver, the proportions of isoenzymes II and III were increased, whereas that of type IV was decreased (Sato *et al.*, 1969).

The isoenzyme patterns in normal and in neoplastic human uterine tissues have recently been studied by Kikuchi *et al.* (1972), using cellulose acetate membrane electrophoresis. Table 18-5 shows that normal uterine cervical epithelium and endometrium contained type I in substantial quantities in every subject, and type II was present in weak or moderate amounts in only a few specimens. In contrast, specimens of cervical and corpus carcinomas had both types I and II present in substantial and relatively equal amounts. The emergence of type II paralleled an increased hexokinase activity and, indeed, the mean values for total hexokinase activity of cervical and corpus carcinomas were substantially and significantly elevated, as compared with the mean values for the

TABLE 18-5

Hexokinase Isoenzyme Electrophoretic Patterns of Human Uterine Neoplasms[a]

Tissue	No. of patients	Isoenzyme I Number of bands			Isoenzyme II Number of bands			Total activity (units/gm protein)
		Strong	Medium	Weak	Strong	Medium	Weak	
Cervical epitherlium	10	10	0	0	0	0	3	6.3 ± 2.3
Cervical carcinoma	10	9	1	0	10	0	0	25.1 ± 8.6[b]
Endometrium	14	14	0	0	0	5	6[c]	7.4 ± 3.3
Corpus carcinoma	5	5	0	0	4	1	0[d]	21.2 ± 9.8[b]

[a] Based on data of Kikuchi *et al.* (1972).
[b] Significantly higher ($p < 0.01$) than in control normal tissue.
[c] Weak isoenzyme III band in addition to I and II in 1 patient.
[d] Medium isoenzyme III band in addition to I and II in 1 patient.

activity of normal cervical epithelium and endometrium, respectively. Both myometrium and myoma had predominantly type I isoenzyme, and their total hexokinase activities were essentially the same.

5. Oxygen Consumption

The oxygen consumption of neoplastic tissue has been of interest for over 40 years, particularly in connection with Warburg's (1930) thesis that the energy for cancer cellular activity was obtained chiefly by anaerobic glycolysis and that the aerobic respiratory component played a smaller role. As was noted in Chapter 2 (Section I,A), Macbeth and Bekesi (1962) found that the mean values for the rate of oxygen consumption in a large series of human adenocarcinomas of the stomach, sigmoid, and colon were higher than those of adjoining corresponding normal tissue. In contrast, in 2 cases of hypernephroma, one of Wilm's tumor and one of papillary carcinoma, the rate of oxygen consumption was lower than that of the normal renal cortex. With regard to the uterus, Alvarez *et al.* (1971), using a Beckman oxygen analyzer, found that the mean value for oxygen consumption for 15 cases of endometrial carcinoma was 2.17 ± 0.30 (SE) μl/mg dry weight per hour, significantly higher than the mean value, 1.22 ± 0.08 (SE) μg/mg dry weight per hour, for 43 cases of normal endometrium, or than the mean values for proliferative or secretory endometrium.

The discrepancies, few as they are, between the findings of increased oxygen consumption for some neoplastic tissues and decreased oxygen consumption for others may, of course, result from the type of tumors but, more specifically, other factors such as functional activity, mitochondrial content, hormones, blood supply, and degree of malignancy may be involved. Technical factors such as the type of instrumentation used, the nature of medium employed, and the length of the period used for measurement may also play a role (Alvarez *et al.*, 1971).

D. Vaginal Fluid Enzymes as Diagnostic Aids

1. General

Just as the vaginal fluid provides a source for cells which are of aid in the diagnosis of cancer of the cervix, so it is possible that it may contain enzymes which serve a similar purpose. Indeed, in 1950, Odell and Burt suggested that the β-glucuronidase activity in the vaginal fluid might be used toward that end. As we shall presently note, Fishman (1956) and Kasdon (1956) explored this possibility in greater detail. Vaginal fluid 6-phosphogluconic dehydrogenase activity has also been studied in this connection.

2. β-Glucuronidase

Fishman (1956) found that the β-glucuronidase activity of the neoplastic tissue in invasive cancer of the uterus ranged from 640 to 2020 and averaged 1125 units/gm tissue. These values were substantially higher than the range of 51–482 and the average of 168 units/gm of normal cervical tissue. It may be postulated that the β-glucuronidase would leak out of the cervical tissue, particularly the neoplastic tissue and, thus, increase the enzyme content of the vaginal fluid. Kasdon (1956) found that 11.4% of 542 observations on 386 premenopausal women without cancer of the cervix had vaginal fluid β-glucuronidase activities about 400 units/ml. In contrast, 80% of 25 observations in a series of 16 premenopausal patients with untreated primary invasive cancer of the cervix had values above this level. Lawson (1969) has more recently reviewed his findings in Wales. High readings for vaginal β-glucuronidase were obtained in 20% of 207 premenopausal controls, 37% of 49 postmenopausal controls, 76% of 72 premenopausal cervical cancer patients, and 80% of 84 postmenopausal cervical cancer patients. Five cervical carcinomas *in situ* gave one low and four high readings.

But conditions unrelated to cervical cancer, such as trichomonas vaginitis, estrogen deficiency states, or gonorrhea, may yield high read-

ings (Kasdon, 1956). Lawson (1969) has pointed out other complications in the performance of the vaginal β-glucuronidase assay such as the occasional inability to aspirate enough material for accurate weighing and assay and the instability of the aspirate at temperatures higher than 4°C. These factors, and, in addition, the occurrence of high vaginal β-glucuronidase activities in about 20–30% of normal individuals and an equivalent fraction of normal activities in patients with cancer of the uterus have limited greatly the further consideration of this vaginal fluid enzyme as a diagnostic aid.

3. 6-Phosphogluconic Dehydrogenase

6-Phosphogluconic dehydrogenase is one of the enzymes in the phosphogluconate oxidative pathway. It is Mn^{2+}-dependent and catalyzes the interaction of 6-phosphogluconic acid with NADPH to form CO_2 and D-ribulose 5-phosphate. Following various reports that the activity of this enzyme was elevated in regenerating liver cells and other regenerating tissues, Bonham and Gibbs (1962) found the following distribution of activity, expressed as units per gram dry weight, in 93 patients without any uterine malignancy: 0–30 units, 97%; 30–100 units, 0%; and over 100 units, 3%. In contrast, 100% of 46 patients with uterine malignancies had vaginal fluids with activities of over 100 units/gm dry weight. These uterine neoplasms included carcinomas of the corpus and of the cervix as well as a few cases of uterine and vaginal sarcoma and squamous carcinomas of the vulva and vagina.

However, this promising distinctiveness failed to be confirmed by subsequent reports (Table 18-6). The fraction of 6-phosphogluconic dehydrogenase activities above a defined upper limit ranged from 3 to 31% in women without evidence of malignant tumors of the uterus. In patients with uterine malignancies, the fraction of activities above the upper limit, that is, of "positive tests" ranged from 100 to 57%. Spuriously high values may be given by several non-neoplastic conditions. For example, it can be calculated from the data of Hoffman and Merritt (1965) that 47% of patients with trichomonas vaginitis; 53% of women with cervicitis, erosions, or polyps; and 56% of patients with menopausal vaginitis resulting from estrogen deficiency gave "positive" tests or enzyme activities above the upper limit of normal. Other conditions or non-neoplastic diseases that yield high values are monilial infection, nonspecific vaginal discharge, ingestion of contraceptive pills, and amenorrhea (Bell and Egerton, 1965).

Several attempts have been made to express vaginal 6-phosphogluconic dehydrogenase activity in such a manner as to accentuate the differences

TABLE 18-6

Activities of 6-Phosphogluconic Dehydrogenase in Vaginal Fluid of Normal Women and Women with Uterine Malignant Neoplasms[a]

Investigators	Upper limit of normal (as units/gm dry wt)	No malignancy			Uterine malignancy		
		No. of cases	With normal values (%)	With values above upper limit of normal (%)	No. of cases	With normal values (%)	With values above upper limit of normal (%)
Bonham and Gibbs (1962)	100	93	97	3	46	0	100
Longnecker and White (1965)	100	108	76	24	74	20	80
Cameron and Husain (1965)	80	515	69	31	66	11	89
Nerdrum (1964)	100	106	72	28	50	12	88
Hoffman and Merritt (1965)	80	840	97	3	223[b]	43	57

[a] The percentages were calculated in most instances from the data of the various investigators.
[b] Represents the number of tests on 126 cancer patients.

between normal subjects and those with uterine neoplasms. Lawson and Watkins (1965) described the activity in units per protein unit, rather than as other investigators had done, in terms of the dry weight. They obtained a positive test in 90% of 49 cases of cervical cancer, but the incidence of positive tests was also high in the controls, namely, 47% of 64 cases. Gibbs *et al.* (1968) pointed out that the determination of potassium in the vaginal fluid specimen would not only be easier to perform but, since this ion is chiefly intracellular, would also give a reasonable estimate of the cell content of the specimen. Expression of the enzyme activity as the enzyme–potassium ratio rather than as the enzyme–dry weight ratio appeared to possess no advantage in distinguishing between premenopausal normal women and women with uterine carcinomas, but was more successful in comparable postmenopausal groups.

Cytological tests in the detection of early stages of uterine cancer have proved their value, but require, of course, highly trained technologists and the supervision by pathologists. Not all areas of the world are well equipped in this respect. The aim in devising a reliable, rapid, and simple method for determining enzyme activities in vaginal fluid is indeed praiseworthy, for such a test would tend to reduce time-consuming and subjective aspects and facilitate still further the screening of large populations. However, the development of a clinical enzyme procedure demands scrupulous attention to the conditions necessary for precise and valid measurement of enzyme activity in general. For example, Sanner *et al.* (1970) have shown that the simple process of diluting the vaginal fluid and storing it at $-20°$ may introduce considerable error. Spurious values for the upper limit of normal may thus be set and, spurious or not, such values may not hold for another investigator who introduces some variation into his method. We have also seen that the activity of vaginal 6-phosphogluconic dehydrogenase is dependent not only on the neoplastic change in the uterine cell but may also be influenced by the presence of local infections or by altered hormonal states. In view of these considerations, it is not surprising that initial reports, optimistic in their diagnostic outlook, have tended to be counteracted by subsequent reports.

VI. Hormonal Aspects of Uterine Neoplasms

A. General

For over 40 years, the literature has contained the recurring concept that a basic endocrinological disturbance underlies the development of

endometrial carcinoma (Charles *et al.*, 1965; Dunn and Bradbury, 1967). Specifically, the existence of abnormal estrogenic stimulation, abnormal pituitary function, decreased glucose tolerance, obesity, and infertility have been taken to support this concept, although many opposing data have also been obtained. We shall briefly consider the present status of each of these endocrine aspects in the development of endometrial carcinoma.

Charles *et al.* (1965) found that pituitary gonadotropin excretion was within the normal range in 6 patients with endometrial carcinoma, abnormally low in 2 others prior to hysterectomy and salpingo-oophorectomy and in 1 patient prior to radium implantation. Operation was followed by an increase in pituitary gonadotropin excretion in 3 of the 6 patients who were studied. Estrogen levels were within normal levels preoperatively, and increased following operation in 5 of the 6 cases and in the patient given radium therapy. Urinary excretions of 17-ketosteroids and 17-hydroxycorticosteroids were normal in 4 patients in whom these were studied, and increased postoperatively in 2 of these cases. In general then, it seemed that the presence of endometrial carcinoma did not alter the gonadotropin, estrogen, or ketosteroid excretion and, according to Charles *et al.* (1965), the effects following operation appeared to be those of adrenocortical stimulation rather than of the removal of the carcinoma.

B. Estrogen Metabolism

The problem of estrogen metabolism in endometrial carcinoma has been treated more definitely by Hausknecht and Gusberg (1969). Table 18-7 shows that there were no significant differences between normal postmenopausal women and those having endometrial carcinoma, either with respect to excretion of the total amount of each of the estrogens or with respect to the metabolism of intravenously infused labeled estradiol. It is possible that the patients with endometrial carcinoma might have differed from their normal controls with respect to other urinary metabolites of estradiol, such as methoxyestrone, epiestriol, or α-ketols, but this aspect was not investigated. After administration of labeled estradiol, the specific activity of the excreted estriol was significantly less than that of the excreted estrone in both the control group and in the patients with endometrial carcinoma. This difference probably resulted from one or more other precursors of urinary estriol, and was consistently smaller in patients with endometrial carcinoma than in the control group. This finding indicated the possibility that the patients with endometrial carcinoma produced less precursor.

TABLE 18-7

Estrogen Metabolism in Normal Postmenopausal Women and Those with Endometrial Carcinoma[a]

Excretion	Estrone excretion in		Estradiol excretion in		Estriol excretion in	
	Normals	Patients with endometrial carcinoma	Normals	Patients with endometrial carcinoma	Normals	Patients with endometrial carcinoma
Urinary recovery (in %) of intravenously infused [H^3]estradiol[b]	9.6 ± 0.9	8.1 ± 0.6	4.4 ± 0.5	4.4 ± 0.5	14.8 ± 1.0	12.2 ± 0.8
Urinary excretion (as μg/24 hours) of unlabeled estrogens[c]	3.5 ± 0.5	3.4 ± 0.5	0.9 ± 0.1	1.1 ± 0.1	7.8 ± 0.8	6.9 ± 0.6

[a] Based on data of Hausknecht and Gusberg (1969).

[b] Represents mean values ± SE from 17 normal postmenopausal women with an average age of 63 years and of 34 postmenopausal women with endometrial carcinoma and an average age of 61 years.

[c] Represents mean values ± SE from 16 normal postmenopausal women and of 21 postmenopausal women with endometrial carcinoma.

The urinary excretion of other steroid hormones has also been explored. We have already noted the results of Charles *et al.* (1965). De Waard *et al.* (1968) found that the excretion of etiocholanolone relative to that of 17-hydroxycorticosteroids (17-OHCS) or to estrogens in a group of 27 patients who had been operated on for endometrial carcinoma was statistically significantly less than that in a control group of 34 normal women. We have previously described the attempts to develop a discriminant function for breast carcinoma (Chapter 17, Section II,C,1). In a similar attempt to develop such a function for endometrial carcinoma, de Waard *et al.* (1968) determined the urinary excretion of estrone, estradiol, estriol, DHA, etiocholanolone, androsterone, total 17-KS, and 17-OHCS, and attempted various combinations. The most useful for classifying patients into those with and without endometrial carcinoma appeared to be equations involving the excretions of etiocholanolone and 17-OHCS, and of etiocholanolone and estriol. In general, women with endometrial carcinoma excreted less etiocholanolone relative to 17-OHCS or to estrogens, chiefly estriol, than did healthy controls.

C. Hypothalamic-Pituitary Function

The relationship between hypothalamic-pituitary function and the presence of endometrial carcinoma has been investigated chiefly by 4 groups of workers (Dilman *et al.*, 1968; Wynder *et al.*, 1966; Glick *et al.*, 1965; Benjamin *et al.*, 1969, 1970; Dilman, 1970). In the study of Dilman *et al.* (1968), the average age of the patients with endometrial carcinoma and of their normal controls was close to 60 years. Patients with endometrial carcinoma, as compared with the control groups, had a significantly lower excretion of total gonadotropins, 121 ± 17.9 (SE) versus 220 ± 15.0 (SE) MUU per 24 hours; a significantly higher excretion of total phenolic steroids, 52.8 ± 2.5 (SE) versus 38.4 ± 2.66 (SE) μg per 24 hours; and a significantly higher concentration of plasma free fatty acids, 915 ± 9.1 (SE) versus 480 ± 15.6 (SE) μEq/liter. The excretions of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and the classic estrogens, namely, estrone, estradiol, and estriol, were the same in both the normal control group and in the cancer group. The observation concerning the estrogens is in agreement with the results of Hausknecht and Gusberg (1969) which we have already described. Thin layer chromatography of the phenolic urine extracts from the patients with endometrial carcinoma showed the presence of two additional spots between 16-ketoestradiol and 16-epiestriol (Dilman *et al.*, 1968), a finding in general agreement with that of Hausknecht and Gusberg (1969) which we noted earlier.

As we noted in Chapter 1, there have been several reports in the literature that carbohydrate metabolism is impaired in patients with endometrial carcinoma. We shall presently discuss this in greater detail, but this finding as well as other clinical and biochemical observations recorded in the literature (Wynder *et al.*, 1966; Glick *et al.*, 1965) suggested the presence of an underlying pituitary disorder in patients with endometrial carcinoma. Benjamin *et al.* (1969) undertook to study the secretion of growth hormone (HGH) in response to glucose administration in 10 such patients as well as in 10 controls, suitably matched with respect to age, degree of obesity, and exhibition of abnormal glucose tests with normal fasting blood glucose levels. All controls and patients were placed on a diet containing 300 gm or more of carbohydrate for at least a week before the tests. Figure 18-5 shows a triphasic curve in patients with endometrial carcinoma for the levels of serum HGH during the 6 hours following the oral ingestion of a test dose of glucose. The mean values for serum HGH were significantly higher in the patients with endometrial carcinoma at 1, 2, and 3 hours than in the control subjects at these times. The levels of serum insulin rose sharply in both groups from a fasting level of about 20–25 μU/ml to peak values of about 150–180 μU/ml between the first and second hours, then declined. There were no statistically significant differences between the values at any time. Although Benjamin *et al.* (1969) offered no specific mechanism for the abnormal serum HGH response, they suggested that their findings lent support to the hypothesis of an underlying hypothalamic-pituitary disorder in cases of endometrial carcinoma.

The findings of Dilman *et al.* (1968) that patients with endometrial carcinoma had a significantly lower excretion of total gonadotropins, a significantly higher excretion of total phenolic steroids, and a significantly elevated level of plasma free fatty acids led them to postulate that hypothalamic-pituitary hyperactivity plays a role in the development of endometrial carcinoma. However, it was pointed out that this phenomenon develops normally with advancing age, and later Dilman (1970) noted that the loss of suppression of the level of HGH had been observed by his group not only in endometrial carcinoma but in patients with breast cancer and, more importantly, in patients with coronary heart disease. In rebuttal, Benjamin *et al.* (1970) pointed out that age alone cannot explain the abnormal growth hormone-response, since, as has already been noted, both their groups of patients and controls were matched with respect to age, degree of obesity, and glucose tolerance tests. The question remains whether patients with nonuterine cancers or, indeed, with other grave disease would show similar or even equivalent manifestations of hypothalamic-pituitary disturbances.

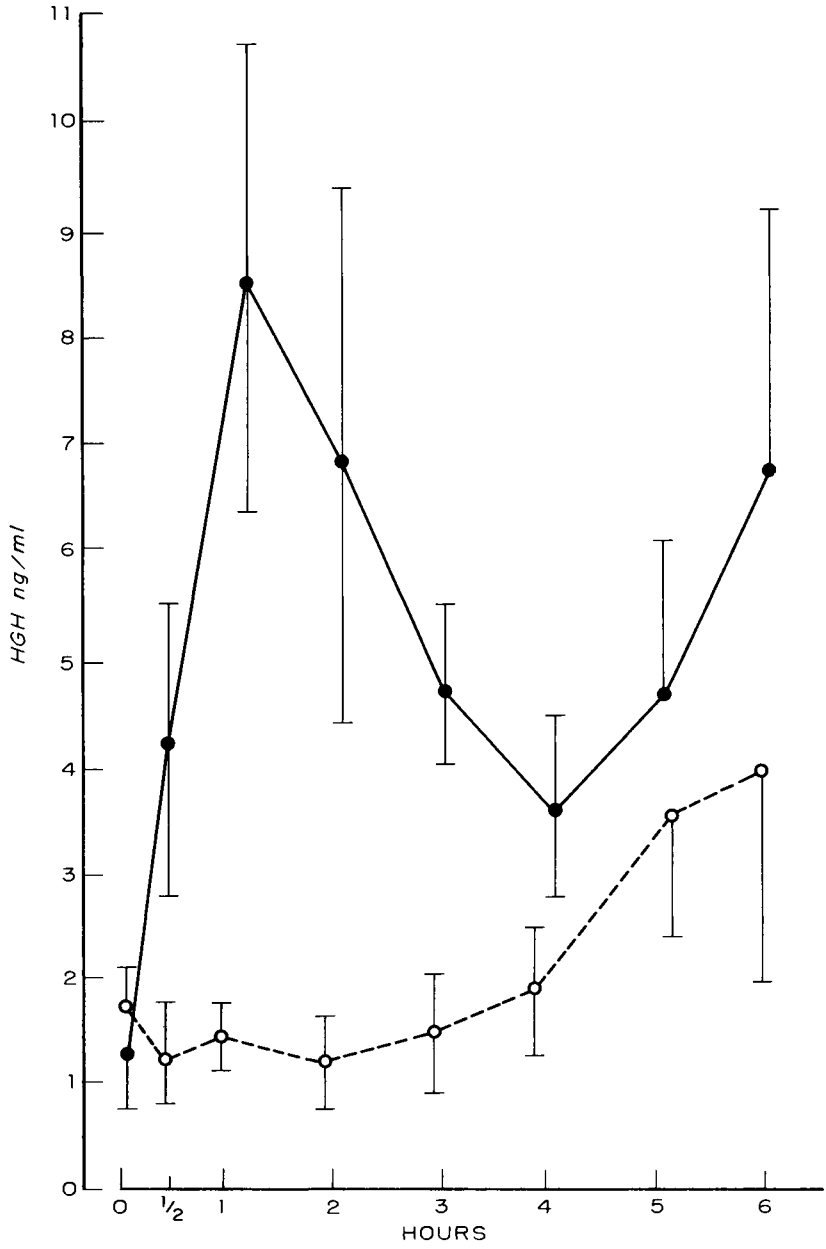

Fig. 18-5 Levels of serum HGH during oral glucose tolerance tests in patients with (●) endometrial carcinoma and (○) controls (mean ± SE). From Benjamin *et al.* (1969). Reproduced by permission of the *New England Journal of Medicine.*

D. Carbohydrate Metabolism

In Chapter 1 (Section III,B), we pointed out that, since the report of Freund in 1885, the literature had contained reports which indicated a substantial frequency of elevations of fasting blood glucose and of decreased glucose tolerance in patients with cancer. It was also noted that a similar incidence of these effects had been reported in patients with noncancerous disease such as tuberculosis, chronic vascular or chronic neurological disease (McBrayer, 1921; Weisenfeld *et al.*, 1962). It was possible that factors common to all grave disease, such as inanition or carbohydrate deprivation, might have been the basis for the hyperglycemia and hyperglycemic response reported in the earlier studies in the literature. More recent studies have, however, attempted to eliminate the various factors that may influence the glucose tolerance curve. Subjects have been allowed either measured or unrestricted carbohydrate diet for several days before the performance of the test, and an attempt has been made to eliminate other exogenous factors such as infection or undue emotional stress (Benjamin and Romney, 1964).

Of the various types of malignancies in which carbohydrate metabolism has been studied, endometrial carcinoma has perhaps received most attention. Benjamin and Romney (1964) compiled a list of 13 such studies in which the frequencies of diabetes mellitus ranged from 3 to 72% in the patients with this type of carcinoma. Dunn *et al.* (1968) have listed 25 studies of patients with this neoplasm during the years 1937 to 1964 in which the frequency of diabetes mellitus ranged from 1.3 to 22.0%. Such discrepancies have been ascribed by Benjamin and Romney (1964) to the criteria used for the diagnosis of diabetes mellitus. In many cases, a note in the medical chart that the patient had a history of diabetes constituted the diagnosis and might be reliable or not. In other instances, glycosuria or a high fasting blood sugar was the basis for the diagnosis. In those studies in which glucose tolerance tests had been performed, the incidence tended to be high since borderline cases were revealed. However, many of these studies had no adequate control groups.

But even recent studies with more rigid criteria for abnormalcy of glucose tolerance curves and with more careful selection of control groups have failed to yield any consistent results in the incidence of disturbed carbohydrate metabolism in patients with endometrial carcinoma. For example, Benjamin and Romney (1964) obtained decreased tolerance curves in 52% of 50 patients with endometrial carcinoma from Capetown, in 64% of 25 such patients from New York City, and in 20% of 50 patients with cervical carcinoma. These may be contrasted with

the incidences of 22% in 100 age-matched control gynecological patients with normal endometria from Capetown and of 20% in 25 similar control patients from New York City.

Although this study appeared to yield at last a high and statistically significant frequency of disturbed glucose tolerance in patients with endometrial carcinoma yet, only a few years later, Dunn *et al.* (1968) failed to obtain such a distinction between a group of 55 patients with endometrial carcinoma and a control group of 114 patients. Factors which might possibly influence glucose tolerance, such as obesity, age, parity, hypertension, or anterior pituitary function, were evaluated. The incidences of probable and definite diabetic glucose tolerance curves in the control groups were high, namely, 37 and 45%, respectively, and were not significantly different from the corresponding incidences, 33 and 47%, respectively, in the 55 patients with endometrial carcinoma. It does not appear possible at the present time to offer a firm basis for the widely differing incidences of altered carbohydrate metabolism in endometrial carcinoma reported by various investigators.

E. Biochemical Effects of Treatment of Endometrial Carcinoma with Progesterone and Other Hormones

Some of the useful chemotherapeutic agents in the treatment of metastatic carcinoma of the endometrium are progestational agents, such as 17α-hydroxyprogesterone-*N*-caproate (Delalutin) and 17α-hydroxy-6α-methylprogesterone acetate (Provera). Approximately one-third of metastatic endometrial carcinomas temporarily regress on such treatment (Kelley and Baker, 1965). This effect has permitted a study of the changes in some biochemical components of the neoplasms.

The enzyme profile of endometrial carcinoma was studied histochemically during treatment of patients with substituted progesterone caproates (Thiery and Willighagen, 1968). Serial samples of the primary neoplasms were obtained by aspiration or curettage before and during treatment, and appropriate serial sections were treated to demonstrate several enzyme activities. The resulting enzyme profile was complicated, but increases in activities of acid phosphatase, decreases in 5′-nucleotidase, and decreases in activities of various dehydrogenases appeared frequently.

Nordquist (1970a,b) has studied the synthesis *in vitro* of DNA and RNA in normal carcinomatous human endometrium. The synthesis of DNA and RNA in the normal endometrium was dependent on the phase of the menstrual cycle, increasing during the proliferative phase, reaching

a maximum on the sixteenth day of the cycle, and then decreasing again. Preincubation of the normal endometrium with 80 μg progesterone per ml led to a mean reduction of DNA synthesis to 64% and/or RNA synthesis to 52% of the corresponding control values (Nordquist, 1970a). Progesterone at this concentration, showed a more marked effect on the carcinomatous endometrium. DNA synthesis was reduced to a mean value of 46%, and RNA synthesis to 39% of the control values (Nordquist, 1970b). Estradiol, at a concentration of 2 μg/ml, had negligible effects on the synthesis of DNA or RNA in either normal or carcinomatous endometrium.

VII. Receptors in Uterine Carcinomas

A. Receptors in Normal Rat Uterus

As a preliminary to a discussion of receptors in breast carcinoma, we considered briefly in Chapter 17 (Section II,D,1) some of the general aspects of the binding and fate of sex steroids in target organs. It seems appropriate to consider these aspects more fully in the uterus. The existence of estrogen receptors was first indicated in 1958 by Jensen, and described in detail in 1962 (Jensen and Jacobson, 1962). When [^{3}H]estradiol-17β was administered subcutaneously to 23-day-old female rats, it was found that the levels of radioactivity in the liver, kidney, muscle, adrenals, and bone ran parallel to those in the blood, reaching a maximum activity within 15 minutes, and then declining rapidly. In contrast, the radioactivity in the uterus and vagina reached a maximum at about 1–2 hours after injection, and persisted at high levels for several hours.

The nature of the macromolecules and, more specifically, of the uterine proteins that combine with the various estrogens is obviously of interest. Noteboom and Gorski (1965) reported that the estrogen receptor was stereospecific and probably a protein. Subcellular fractionation of rat uterine cells following injection of tritium-labeled estradiol indicated that approximately 30% of the receptor was in a soluble (105,000 *g* supernatant) fraction and 50% was in a heavy nuclear-myofibrillar fraction. Further definition of the receptor–estradiol complex was achieved by Toft and Gorski (1966) who found that the 105,000 *g* supernatant fraction (cytosol) contained a component that migrated with a sedimentation coefficient of 9.5S. Disruption of the complex by proteases, but not by nucleases, indicated that the complex was largely protein. Jensen *et al.* (1968) confirmed this observation and, in addition, found that the uterine nuclei contained another estradiol–receptor complex

which could be extracted by cold 0.3 *M* KCl and sedimented at about 5S.

The dynamic aspects of these findings were elaborated by Shyamala and Gorski (1969) who showed that the estrogen first moves into the cytosol where it is bound to a large, approximately 9S protein, and then subsequently migrates into the nucleus where it is bound to a 5S protein which can be extracted from the chromatin. The nature of the complexes in the natural intracellular state has been further investigated by Chamness and McGuire (1972) who found that, at physiological ionic strength (0.15 *M* KCl), the cytoplasmic form sediments at 6S, in contrast to values at 8–10S obtained by previous investigators at low salt concentrations and 4S obtained at high salt concentrations, usually 0.3–0.4 *M* KCl. In contrast, receptor extracted from nuclei sediments at 4.5S, either at physiological or higher salt concentration. This behavior appears to provide a criterion for distinguishing between the nuclear and cytoplasmic receptors, although either form can be made to assume any value between 4S and 9S by adjusting the concentration of the polyanion heparin in the sucrose gradient medium.

B. Receptors in Chick Oviducts

The chick oviduct system has been used to characterize progesterone-binding components. Without reviewing the substantial literature that is already present in this field (O'Malley *et al.*, 1971a), a few investigations may be cited in illustration. O'Malley *et al.* (1969) found that, after injection of radioactive progesterone, a major fraction of the labeled steroid was present in the cytoplasmic supernatant fraction.

Employing an aqueous extract of chick oviduct, Sherman *et al.* (1970) studied the *in vitro* interaction of the cytoplasmic components with the labeled progesterone. The resultant complex was isolated by sucrose gradient centrifugation, polyacrylamide gel electrophoresis, enzymic digestion, and gel filtration on Agarose. The radioactive steroid in the isolated complex was progesterone, and not a metabolite. The presence of protein in the complex was inferred from the destruction of the complex by *p*-hydroxymercuribenzoate and by a protease preparation. The apparent number and size of the cytoplasmic binding components varied with the concentration of KCl used in the isolation and with other features of the technique of isolation and detection. These binding components were shown to be tissue-specific, heat-labile acidic proteins with an average dissociation constant for progesterone of about 8×10^{-10} *M* at 1°C in the presence of 0.3 *M* KCl.

The nuclear components of chick oviduct that bind progesterone were

studied by O'Malley *et al.* (1971b). The nuclear pellet was obtained by centrifuging chick oviduct homogenate at 850 *g* and extracting with Tris-EDTA buffer. The gradual but steady increase of nuclear binding components with time following injection of [^{3}H]progesterone *in vivo* or incubation of oviduct slices *in vitro* with [^{3}H]progesterone indicated a transfer of the protein–progesterone complex from cytoplasm to nucleus. The cytoplasmic and nuclear components appeared identical. These findings support the hypothesis that the cytoplasmic macromolecular–steroid complex is transferred, probably under the inflence of hormone, to the nucleus.

C. Receptors in Human Uterine Carcinomas

The studies on rat uterus and chick oviduct, which we have just described, have served as excellent models for determining the various aspects of steroid binding in these tissues. The situation with human material is much less satisfactory and has not yet reached the levels of insight provided by the animal material. Brush *et al.* (1967) found that normal human endometrium could take up intravenously administered [^{3}H]estradiol at all phases of the menstrual cycle. In the follicular phase, 79% of the [^{3}H]estradiol which gained access to the cell was transferred to the nuclei as contrasted with the transfer of a much smaller fraction, 22%, in the luteal phase (Taylor *et al.*, 1971).

Similar studies involving a preoperative intravenous injection of [^{3}H]estradiol into patients with various genital tract malignancies yielded the following results (Taylor *et al.*, 1971). A mixed mesodermal tumor and a carcinosarcoma took up some administered estradiol, but there was no transfer to the nuclear fraction of the tumor tissue. A squamous carcinoma of the vagina, an adenocarcinoma of the cervix, and 3 cases of poorly differentiated carcinoma of the endometrium all took up estrogen avidly, and significant proportions of these, namely, 28%, 75%, and an average of 35% for the 3 carcinomas of the cervix, respectively, were present in the nuclear fraction of the cells. In 18 cases of well-differentiated carcinoma of the endometrium, an average of 41% of the radioactivity was transferred to the nuclear fraction. Although the extent of transfer of radioactivity in this group of patients with endometrial carcinoma was substantial, it was midway between that occurring in the follicular and luteal phases of the normal endometrium, and was not particularly distinctive. In all tumors where appreciable transfer to the nuclear occurs, the process may be considered hormone-influenced.

The *in vitro* binding of progesterone by normal and neoplastic human endometrium has been recently studied by Haukkamaa *et al.* (1971).

Specimens of endometrium were obtained, in most cases, as a result of hysterectomies and, in some, for diagnostic curettage. The tissues were homogenized and diluted to a protein concentration of 100 mg per 100 ml, and an equilibrium dialysis method was used for determining the binding. To evaluate the extent of nonspecific binding by the endometrial proteins, the samples were also dialyzed against human albumin solution.

With regard to endometrium from normal women, the highest specific binding rates were observed during the late proliferative and secretory phases of the menstrual cycle. Atrophic endometrium appeared to be completely devoid of specific progesterone binding. Hyperplastic endometrium yielded specific values ranging from 0 to 73%, but no correlation could be elicited between these values and the degree of hyperplasia as judged by morphological criteria. Six cases of endometrial carcinoma yielded very irregular results with the specific binding fraction being zero in 2 cases, and ranging up to 60% in a poorly differentiated carcinoma. In general, there was no correlation between the degree of differentiation of the tumor and the progesterone-binding capacity.

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Subject Index

A

Acetylkynurenine
  structure of, 136
  urinary excretion of
    in cancer of the bladder, 140
    in normal persons, 140
Acid phosphatase, 61–71, 233–237
  in human semen, 65
  leukocytic, 233–235
    isoenzymes, 233–235
    isoenzymes in leukemias, 233–235
    in leukemias, 233
    in leukemic reticuloendotheliosis, 234, 235
  of placenta, 63
    isoenzymes of, 63
    properties of, 63
    purification of, 63
  of prostate, 62
    isoenzymes of, 62
    kinetics of, 63
    in serum, 65–71
  of tissues, 62–65
    isoenzymes of, 62, 63
    kinetics of, 63
    purification of, 62
Acromegaly
  bone formation rate in, 340
  bone resorption rate in, 340
  calcium phosphorus metabolism in, 339–341
  carbohydrate metabolism in, 337–339
  clinical symptoms of, 334, 341
  and effect on other hormones, 341–343
  exchangeable calcium in, 340
  hypertension in, 543, 544
  incidence of, 334
  nature of tumor in, 333–334
  renal function in, 342
  serum GH levels in, 335–337
  and size of pituitary tumor, 337
Adenosine 3′,5′-cyclic monophosphate (Cyclic AMP)
  in calcium metabolism, 287
  in phosphorus metabolism, 287
  in primary hyperparathyroidism, 301
Adrenal rest tumors of testis, 479
Adrenocortical steroids, 357–372
  and biochemical effects, 372
  and dihydroxycholesterol, 361
  normal biosynthesis of, 359–361, 363
  normal concentrations in plasma, 364, 365, 367–370
  normal metabolism of, 361–363

and plasma cortisol, 365
episodic secretion of, 366
secretion of, 359
structures of, 358–359
systematic names of, 360
systematic names of related steroids, 360
Alanine aminotransferase, 98–102
action of, 100
Aldolase
action of
on fructose 1,6-diphosphate (FDP), 43
on fructose 1-phosphate (F-1-P), 43
FDP/F-1-P ratio of, 44
in brain tumors, 47
in fetal brain, 47
in neoplastic tissues, 46, 47
in normal brain, 47
in normal tissues, 46, 47
isoenzymes of, 43–44
hybrids of, 47
in various tissues, 44, 45
Aldosterone-producing tumors, 391–398
and adrenal venography, 397
and biochemical diagnostic procedures, 396–398
clinical symptoms of, 391
and photoscanning, 397
and plasma aldosterone, 392–393
and plasma electrolytes, 392–393
and plasma renin, 392–393
and primary aldosteronism, 394–396
and adrenocortical carcinoma, 395
types of, 394
and secondary aldosteronism, 396
steroid content in, 392
urinary excretion of aldosterone, 393
Alkaline phosphatase, 71–83
of bone, 73, 74, 79
electrophoretic properties of, 76, 79
of intestine, 73, 74, 79
isoenzymes of, 72–75
effect of low pH, 73
effect of neuraminidase on, 73, 75
effect of L-phenylalanine on, 74
effect of urea on, 73
immunochemical properties of, 73, 74
inhibition by bile acids, 72, 73
methods of differentiation, 72, 73
resistance to denaturation by heat, 73
isoenzymes in serum, 77
kinetic properties of, 75, 76
leukocytic
in leukemias, 237
new type of, 237
in normal persons, 236, 237
of liver, 73, 74, 79
of placenta, 73, 74, 79
Allopurinol, effect on uric acid excretion, 260–262
Amino acids
metabolism of, 3
enzymes in, 3
Amylase, types of, 93, 94
α-Amylase
action of, 94
activity in tissues, 94
Androblastoma of testis, 475–477
hormonal studies in, 477
incidence of, 476, 477
Androgens
adrenocortical production of, 458
metabolic clearance rate of, 457
metabolism of, 385
secretion rate of, 457
structure of, 457
testicular production of, 457
Antibody formation, 111
mechanism involved in, 112
Antidiuretic hormone (ADH), 541–542
effects of, 541, 542
structure of, 542
Arrhenoblastoma, 505–509
and estrogen excretion, 507, 508
and excretion of other steroids, 507, 508
histology of, 505, 506
and 17-ketogenic steroids, 507
and 17-ketosteroid excretion, 506
and plasma steroids, 509
and plasma testosterone, 507
and pregnanetriol excretion, 507
Arteriovenous differences, 35
for glucose, 35
for lactate, 35
in tumor-containing tissues, 35

Arylamidase, 98–99
isoenzymes of, 98
electrophoretic behavior of, 99
Aspartate aminotransferase, 99–104
action of, 100
in human tissues, 100–102
isoenzymes of, 101, 103
properties of, 100
in serum, 102, 103
in cancer, 103, 104
Aspartate carbamyltransferase, 247
Autopsy tissue, 38
for enzyme analysis, 38

B

Bence Jones protein, 122, 123
in macroglobulinemia, 124
in multiple myeloma, 122, 123
physical properties of, 123
structure of, 123
Blood–brain barrier, 17, 19
Blood components, *see* Serum
Blood glucose, *see also* Serum
in normal individuals, 14, 35
in patients with cancer, 14, 35
recycling of, 35
Bone
accretion rate, 283, 284
calcium in, 273
chemical composition of, 273
fluoride in, 274
magnesium in, 273
organic composition of, 275
phosphorus in, 273
potassium in, 273
resorption rate, 284–285
sodium in, 273
strontium in, 274
structure of, 276
Brain tumors, 48
aldolase isoenzymes in, 48
Breast cancer tissue
adenosinetriphosphatase, 584
alkaline phosphatase, 584
calcium content, 584
dehydrogenases, 583
glycolytic enzymes, 583, 584
in vitro sensitivity to hormones, 572–574
determination of hormonal dependency, 573
as a prognostic aid in treatment, 574
technique of, 572
types of hormonal dependency, 573
phosphohexose isomerase, 583
uridinetriphosphatase, 584
Brenner tumors, 523–524
masculinization in, 523–524

C

Cachexia, 27–29
biochemical aspects of, 27, 28
catabolism of amino acids in, 28
lysosomal enzymes in, 29
tissue lysosomes in, 28
Calcification, 276, 277
and alkaline phosphatase, 277
osteoblasts in, 276
osteocytes in, 277
Calcitonin, 435
action of, 435
and histamine wheal, 443
in medullary thyroid carcinoma, 434–440
purification of, 435
in serum, 436–439
Calcium
content in body, 274
daily intake of, 278
relation to excretion, 278, 279
intestinal absorption of, 278
metabolism of, 277–279
and bone accretion rate, 283, 284
and bone resorption rate, 284–285
cellular mechanisms in, 287–289
cyclic AMP in, 287
dynamic aspects of, 282–283
and exchangeable calcium pool, 283
regulation of, 286–289
and renal calcium clearance, 285, 286
and renal phosphorus clearance, 285, 286
requirement in man, 277–278
Cancer, *see* Carcinoma, Neoplasms
Cancer tissue, 34
glycolysis in, 34

Carbohydrate metabolism, 13–21
  blood glucose in, 14
Carcinoembryonic antigen (CEA), 198–199
  in colonic carcinoma, 198
  in nonneoplastic disease, 198–199
  normal values for, 198
  in other neoplasms, 198
Carcinoma
  of the breast
    and the Bulbrook discriminant, 501–563
      in early cancer, 563, 564
      in metastatic cancer, 562–563
      in prospective studies, 564–566
      and response to adrenalectomy, 562–563
      and response to hypophysectomy, 562–563
    estrogen metabolism in, 554–556
      clearance of estrogen from plasma, 554
      formation of estriol from estradiol, 555
      infusion of radioactive estrogen, 554, 555
      urinary excretion of estrogens, 556
    incidence of, 553
    and the incremental ratio discriminant, 566, 567
    mortality rate of, 553
    receptors in, 567–575
      assays for, 568–570
      and *in vivo* binding of estradiol, 568
      as a prognostic aid in treatment, 571
    serum fucose in, 586
    steroid conversions in, 557
    sulfurylations of steroids in, 556–557
    tissue lipid content of, 24, 586
    tryptophan metabolism in, 142, 584, 585
    urinary excretion of 17-OHCS and etiocholanolone, 561–563, 567
    urinary excretion of steroids, 560–567
  of uterus
    incidence of, 592
    mortality from, 592–593
    receptors,
      in neoplastic human uterus, 631–632
        of estradiol, 631
        of progesterone, 632
      in normal chick oviduct, 630, 631
      in normal rat uterus, 629, 630
Carcinoid syndrome, 145–156
  clinical symptoms and biochemical findings in, 151, 154
  kinins in, 153
  metastases in, 151
  prostaglandins in, 154
  variants of, 150, 151
Carcinoid tumors, 145–146
  bronchial, 151, 152
  clinical symptoms in, 146
  genesis of, 145
  incidence of, 145
  metabolites in tissues, 148
  of ovary, 522–523
  tryptophan metabolism in, 146, 147
Catecholamines, *see also* Epinephrine, Norepinephrine
  in neuroblastoma, 159, 174–180
  in pheochromoblastoma, 171–173
  in pheochromocytoma, 169, 170
Cerebrospinal fluid, 17–18
  circulation of, 17
  glucose in neoplastic involvement of meninges, 17–19
  protein in brain tumors, 17
Chondrosarcoma, 306
  serum alkaline phosphatase in, 306
Choriocarcinoma, 603–613
  effect of methotrexate, 609, 610
  estrogen excretion in, 608, 609
  extragenital, 612–613
  gonadal, 610–612
    biochemical findings in, 611
    incidence of, 611
  HCG excretion, 604, 606
  histology of, 604
  incidence of, 604
  serum HCG in, 608, 609
  serum HPL in, 606, 609
  serum progesterone in, 606
Chorionic (human) gonadotropin (HCG), 467
  administration of, 467

content in hydatidiform mole, 596
serum levels
in choriocarcinoma, 605
in hydatidiform mole, 596
in pregnancy, 596
urinary excretion
in hydatidiform mole, 596
in invasive mole, 603
in pregnancy, 596
Chorionic (human) somatotropin (HCS), *see* Ectopic syndrome
Chromophobe adenoma, 347–351
and aldosterone secretion, 351
clinical aspects of, 347–348
and Cushing's syndrome, 348–349
and hyperthyroidism, 349–351
biochemical parameters in, 350
incidence of, 347
Collagen, 275
structure of, 275
Colorectal carcinoma
and CEA, 198–199
and diet, 200
differentiation of colonic cells in, 193, 194
incidence of, 185
mortality in, 185
proliferation of colonic cells in, 193, 194
purine metabolism in, 194–198
pyrimidine metabolism in, 194–198
serum alkaline phosphatase in, 200
thymidine kinase in, 197
tRNA methylase in, 200
types of, 193
Craniopharyngioma, 351–353
biochemical parameters in, 352
clinical symptoms of, 352
histology of, 352
incidence of, 352
Cushing's syndrome, 345–347, 373–384
and adrenal cortex, 373
biochemical findings in, 346–347
and Cushing's disease, 373
dexamethasone test in, 378–380
incidence of, 373, 374
in infants and children, 382–384
metyrapone test in, 380, 381
serum corticosteroids in, 375
symptomatology of, 374
types of, 373
urinary excretion
of corticosteroids, 377
of 17-ketogenic steroids, 378
Cystadenomas of ovary, 520–522
cystic fluid of, 521–522
glycoproteins in, 521
protein contents of, 521
plasma steroids in, 522
types of, 520, 521
urinary excretion of steroids in, 522
Cystathionine
excretion in neuroblastoma, 180
in methionine metabolism, 164, 165
in neuroblastoma tissue, 175
in normal tissues, 175

D

Deoxythymidine synthetase, 248
Dexamethasone, 378–381
effect on plasma 17-hydroxycorticosteroids (17-OHCS)
in Cushing's syndrome, 380
in hirsuitism, 379
in normal persons, 379
in obesity, 380
structure of, 378
Diiodotyrosine (DIT), structure of, 409
Diphosphopyridine nucleotide (DPN^+), 253
Dysgerminoma, 517–520
and clinical symptoms, 518
histology of, 518
incidence of, 518
and urinary excretion
of HCG, 519–520
of 17-hydroxycorticosteroids, 519
of 17-ketosteroids, 519
of other steroids, 519
of testosterone, 519

E

Ectopic adrenocorticotropic hormone (ACTH), structure of, 536, 537
Ectopic ACTH syndrome, 530–537
clinical features of, 530
and dexamethasone test, 530, 536
incidence of, 530

serum (plasma) ACTH in, 531–533
serum (plasma) bicarbonate in, 531
serum (plasma) cortisol in, 531
serum (plasma) protein in, 531
tumor ACTH content in, 532–535
urinary steroid excretion in, 536
Ectopic antidiuretic hormone (ADH) syndrome, 541–543
arginine vasopressin (AVP) in, 543
serum electrolytes in, 542
serum osmolality in, 543
sites of tumors in, 542
Ectopic erythropoietin syndrome, 549
Ectopic gastrin syndrome, *see* Zollinger-Ellison syndrome
Ectopic gonadotropic hormone syndrome, 539–541
and chorionic gonadotropic excretion, 541
gynecomastia in, 540
with hepatic carcinoma, 540
with lung carcinoma, 540
and testosterone excretion, 541
Ectopic human chorionic somatotropin (HCS) syndrome, 545–547
and acromegaly, 546
and osteoarthropathy, 546
relation to pituitary growth hormone activity, 545
relation to placental lactogen formation, 545
serum (plasma) growth hormone in, 546
Ectopic humoral syndromes
mechanism of, 529
types of, 529
Ectopic hyperparathyroidism
calcium and phosphorus metabolism in, 319, 321
incidence of, 317, 318
mechanisms of, 320–324
parathyroid-like hormone, 321–323
and immunoreactive parathyroid hormone, 322
and radioimmunoassay, 322, 323
structure of, 323, 324
serum calcium in, 318–320
serum chloride in, 318
serum phosphorus in, 318–320
Ectopic melanocyte-stimulating hormone (MSH) syndrome, 537–539
ACTH and MSH in, 538
of pituitary tissue, 538
of serum (plasma), 538
of site of tumor tissue, 538
Ectopic thyrotropin syndrome, 543–544
and trophoblastic tissue, 544
TSH activity in, 543, 545
Endometrial carcinoma, 621–629
biochemical effects of treatment, 628, 629
estrogen metabolism, 622–624
excretion of total gonadotropins, 624
excretions of estrone, estradiol, and estriol, 623
glucose tolerance test, 625–628
and serum HGH, 625, 626
hypothalamic-pituitary function, 624–626
serum free fatty acids, 625
Endometrium
and menstrual cycle, 486
and ovarian cycle, 486
Enzyme equilibrium constants, 37
Enzymes
in breast cancer tissue, 583–584
classification of, 33
Enzyme Commission number, 33
trivial name, 33, 34
Epinephrine
biosynthesis of, 159–161, 164
cellular localization of, 161
metabolism of, 161–164
in serum (plasma), 168, 169
in normals, 168
in pheochromocytoma, 169
Esophageal neoplasms
incidence of, 185
mortality in, 185
Extrapancreatic neoplasms, 223–225
hypoglycemia in, 224–225
mechanisms of, 224–225
incidence of, 223
types of, 223

F

Feminizing tumors
of adrenal cortex, 390–391
of testis, 473, 477

Folic acid metabolism
and formate-activating enzyme, 252
in leukemias, 250–252
and N^5,N^{10}-methylenetetrahydrofolic dehydrogenase, 252
and tetrahydrofolic dehydrogenase, 252
Follicle-stimulating hormone (FSH)
action of, 539
in menstrual cycle, 487–489
plasma (serum) levels, 489
Fructosediphosphate aldolase, 36

G

Ganglioneuroma (sympathocytoma), 159, 173–176
biochemical diagnostic procedures in, 180–181
catecholamines in tissues of, 175
histology of, 173
urinary excretion of
cystathionine, 180
dopamine, 178
homovanillic acid (HVA), 179
3-methoxy-4-hydroxymandelic acid (VMA), 179
Gastric neoplasms
incidence of, 185
mortality in, 185
Gastric secretion
in gastric carcinoma
acidity of, 187
enzymes of, 188, 189
glycoproteins in, 189
lactate dehydrogenase of, 188
normal volume, 186
acidicity of, 186
enzymes in, 187–189
glycoproteins in, 189–190
lactate dehydrogenase in, 188
Germinal cell tumors, 464–468
androgen excretion in, 464
and estrogen excretion, 468
gonadotropin excretion, 464, 465
17-ketosteroid excretion in, 464
Gigantism
cerebral, in childhood, 344–345
clinical features of, 334
Glucokinase, 36
Glucose
administration in tumor-bearing animals, 35
in blood
of normal individuals, 14, 35
in patients with cancer, 14, 35
in cerebrospinal fluid, 17
Glucose-6-phosphatase, 36
Glucosephosphate isomerase, 36
action of, 41
activities
in body fluids, 43
in serum, 42, 43, 57
in spinal fluid, 43
in tissues, 42
electrophoresis of, 42
properties of, 41, 42
purification of, 41
Glucose tolerance
in cancer, 15
factors in, 16
mechanisms of, 15, 16
Glutamic-oxaloacetic transaminase, *see* Aspartate aminotransferase
Glutamic-pyruvic transaminase, *see* Alanine aminotransferase
Glycerate phosphomutase, 36
Glyceraldehydephosphate dehydrogenase, 36
Glycolytic enzyme pattern
in human cancer tissue, 36
in leukemic leukocytes, 36, 38
in neoplastic counterparts of normal human tissue, 38
in normal human tissue, 38
in normal leukocytes, 36, 38
Glycolytic rates
aerobic and anaerobic, 34
in carcinoma, 35
of rectum, 35
of sigmoid colon, 35
of stomach, 35
of normal rat retina, 35
Glycolytic sequence, respiration in, 34
Glycosphingolipids
in human tumors, 25–26
structure of, 25
Gonadotropins, 461–466, *see also* Chorionic (human) gonadotropin, Pituitary, anterior

excretion in testicular neoplasms, 465–466
molecular weights of, 461
in serum of normal male adults, 462
Granulosa cell tumors
of ovary, 499–504
and estrogen excretion, 500–503
and estrogen metabolism, 500–504
and feminization, 500
histology of, 500
incidence of, 500
of testis, 477–478
hormonal studies of, 478
Growth hormone (GH), *see* Acromegaly
Gynandroblastoma, 509–510
and plasma steroids, 510

H

Heavy-chain diseases, 124–126
clinical findings in, 125
formation of L and H chains in, 124
types of, 125, 126
α-chain, 125
γ-chain, 126
μ-chain, 126, 127
Hepatomas in mice, 35
pH levels in, 35
Hexosediphosphatase, 36
Human chorionic gonadotropin (HCG)
content in moles, 596
production of, 595
serum concentration
in hydatidiform mole, 596, 597
in normal pregnancy, 596
urinary excretion
in hydatidiform mole, 596
in normal pregnancy, 596
Hydatidiform mole, 593–607
average age after amenorrhea, 594
estrogen excretion, 597
incidence of, 593
invasive, 602–603
HCG excretion, 602
serum HPL, 603, 607
and the normal trophoblast, 593, 594
origin of, 594
progesterone excretion, 597, 599
serum HPL in, 607
serum progesterone in, 599, 600
17-Hydroxycorticosteroids (17-OHCS)
in dexamethasone test, 379–381
in plasma
in Cushing's syndrome, 372, 376
of normal individuals, 372
urinary excretion
in adrenogenital syndrome, 388
in Cushing's syndrome, 377
in normal individuals, 371, 372
5-Hydroxyindole acetic acid (5-HIAA)
urinary excretion of, 149, 150
3-Hydroxykynurenine, 137
structure of, 136
urinary excretion of
in cancer of the bladder, 140
in normal persons, 140
Hydroxyproline, 289–290
assay of, 289
in cancer of bone, 301, 302
normal urinary excretion of, 288, 289
structure of, 289
5-Hydroxytryptamine (5-HT)
in blood, 148
in carcinoid tissue, 148
urinary excretion of, 150
Hyperglycemia in cancer, 15
Hypoglycorrachia, 17, 18, *see also* Cerebrospinal fluid
mechanisms of, 18, 19

I

Immune mechanisms, 111
Immunoglobulinopathies, 127
amyloidosis in, 127
cryoglobulinemia in, 128
renal function in, 127–128
serum sodium in, 128
viscosity syndrome in, 128
Immunoglobulins, 110–117
bursal (B) cells in formation of, 110, 114
cell types in formation of, 110
distinction between, 110
electron microscopy in, 110
electrophoretic mobility in, 110
serological criteria in, 110
cellular basis for formation of, 110, 114
macrophages in formation of, 114

metabolic properties of, 116
metabolism of, 113, 114
molecular weights of, 116
monocyte cells in formation of, 110
physical properties of, 116
rate of synthesis of, 115
sedimentation coefficients of, 116
structures of, 112, 113
synthesis of, 113, 115
heavy (H) chain in, 115
light (L) chain in, 115
thymus (T) cells in formation of, 110, 114
types of, 113
Immunoglobulins in cancer, 117–118
biochemical characteristics of, 118
Immunological deficits, 129–131
and agammaglobulinemia, 129
in chronic lymphocytic leukemia, 129
and incidence of malignancy, 130
in ataxia-telangiectasia, 130
in Bruton's disease, 130
in Wiskott-Aldrich syndrome, 130
Insulin, 212
activity of, 211
mechanisms of action, 212
Insulinomas (Beta-cell islet tumors), 211–215
blood sugar in, 213
glucose assimilation coefficient, 214
mechanisms of hypoglycemia, 212
plasma insulin in, 215
Interstitial cell-stimulating hormone (ICSH), action of, 539
Interstitial cell tumor of testis, 468–475
clinical manifestations, 469, 472, 473
and estrogen excretion, 474
and excretion of other steroids, 473, 475, 476
effect of chemotherapeutic agents on, 475
feminization in, 473
incidence of, 469
and isosexual precocious development, 469–470
17-ketosteroid excretion
in adults, 473, 475
in boys, 470
effect of chemotherapeutic agents on, 475
metabolism of testosterone and related steroids in, 475
and serum androstenedione, 471
and serum dehydroepiandrosterone, 471
and serum testosterone, 471
Iodine
incorporation into organic compounds, 408–409
metabolism of, 405–407
oxidation of, 408
transport into thyroid, 408
17-Ketogenic steroids
urinary excretion of
in Cushing's syndrome, 378
effect of metyrapone, 382
in hyperaldosteronism, 395
normal, 371, 460, 461
17-Ketosteroids (17-KS)
urinary excretion
in Cushing's syndrome, 388
in feminizing adrenal tumor, 390
in hyperaldosteronism, 395
normal, 364
in virilizing adrenogenital syndrome, 384, 386
Kinins, in carcinoid syndrome, 153
Krukenberg tumor, 524
and excretion of estrogens, 524
and excretion of gonadotropins, 524
Kynurenic acid
structure of, 136
urinary excretion of
in cancer of the bladder, 140
in Hodgkin's disease, 142
in normal persons, 140
Kynurenine
structure of, 136
urinary excretion of
in cancer of the bladder, 140
in normal persons, 140
in other diseases, 141

L

Lactate, 35–36
concentration in tumors, 35
recycled to glucose, 36
Lactate dehydrogenase, 36, 50–56

isoenzymes of, 50, 51
in breast cancer, 53
in colon cancer, 53
in gastric cancer, 53
in lung cancer, 53
in neoplastic tissues, 51
nomenclature of, 50, 51
in normal tissues, 50, 52, 53
Leucine aminopeptidase, 96–98
action of, 96
determination of, 96, 97
isoenzymes of, 96
in tissues, 96
Leukemias, 127
abnormal serum proteins in, 127
excretion of L chains in, 127
incidence of, 232
mortality rates for, 232, 233
and purine biosynthesis, 243–251
and pyrimidine biosynthesis, 246–247
types of, 232
Leukocytic enzyme activities in leukemia
acid phosphatase isoenzymes, 234–236
alkaline phosphatase in, 236–237
glucose-6-phosphate isomerase, 38
glyceraldehydephosphate dehydrogenase, 38
fructosediphosphate aldolase, 38
hexokinase, 38
lactate dehydrogenase, 38
phosphofructokinase, 38
pyruvate kinase, 38
triosephosphatase, 38
Leydig cell tumor, *see* Interstitial cell tumor of testis
Lipid metabolism, 21, 22–27
body storage in cancer, 21, 22
in cancer, 23
in carcinoma of the breast, 586–587
Lipoid cell tumors of ovary, 514–517
histology of, 515
incidence of, 515
plasma steroids in, 515, 517
steroid excretion in, 515, 516
Luteinizing hormone (LH)
in pituitary, 331–332
in serum, 462–463
Luteoma of pregnancy, 512–514
histology of, 513
incidence of, 513
metabolism of, 514
plasma testosterone in, 513
steroid content of, 514
urinary steroid excretion in, 513
Lymphomas, 126–127
abnormal proteins in serum, 127
of testis, 478
urinary excretion of proteins, 127
Lysozyme, 237–243
action of, 238
in bone marrow, 240
method of determination of, 238
relation between serum and leukocytic, 240
in normal persons, 241
in patients with leukemia, 241
in serum, 238
in actue monocytic leukemia, 239
in acute myelocytic leukemia, 239
in acute myelomonocytic leukemia, 239
in chronic lymphocytic leukemia, 239
in chronic myelocytic leukemia, 239
in nonneoplastic disease, 242
in normal persons, 239
in other types of cancer, 242
in urine, 239–243
of nonneoplastic disease, 242, 243
of patients with leukemia, 239–240
of patients with other types of cancer, 242, 243

M

Macroamylasemia, 95
Magnesium
in body, 280
in bone, 273
metabolism in hyperparathyroidism, 299
requirement in man, 280
serum
in hyperparathyroidism, 299–300
in normals, 299
Melanocyte-stimulating hormone (MSH)
in ectopic tumor, 538
in pituitary, 537
structure of, 539
Methionine, metabolism of, 164–165

Metyrapone, 380, 381
effect on 17-OHCS, 380
in Cushing's syndrome, 381, 382
effect on 17-KS, 382
Monoiodotyrosine (MIT)
in organification, 408, 409
structure of, 409
Mucopolysaccharides, 19–20
structure of, 19, 20
Multiple myeloma (myelomatosis), 119–123, 306–310
amyloidosis in, 121
Bence Jones proteins in, 112, 123
and bone formation rate, 308, 309
and bone resorption rate, 308, 309
and calcium metabolism, 308–309
clinical findings in, 119, 120
cryoglobulinemia in, 121
and exchangeable calcium pool, 308, 309
incidence of, 119, 306
and phosphorus metabolism, 308–309
renal function in, 309–310
serum alkaline phosphatase in, 120, 307, 308
serum calcium in, 120, 307
serum immunoglobulins in, 120
serum phosphorus in, 307
serum proteins in, 120, 121
serum urea in, 121
sites of, 119
viscosity syndrome in, 121
Myelocytic leukemia, *see also* Leukocytic enzyme activities in leukemia
leukocytic enzymes, 38

N

Neoplasms
of adrenal cortex, 356–401, *see also* Aldosterone-producing tumors, Cushing's syndrome, Neoplastic adrenogenital syndrome
of bladder
incidence of, 135
and tryptophan metabolism, 138–141
(primary) of bone, 303–310
benign, 304
other tissue enzymes in, 304
serum alkaline phosphatase in, 304
tissue alkaline phosphatase in, 304
chondrosarcoma, 306
classification of, 303
incidence of, 272, 273, 303
multiple myeloma, *see* Multiple myeloma
osteogenic sarcoma, 304, 306
serum alkaline phosphatase in, 305–306
serum calcium in, 306
(secondary) of bone, *see* Skeletal metastatic disease
of colon, *see* Colorectal carcinoma
extrapancreatic, 223–225
of liver
incidence of primary, 185, 201
incidence of secondary, 185
mortality in primary, 185
mortality in secondary, 185
primary, 200–207
cholesterol synthesis in, 203, 204
hypoglycemia in, 201–203
mechanism of, 202
polycythemia in, 203
serum α-fetoprotein in, 204–206
types of, 200, 201
secondary, 207
hypercholesterolemia in, 207
intrahepatic metastases, 207
serum alkaline phosphatase in, 207
of small intestine
benign types, 192
incidence of, 185, 191
and the malabsorption syndrome, 192
and microsomal enzymes in, 191
mortality in, 185
and nature of intestinal contents in, 191
serum alkaline phosphatase in, 192, 193
and sterility of contents, 191
of ovary, *see also* Androblastoma, Granulosa cell tumor, Gynandroblastoma, Luteoma of pregnancy, Thecoma
biochemistry of, 499–524
classification of, 485
incidence of, 483

Neoplasms of ovary (*continued*)
mortality rate of, 483
of stomach, *see* Gastric neoplasms
of testis, 451, 457, *see also* Adrenal rest tumor, Androblastoma, Germinal cell tumor, Granulosa cell tumor of testis, Interstitial cell tumor, Testicular steroids, Testis
classification of, 452–453, 463, 464
incidence of, 451
mortality in, 451
of thyroid gland, 403–450, *see also* Thyroid medullary carcinoma, Thyroid papillary and follicular carcinomas
classification of, 403
incidence of, 404–405
and nodules, 405
of uterus, 613–618, 621–629, *see also* Endometrial carcinoma
fibrinolytic activity, 616
hexokinase isoenzymes, 616, 617
lactate and other dehydrogenases, 614, 615
LDH isoenzymes, 614, 615
oxygen consumption, 617, 618
serum alkaline phosphatase, 615, 616
Neoplastic adrenogenital syndrome, 384–391
in infancy and childhood, 388–390
urinary 17-ketosteroids in, 389
types of, 384
virilizing in females, 384–388
plasma androstenedione in, 387
plasma cortisol in, 387
plasma testosterone in, 386, 387
urinary 17-ketosteroids in, 384, 386
Neoplastic gammopathies, 109, 118
Neural crest tumors, 158–183, *see also* Ganglioneuroma, Neuroblastoma, Pheochromoblastoma, Pheochromocytoma, Sympathocytoma
Neuroblast, 159
Neuroblastoma (sympathoblastoma), 159, 174–180
biochemical diagnostic procedures in, 180–181
catecholamines in tissues of, 174, 175
cystathionine in tissues of, 175
incidence of, 174
serum dopamine β-hydroxylase in, 179
urinary excretion of
catecholamines, 177
cystathionine, 180
dopamine, 178
dopamine metabolites, 179
homovanillic acid (HVA), 178, 179
3-hydroxy-4 mandelic acid (VMA), 179
Nicotinamide adenine dinucleotide (NAD), *see* Diphosphopyridine nucleotide
Nicotinamide adenine dinucleotide phosphate (NADP), *see* Triphosphopyridine nucleotide
Nitrogen balance
in cancer, 7–8
in gastrectomized patients, 9
with high protein intake, 6
with low protein intake, 5
in noncancerous disease, 8
in normal persons, 9
"trap" theory, 7
Nonfunctioning adrenocortical neoplasms, 398
definition of, 398
urinary steroids in, 398
Norepinephrine
biosynthesis of, 159–161, 164
cellular localization of, 161
in ganglioneuroma tissue, 175
metabolism of, 161–164
in neuroblastoma tissue, 175
in pheochromoblastoma, 171–173
in pheochromocytoma, 166, 168, 169
in serum of normals, 168
urinary excretion
in ganglioneuroma, 176–178
in neuroblastoma, 176–178
Nucleic acid metabolism
and acid phosphatase, 258
and alkaline phosphatase, 268
and aspartate carbamyltransferase, 268
and dihydrofolate reductase, 268
and DNA polymerase, 267
and lactate dehydrogenase, 268
in leukemia, 263–268

in neoplastic colonic cells, 194
in normal colonic cells, 194
and patterns of transfer RNA's, 265–266
and RNA-dependent DNA polymerase, 263–264
and RNA homologies, 264, 265
and tRNA methylases, 266
5′-Nucleotidase
distribution of, 85
isoenzymes of, 86, 87
kinetics of, 85, 86
preparation and properties of, 85
purification of, 85
serum activity, 87–89
normal values, 87
in carcinoma of liver, 88
in osseous disease, 88

O

Osteogenic sarcoma, 304–306
serum alkaline phosphatase in, 80, 81, 305–306
serum calcium in, 306
types of, 305
Ovarian Leydig (hilus) cell tumor, 510–512
and estrogen excretion, 511
and gonadotropin excretion, 511
histology of, 510
incidence of, 510
and 17-ketosteroid excretion, 511
and pregnanediol excretion, 511
and virilism, 510
Ovary
and biosynthesis of progesterone, 491–492
and metabolism of progesterone, 492–497
normal
and estrogen biosynthesis, 480–481
and the hypothalamic–hypophysial-ovarian axis, 485, 487
mature follicles in, 484
and menstrual cycle, 484, 487
metabolism, 490
primordial follicles in, 484
structure of, 484
Ovulation
and plasma FSH, 488–489
and plasma LH, 488–489
Oxygen consumption, 35
in carcinoma, 35
of breast, 35
of large bowel, 35
of stomach, 35

P

Pancreatic carcinoma
alpha-cell type (glucagonoma), 215–218
beta-cell types (insulinoma), 211–215
of ducts, 208–211
carbohydrate metabolism in, 210, 211
location of, 208
secretion in, 209
tests in, 210
glucagonoma, 215–218
blood glucose in, 215–217
effect of intravenous glucagon in, 217
serum insulin in, 217
tests for, 217, 218
incidence of, 185, 208
insulinoma, 211–215
glucose assimilation coefficient in, 214
hypoglycemia in, 212–214
serum insulin in, 215
mortality in, 185
and watery diarrhea–hypolakemia-achlorhydria (WDHA) syndrome, 221–223
and Zollinger-Ellison syndrome, 218–221
Paraprotein, *see* Immunoglobulins in cancer, Neoplastic gammopathies
Parathyroid adenoma, 290–302
excretion of calcium in, 296
excretion of phosphorus in, 296
incidence of, 291, 292
and primary hyperparathyroidism, 297–301
bone formation rate in, 298
bone resorption rate in, 298

Parathyroid adenoma and primary hyperparathyroidism (*continued*)
collagen metabolism in, 300
magnesium metabolism in, 299–300
serum alkaline phosphatase in, 298
serum calcium in, 298
types of, 298
urinary cyclic AMP in, 301
urinary hydroxyproline in, 300
and renal effects, 291
and serum alkaline phosphatase activity, 297
and serum calcium, 293–296
and serum phosphorus, 293–296
and skeletal calcium, 297–298
and skeletal effects, 291, 293
symptoms in, 294, 298
Parathyroid carcinoma, 308–309
incidence of, 308
parathyroid hormone production in, 308
serum calcium in, 308
Pheochromoblast, 159, 171
Pheochromoblastoma, 159, 171–173
catecholamines, 171–173
in plasma of, 172
in tissues of, 171, 172
urinary excretion of, 172
Pheochromocyte, 159
Pheochromocytoma, 159
catecholamines
metabolites of, 169
in urine, 169
urinary excretion of, 169, 170
clinical manifestations of, 166
epinephrine
in blood of patients with, 168, 169
in tissues of, 166
incidence of, 166
mortality of, 166
norepinephrine
in blood of patients with, 168, 169
in tissues of, 166
urinary excretion
of catecholamines in, 169, 170
of 3-methoxy-4-hydroxymandelic acid in, 170
Phosphofructokinase, 36
Phosphoglycerate kinase, 36
Phosphohexose isomerase, *see* Glucose-phosphate isomerase
Phosphopyruvate hydratase, 36
Phosphorus
content in body, 274
daily intake of, 280
relation to excretion, 280
normal metabolism of, 279–280
renal excretion of, 280
requirement in man, 280
in serum, *see* Serum phosphorus
Phosphorus metabolism
and regulation of, 286–289
cellular mechanisms in, 287–289
and role of cyclic AMP in, 287
Pituitary, anterior
anatomy of, 330
histology of, 331
hormones in, 331, 332
Pituitary tumors, 330–335
acidophilic, *see* Acromegaly, Gigantism
classification of, 332, 333
Cushing's disease, 345–347
and hypopituitarism, 353
incidence of, 332, 333
Placental (human) lactogen (HPL), 600, 601–602
in serum, 601–602
in hydatidiform mole, 602
in normal pregnancy, 601
structure of, 601
Plasma, *see also* Serum
estradiol
in granulosa cell tumors, 501
during puberty, 502
estrogens in, during menstrual cycle, 479–489
FSH in, during menstrual cycle, 487–490
17-hydroxyprogesterone in, during menstrual cycle, 495
luteinizing hormone (LH), during menstrual cycle, 487–490
progesterone in, during menstrual cycle, 493, 495, 496
testosterone
in normal women, 513, 514
in patients with lipoid cell tumors, 515

Prostaglandins
in carcinoid syndrome, 154
in thyroid medullary carcinoma, 444, 445
Prostatic carcinoma, 66–69, 315–316
mortality from, 66
serum acid phosphatase in, 66–69
effect of treatment on, 69
with metastases to soft parts, 67, 68
with skeletal metastases, 67, 68
serum alkaline phosphatase, 316
skeletal metastases
metabolic studies, 312
serum calcium in, 312
Prolactin, human (HPRL), 575–579
and effect of ergot drugs, 579
and effect of levodopa, 578
and factors regulating secretion, 576
isolation, 575
molecular weight, 575
and prolactin-inhibiting factor, 576
and prolactin-releasing factor, 576
role in breast cancer, 577
in serum, 576, 577
structure of, 575
Protein metabolism, 1–9
in cancer, 6
digestive enzymes in, 2
nitrogen excretion in, 3
nitrogen intake in, 3
Pseudohyperparathyroidism, *see* Ectopic hyperparathyroidism
Purine metabolism
in neoplastic colon, 194–198
in normal colon, 194–198
Purines, 243–251
biosynthesis of, 244
and folic acid metabolism, 250
in leukemic leukocytes, 244–245
in normal leukocytes, 244, 245
catabolism of, 255–263
and urinary excretion in leukemia, 257
Pyridine nucleotides, 251–255
in leukemic leukocytes, 254
in normal leukocytes, 254
structure of, 253
transhydrogenation of, 254
Pyrimidines, 246–257
biosynthesis of
and folic acid metabolism, 250
in leukemic leukocytes, 246–248
in normal leukocytes, 246–248
and biosynthesis of orotic acid, 246
catabolism of, 255
in leukemias, 255
normal, 255
and deoxythymidine synthetase, 248
metabolism
in neoplastic colon, 194–198
in normal colon, 194–198
and thymidine kinase, 249
and thymidylate synthetase, 246, 247
Pyruvate, 36
recycled to glucose, 36
Pyruvate kinase, 36

R

Rectal carcinoma, *see also* Colorectal carcinoma
incidence of, 185
mortality in, 185
RNA homologies
between human breast cancer and MMTV
and human breast cancer virus, 581–582
and human milk virions, 582, 583
and the hybridization technique, 580
and reverse-transcriptase, 579
between human leukemias and Rauscher leukemia virus, 264–265

S

Sertoli-cell tumor, *see* Androblastoma
Serum, *see also* Plasma*
acid phosphatase, 65–71
in leukemias, 71
methods of determination, 65, 66
in nonprostatic cancer, 69
normal values for, 65, 66
in patients with carcinoma of prostate, 66–68

* Some of the following components were determined in plasma, and some in either serum or plasma. For convenience, they are all listed under "Serum."

Serum acid phosphatase (*continued*)
with metastases to distant soft parts, 67, 68
with skeletal metastases, 67, 68
without skeletal metastases, 67, 68
prostatic moiety of and treatment of prostatic carcinoma, 68
in thrombocytopenia, 71
in thrombocytosis, 70
adrenocorticotropic hormone (ACTH)
in ectopic ACTH syndrome, 531–533
alanine aminotransferase in cancer, 57
aldolase, 49
isoenzyme patterns of, 49
in cancer of liver, 50
in normal individuals, 50
in primary hepatoma, 50
in neoplastic disease, 49
aldosterone
episodic secretion of, 367
in normal individuals, 467
in primary aldosteronism, 392–393, 397
alkaline phosphatase, 70–85
activities in
carcinoma of breast, 80, 81
carcinoma of prostate, 80, 81
carcinoma of uterus, 615, 616
chondrosarcoma, 306
hyperparathyroidism, 80, 81, 297–299
intrahepatic metastases, 80, 207
malignant biliary obstruction, 80
multiple myeloma, 80, 82, 120, 307, 308
neoplasms of small intestine, 192, 193
nonneoplastic disease, 82
osteogenic sarcoma, 80, 81, 305–306
isoenzymes of, 82
in bronchogenic carcinoma, 83
in metastatic disease, 82, 83
normal values of, 78
Regan isoenzyme, 79
in bronchogenic carcinoma, 83
in noncancerous disease, 84, 85
in patients with cancer, 84
amylase, 94–96
assay for, 94
in bronchogenic carcinoma, 95, 96
in carcinoma of pancreas, 95
in liver disease, 95
in pancreatitis, 95
androstenedione
in adrenogenital syndrome, 387
in interstitial cell testicular tumor, 471
in normal male adults, 457
angiotensin
in normal individuals, 369
in secondary aldosteronism, 396
arylamidase, 98–99
action on substrate of, 98
isoenzymes of, 98, 99
in cancer of pancreas, 98
in pancreatic disease, 98
aspartate aminotransferase, 102–103
isoenzymes of, 103–104
in cancer patients, 103
in metastatic involvement of liver, 102
in primary cancer of liver, 102
calcitonin
in familial medullary thyroid carcinoma, 438, 439
in medullary thyroid carcinoma, 436–439
in normal individuals, 436
calcium
in carcinoma of breast, 312, 313
in carcinoma of prostate, 312
in multiple myeloma, 120, 137
in osteogenic sarcoma, 306
in parathyroid adenoma, 298
in parathyroid carcinoma, 308
cholinesterase, in cancer of the liver, 105
corticosterone
in Cushing's syndrome, 377
in normal individuals, 369, 370, 377
cortisol, 364–366, 531
in adrenogenital syndrome, 387
in Cushing's syndrome, 376, 377
in ectopic ACTH syndrome, 531
episodic secretion of, 366, 375
in normal individuals, 364–366, 369, 370, 375, 377
cortisone
in Cushing's syndrome, 375

in normal individuals, 370
dehydroepiandrosterone
in interstitial cell testicular tumor, 471
in male adults, 457
dehydroepiandrosterone (DHEA) sulfate
in carcinoma of breast, 560
in normal men and women, 560
deoxycortisol, in normal individuals, 370
deoxyribonuclease, in cancer, 105
electrolytes
in aldosteronism, 392, 396
in Cushing's syndrome, 96–99, 374
enzymes, elevations in cancer, 41
factors involved in, 41
epinephrine
in normal individuals, 168
in pheochromocytoma, 169
follicle-stimulating hormone (FSH)
during sexual development, 463
in male adults, 462
free fatty acids
in carcinoma of breast, 587
in endometrial carcinoma, 625
free thyroxine, in normal individuals, 422, 423, 427
fucose, in carcinoma of breast, 588
gastrin, in ectopic gastrin syndrome, 547–548
glucosephosphate isomerase
in cancer, 43, 57
in carcinoma of breast, 42
in carcinoma of prostate, 42
glutathione reductase, in cancer, 57
glycoproteins
in benign neoplasms, 20
in cancer, 20–22
in normal adults, 22
histaminase
in medullary thyroid carcinoma, 442
in normal individuals, 442
human chorionic gonadotropin (HCG)
in choriocarcinoma, 608, 609
in hydatidiform mole, 596, 597
in normal pregnancy, 596
human growth hormone (HGH)
in acromegaly, 336–337
in ectopic HCS syndrome, 546
in endometrial carcinoma, 625, 626
in normal individuals, 335
and size of pituitary tumors, 337
17-hydroxycorticosteroids
in Cushing's syndrome, 376
in normal individuals, 371, 375
immunoglobulin concentrations
effect of age on, 117
multiple myeloma, 120
normal values for, 117
isocitrate dehydrogenase, in cancer, 57
of the liver, 105
lactate dehydrogenase, 54–56
isoenzymes of, 54
in neoplastic disease, 54–56
in normal persons, 55
leucine aminopeptidase, 96–98
in cancer of pancreas, 96
in liver disease, 97
in pancreatic disease, 97
lipids and lipoproteins
in cancer, 26, 27
in metastatic carcinoma of breast, 587
in normal women, 587
luteinizing hormone (LH)
during female sexual development, 463
in male adults, 462, 463
magnesium, 281–282
in normals, 299
in parathyroid adenoma, 299–300
malate dehydrogenase, in cancer, 57
melanocyte-stimulating hormone (MSH), in ectopic MSH syndrome, 538
mucoproteins, 21
in cancer, 21
5′-nucleotidase, 87–89
in cancer, 88, 89
in hepatic disease, 88
methods for determination of, 87
normal values for, 87
in osseous disease, 88
ornithine carbamylase, in cancer of liver, 104
osmolality, in ectopic ADH syndrome, 543
phosphorus

Serum phosphorus (*continued*)
in hyperparathyroidism, 293–297
in multiple myeloma, 307
in normal individuals, 281
in skeletal metastatic disease, 314–316
placental lactogen (HPL), 601–602
in choriocarcinoma, 606, 609
in hydatidiform mole, 602
in invasive hydatidiform mole, 602–603
in normal pregnancy, 601
polysaccharide levels
in normal adults, 22
in patients with
benign neoplasms, 22
malignant neoplasms, 22
nonneoplastic disease, 22
potassium
in Cushing's syndrome, 347, 374
in hyperaldosteronism, 392
in normal individuals, 369
prolactin (HPRL), 576, 577
in adult males, 577
in breast cancer, 577
in children, 577
in pregnancy, 577
protein-bound iodine (PBI)
in normal individuals, 422
in papillary and follicular thyroid carcinoma, 423
proteins, 10–13
cancer tests and, 12–13
electrophoretic pattern
in cancer, 10, 11
in noncancerous disease, 10, 12
in gastric carcinoma, 190
in multiple myeloma, 120, 121
renin (plasma)
in normal individuals, 367, 369
in primary aldosteronism, 392
ribonuclease in cancer, 105
testosterone
in adrenal and ovarian veins, 387
in adrenogenital syndrome, 386
during sexual developmet, 463, 471
in females, 386, 458
in interstitial cell tumor of testis, 471
in luteoma of pregnancy, 513
in males, 386, 457, 471
thyrotropin
in ectopic thyrotropin syndrome, 544
in normal individuals, 427, 431
in thyroid cancer, 430, 431
total thyroxine, in normal individuals, 422
urea, in multiple myeloma, 121
Skeletal metastatic disease, 310–317
and carcinoma of breast, 311–314
bone formation rate in, 315
calcium metabolism in, 313, 314
exchangeable calcium pool in, 315
nature of skeletal lesions in, 311, 312
phosphorus metabolism in, 313, 314
serum alkaline phosphatase in, 312, 314
serum calcium in, 312–313
serum phosphorus in, 314
and carcinoma of prostate, 315–316
calcium metabolism in, 316
nature of skeletal lesions in, 316
phosphorus metabolism in, 316
serum alkaline phosphatase in, 316
serum calcium in, 316
serum phosphorus in, 316
incidence of, 310, 311
and other primary neoplasms, 316–317
relation to primary sites, 311
Spermatic venous blood serum (plasma)
dehydroepiandrosterone in, 456
steroids in, 456
testosterone in, 456
Steroid sulfate synthesis
in carcinoma of breast, 558
and response to adrenalectomy, 558
Sulfate-activating enzymes, 558–559
Sympathoblast, 159
Sympathoblastoma, *see* Neuroblastoma, 159, 174–180
Sympathocyte (sympathetic ganglion cell), 159
Sympathocytoma (ganglioneuroma), 159, 173–176

T

Testicular steroids
excretion of metabolites, 459
metabolism of, 459

Testis
biochemical constituents of, 454
and biosynthesis of androgens, 455
lipid metabolism of, 454
normal structure of, 451, 452
and production of estrogen, 458
protein metabolism, 454
effect of hormones on, 455
and secretion of androgens, 456
effect of HCG on, 456
rate of, 457
structure of androgens, 457
Testosterone
clearance
in normal men, 388
in normal women, 388
in virilized women, 388
excretion in females, 460
excretion in males, 460
metabolism of, 459, 460
in serum, 386, 387, 463, 471, 513
Tetraiodothyronine (T_4)
binding to plasma proteins, 410, 422
in papillary and follicular thyroid carcinomas, 429
in serum, 410, 411
structure of, 409
Theca cell tumor of ovary, 504–505
and clinical symptoms, 505
and endometrial hyperplasia, 505
estrogens in, 505
histology of, 504
Thymidine kinase, 197, 249
in colorectal neoplasms, 197
Thymidine phosphorylase, 251
Thymidylate synthetase, 246
Thyroid carcinomatous tissue, 432–434
enzymes in, 433
iodotyrosine deodinase in, 432
and uptake of radioiodine, 433, 434
Thyroid gland, 402–403
functions of, 403
normal structure, 402
Thyroid hormone economy
diagnostic tests of, 420–428
basal metabolic rate, 424
creatine tolerance, 424
external scanning, 426
protein-bound radioiodine uptake, 421
radioimmunoassay of serum (plasma) TSH, 427, 428
radioiodine uptake, 420, 421
serum butanol-extractable iodine (BEI), 422
serum protein-bound iodine (PBI), 421, 427
serum T_4 iodine, 422, 427
thyroid-stimulating hormone (TSH) test, 425
in normal individuals, 425, 427
in panhypopituitarism, 425
in Sheehan's syndrome, 425
thyroid-suppression, 425, 427
tyrosine tolerance, 424
Thyroid hormones
activity of, 412–416
diagnostic tests for, 420–428
and feedback effects, 416
and long-acting thyroid stimulator (LATS), 415, 416
and thyrotropin, 414–415
structure of TSH, 414
and thyrotropin-releasing factor (TRF), 314–315
structure of, 414
content in thyroid gland, 405
degradation of, 411–412
effects of, 416–420
on androgen metabolism, 420
on calcium and phosphorus metabolism, 419
on calorigenesis, 416
on carbohydrate metabolism, 417, 418
on creatine metabolism, 419
on epinephrine action, 419
on growth, 417
on insulin action, 419
on lipid metabolism, 418, 419
and oral glucose tolerance tests, 418
and serum cholesterol levels, 419
and synthesis of cholesterol, 418
and oxygen consumption, 417
of tissue, 417
of whole organism, 417
and protein metabolism, 417
and vitamin requirements, 419
Thyroid medullary carcinoma, 434–445
association with other tumors, 440–441

Thyroid medullary carcinoma association with other tumors (*continued*)
in hyperparathyroidism, 440
in the Zollinger-Ellison syndrome, 440, 441
and calcitonin activity, 434, 436
and diarrhea, 444
and prostaglandins, 444, 445
serum calcitonin in, 436–439
serum calcium and phosphorus in, 436
serum histaminase activity in, 441–442
Thyroid papillary and follicular carcinoma, 429–434
basal metabolic rate in, 429
long-acting thyroid stimulator in, 432
serum protein-bound iodine in, 429–430
serum thyrotropin in, 430
Thyroxine, *see* Tetraiodothyronine
Transaminase, *see* Aspartate aminotransferase
Triiodothyronine (T_3)
binding to plasma proteins, 410, 411
concentration of, in serum, 411
structure of, 409
Triosephosphate isomerase, 36
Triphosphopyridine nucleotide (TPN^+), 253
in leukemic leukocytes, 254
and TPNH in leukemic leukocytes, 255
Tryptophan
metabolic pathways, 134, 135–137
metabolism, 135–144
metabolites
in cancer of bladder, 140
in cancer of breast, 142, 143, 584, 585
and carcinogenesis, 141
in carcinoid syndrome, 147–150
effect of hydrocortisone on, 144
effect of pyridoxine-deficient diet on, 144
in Hodgkin's disease, 142–143
and loading dose of, 141
in nonneoplastic diseases, 143–144
in normal persons, 140
and other types of cancer, 142
and recurrence of bladder cancer, 141
urinary excretion of, 138, 139
Tumors, lipids
composition of, 23, 24
content of, 24
desmosterol in, 25
glycosphingolipids in, 25

U

Uric acid, 256–263
production of, 256
urinary excretion of, 256–263
effect of allopurinol on, 260–262
in leukemias, 257–259
Urinary excretion
of corticosteroids
in normal persons, 370–372
of pregnanediol, during menstrual cycle, 496
of steroids
estrogens, granulosa cell tumors, 500–501
estrogens, during menstrual cycle, 497
of patients with ovarian Leydig (hilus) cell tumor, 512
testosterone excretion, 512
of normal females, 512
of normal males, 512
Uterus, *see* Carcinoma of

V

Vaginal fluid enzymes, 618–621
as diagnostic aids in uterine carcinoma, 618
β-glucuronidase, 619–620
6-phosphogluconic dehydrogenase, 619, 620
and potassium, 620

W

Waldenstrom's macroglobulinemia, 123–124
Bence Jones proteinuria, 124
clinical findings in, 124
macroglobulins in, 124
Warburg formulation, 34
Watery diarrhea–hypokalemia–achlorhydria (WDHA) syndrome, 221–223

gastric acidity in, 222
hormones in, 223
hypokalemia in, 222
pancreatic cells in, 221

Z

Zollinger-Ellison syndrome, 218, 221, 547–549
ectopic gastrin syndrome, 547–549
gastric secretion in, 319–220
incidence of, 218, 547
and isolation of gastrin-like substances, 547
mortality in, 548
and pancreatic adenoma, 547
serum gastrin in, 220, 547–548
and tumors of pancreatic islets, 218, 219
presence of gastrins in, 218
structure of gastrins in, 218

A 5
B 6
C 7
D 8
E 9
F 0
G 1
H 2
I 3
J 4