INTERMEDIARY METABOLISM OF THYROID TISSUE AND THE ACTION OF DRUGS[1]

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ABBREVIATIONS

Adenosine mono-, di-, triphosphates	AMP, ADP, ATP
Deoxyribonucleic acid	DNA
2,4-Dinitrophenol	DNP
Flavin mononucleotide	FMN
Flavin adenine dinucleotide	FAD
Hexose monophosphate	HMP
Nicotinamide-adenine dinucleotide	NAD
	NADH
Nicotinamide-adenine dinucleotide phosphate	NADP
	NADPH
Radioactive iodide	I^{131}
Radioactive orthophosphate	P_i^{32}
Ribonucleic acid	RNA
Thyrotropin	TSH

INTRODUCTION

Until the past few years, the investigations of thyroid function have been concerned primarily with iodine metabolism. However, recently, experiments have been carried out to determine some of the basic biochemical reactions in thyroid tissue. Such studies seem crucial if one is to define clearly the various functions of this organ. The current literature will be discussed and attempts will be made to correlate these fundamental reactions with the iodine metabolism of thyroid tissue. A brief review of the cytostructure and histochemistry of the thyroid follicle will precede these biochemical discussions.

A study of the antithyroid drugs has been and continues to be helpful in evaluating thyroid physiology. Recent observations with such compounds have led to new concepts in thyroid function. These will be presented and an effort will be made to define the inhibitory activity of some antithyroid drugs at a molecular level rather than at the level of iodine metabolism.

I. ELECTRON MICROSCOPY

A. Ultrastructure of the thyroid

The integrated morphological and biochemical studies of Palade and Siekevitz (225, 279, 280, 281, 282, 283, 284) have contributed immensely to knowledge concerning the structure and function of the liver and pancreatic cells. Unfortunately, no such composite study is available for thyroid tissue. Nevertheless, electron microscopic studies of mammalian thyroid tissue have been reported. For details of thyroid ultrastructure, the reader is referred to a recent review by Kurosumi (173) which includes contributions made by Japanese workers which are not readily available.

Monroe (215) apparently first reported electron microscopic observations on the thyroid. This work was followed by more elaborate studies of Braunsteiner *et al.* (34), Dempsey and Peterson (65), Ekholm and Sjöstrand (85), Lever (185), and Wissig (339). The observations were made on mice, rats, and guinea pigs, and the results were in general similar. The readily identifiable cellular components are: the microvilli at the luminal surface; the basement membrane and the capillary endothelium at the basal end of the cell; the nucleus; mitochondria; endoplasmic reticulum; Golgi apparatus; secretory droplets, and lysosomes.

1. Microvilli. The microvilli are present on the luminal surface of the follicular cells as fingerlike projections. These were found to be quite irregular in size and shape (*ca.* 0.35 μ tall and 0.07 μ broad) and their number varies from cell to cell in the rat thyroid (339). They frequently appear to be detached from the plasma membrane. This observation should be noted by investigators who state categorically on the basis of radioautographic studies that radioactive iodine is found in the colloid, since I^{131} localized in these structures might be considered as existing in the follicular colloid. The microvilli of the thyroid epithelium appear to be linked together (339), a point which distinguishes them from the clearly individual and more regularly oriented microvilli on the surface of the intestinal lining cells (368) and the proximal convoluted tubule cells of the kidney (244). Calculation of the number of villi reveals there are *ca.* 25×10^6 per square millimeter of follicular cell surface. This is to be contrasted with 200×10^6 villi per same area of intestinal cell surface. Small vesicles (*ca.* 60 mμ in diameter) appear within the substance of the microvilli. Their density is similar to that of follicular colloid. Their origin and fate are unknown. Since thyrotropin (TSH) stimulation led to accelerated colloid resorption, Wissig (338, 340) postulated that the villi are concerned with absorption rather than secretion.

2. Basement membrane and capillary endothelium. At the basal end of the thyroid cell is a definite basement membrane (*ca.* 500 Å in thickness) which was found to contain silver deposits following the ingestion of silver nitrate by rats (65). Below this is a rich network of capillaries lined by an endothelium (*ca.* 200 to 600 Å in thickness). The endothelium is thought to be discontinuous by some authors (85, 215) but continuous by another (65).

3. Nucleus. This is enclosed within a double-walled membrane the layers of which are separated by a distance of *ca.* 200 Å. The nuclear membrane of other glandular epithelial cells contains small porous openings (330), but these are

infrequent in the thyroid (339). These openings have been interpreted as communications between the nucleus and the cytoplasm. The outer lamella of the nuclear membrane in acinar cells of the pancreas (224) and in other cells is coated with ribonucleoprotein particles. This was not found to be so in the thyroid (339). Nucleoli are not prominent in the nuclei of the thyroid cell.

4. Cytoplasmic components. Mitochondria. There are about 1000 mitochondria per liver cell (182). No such count is available for the thyroid, but the mitochondria in the rat thyroid cell appear to be smaller than those in the cells of other tissues (339), and average *ca.* 0.2 μ in diameter compared to 0.3 to 0.7 μ for liver mitochondria. They are uniformly distributed throughout the cytoplasm except for the apical portion of the cell which is usually devoid of mitochondria.

Endoplasmic reticulum. This is the cytoplasmic organelle system most directly concerned with the synthesis of protein. The ergastoplasmic vesicles are about 50 to 200 mμ in diameter and have a membrane *ca.* 70 Å in diameter (339). The outer surface of the membrane is studded with many fine particles, *ca.* 130 Å in diameter (339), which contain ribonucleoprotein (226). These particles appear to correspond to a portion of the basophilic components of the thyroid cell, as observed with light microscopy. The ergastoplasmic vesicles are not arranged in an orderly pattern (339) as in the cell of another secretory gland, the pancreas (286). Their location within the follicular cell varies according to their size (339). The small vesicles appear near the apical surface of the cell and are only partially covered by ribonucleoprotein particles, whereas the large vesicles are located near the base of the cell and are heavily coated by ribonucleoprotein particles.

These structures contain a homogeneous material of granular texture with morphological characteristics (339), staining reaction (68), and spectrophotometric absorption pattern (117) similar to that of follicular colloid. As such, components of these vesicles were termed "colloid droplets" by Dempsey and Peterson (65). On the basis of these findings, it has been assumed that the chemical precursors of follicular colloid are also present in "colloid droplets." However, Wissig (339) found no evidence that the contents of these vesicles were released into the follicular lumen. An iodine-containing protein (54, 207) has been isolated from thyroid "microsomes" and has been found to be different from thyroglobulin, the major protein in follicular colloid, on the basis of its electrophoretic and ultracentrifugal behavior, its iodine content, and its very limited extractability into isotonic saline.

Golgi apparatus. In certain cells, particularly those which have a secretory function, a system of globular bodies which stain with osmic acid is apparent. These bodies in the thyroid cell consist of a group of small vesicles with an agranular membrane. The diameter varies from 50 to 175 mμ (339). They are apparently not as conspicuous in the rat thyroid as in other secretory cells (339). The lumina of the vesicles of the Golgi apparatus do not appear to be in direct continuity with those of the ergastoplasm as seen occasionally in other tissues (227). The content of the vesicles of the Golgi apparatus is homogeneous but the density is highly variable.

Secretory droplets. These structures usually occur in the apex of the cell, have a diameter of 0.050 μ to 3.0 μ and are surrounded by a single-layered agranular membrane, 50 Å in thickness (339). It has been suggested that these droplets originate from the Golgi apparatus (339). However, unlike the vesicles of the Golgi apparatus, these droplets contain numerous minute, dense particles, 75 Å or less in diameter. The composition of these particles is unknown but their appearance is similar to that of iron-containing compounds that have been identified in other tissue (339). Ekholm and Sjöstrand (85) and Lever (185) have described two somewhat ambiguous inclusion bodies in the cytoplasm of the follicular cell. It is difficult to relate these bodies to the secretory droplets described by Wissig (339). Nevertheless, one of these has been reported by Lever (185) to possess an iron-containing compound. This inclusion body is oval, its size ranges from 0.4 to 1.25 μ with an average cross-sectional diameter of 0.7 μ, and it is made up of aggregates of very dense, 70 Å particles. Similar particles may lie free in the cytoplasm and differ markedly from the less dense, larger (200 Å), irregular, ribonucleoprotein particles of Palade. It was also noted that these particles darkened following the staining of thyroid tissue with α-naphthol, suggesting the presence of a peroxidase (185). The darkening was prevented if the tissue was preheated or if thiourea was included with the α-naphthol. Similar studies were not reported for tissues other than the thyroid. Furthermore, the specificity of this reaction for a peroxidase is doubtful (232).

Lysosomes. deDuve (53) has presented evidence supporting the concept that the lysosome is a cytoplasmic particulate body which is distinct from mitochondria or microsomes. Assuming a spherical shape, the mean diameter of a liver cell lysosome is *ca.* 0.4 μ and the average density is *ca.* 1.15. The particle is surrounded by a smooth lipoprotein membrane in which are enclosed inactive, easily soluble hydrolases having in common an acid pH optimum, such as: acid phosphatase, acid ribonuclease, acid deoxyribonuclease, cathepsin, β-glucuronidase, and aryl-sulfatase. Uricase and possibly catalase may also be associated with these particles. deDuve reported (53) that particulate fractions containing the lysosomal enzymes have been isolated from beef thyroid by Neil.

B. Effect of drugs and altered physiological state of the thyroid

Several authors have made electron microscopic observations on the thyroid following the administration of thiouracil or TSH to animals (34, 65, 84, 338, 340), following exposure of the animals to cold (65), and following hypophysectomy (34, 65). Stimulation of the thyroid by TSH, the administration of thiouracil, or exposure to cold led to a general increase in the height of the follicular cell and the microvilli and in the number of cytoplasmic particles (65, 84, 338, 340). With the depletion of the follicular colloid, it appeared that the follicular cells changed the balance of their operations from colloid production to resorption. The data of Wissig (338) suggest a possible reversed polarity of the follicular cell with an emptying of the resorbed colloid by the messenger, the ergastoplasmic vesicles, directly onto the basal surface of the cell. No mention was made of a direct release of this colloid into the capillary endothelium. The changes in the

thiouracil-treated animal were most marked. Changes were noted as early as one hour following the administration of TSH (34, 84, 340). Hypophysectomy led to a decrease in cell size and an overall decrease in the number of cytoplasmic particles; the Golgi apparatus was not apparent in such animals (34, 65).

II. HISTOCHEMICAL ENZYMOLOGY

A. Hydrolytic enzymes

Phosphatases. Dempsey and Singer (64, 66) found both acid and alkaline glycerophosphatases in the follicular epithelium of the rat thyroid. Using as substrates phosphoric esters other than glycerophosphate, they found enzyme systems capable of dephosphorylating yeast nucleic acid, adenylic acid, fructose diphosphate, glucose-1-phosphate, and lecithin. The intensity of the reactions varied in different species of animals, in different follicles within the same thyroid, and even in different sites within a single follicular cell. Recently, Sobel (291), using a new azo dye method (18), showed acid phosphatase activity, localized to the Golgi apparatus of follicular cells, in the thyroid of the rat.

Esterases. Pepler and Pearse (233) investigated the nature and distribution of two esterases in rat thyroid tissue, namely, those which hydrolyze O-acetyl-5-bromoindoxyl and acetylthiocholine. The indoxyl esterase is present throughout the cytoplasm of the individual cells, but is more concentrated toward the luminal border. It is activated by cysteine and inhibited by silver nitrate. The enzyme hydrolyzing acetylthiocholine is thought to be a pseudocholinesterase and is distributed throughout the cytoplasm, in particular around the nucleus and near the luminal border.

B. Oxidation-reduction enzymes

Peroxidase. Dempsey (63) in 1944 described a peroxidase in the follicular cells of the rat thyroid. Blue granules appeared in the follicular cells following immersion of frozen sections in benzidine and peroxide. Thiouracil added to the peroxide-benzidine staining mixture in dilutions of up to 1/10,000 (8.0×10^{-4} M) prevented the formation of the blue granules. Thiouracil at higher concentrations (value not stated) also prevented the blue staining visible in the red blood cells within the thyroid (63). These findings were confirmed and extended by DeRobertis and Grasso (71) and DeRobertis (70). The latter (70) found peroxidase activity not only in the follicular cells, but in the colloid as well. This activity was inhibited by the addition *in vitro* of thiourea, but not of sulfanilamide. To inhibit the reaction completely, thiourea at a concentration of 2×10^{-3} M was required; 10^{-4} M was ineffective.

One must question the validity of these experiments *in vitro* with the thiocarbamides as inhibitors of thyroid peroxidase for several reasons. First, the concentrations of the thiocarbamides were high. Secondly, these compounds may not inhibit peroxidase activity at all *in vitro*, but may simply reduce the colored products formed by the peroxidase-catalyzed reaction (242). Finally, the administration of propylthiouracil to people (134) and to rats (186) does not inhibit the subsequent staining reaction for peroxidase activity in thyroid tissue.

Oxidases. Dempsey (63) noted that the treatment of frozen sections of rat thyroids with the Nadi reagent (*p*-phenylenediamine and α-naphthol) led to the rapid appearance of blue granules uniformly distributed throughout the cytoplasm of the follicular cells. This system, called the indophenol oxidase reaction, is thought to be a measure of the activity of the cytochrome oxidase-cytochrome c system (160). The addition of thiouracil to the Nadi reagent in concentrations up to 1/1000 (*ca.* 8.0×10^{-3} M) failed to inhibit the development of blue granules.

Dehydrogenases. Goddard and Seligman (121) developed a technique for the histochemical detection of succinic dehydrogenase activity in fresh tissue. The method used a ditetrazolium salt, as a hydrogen acceptor, which is converted to a blue insoluble diformazan. This activity is less prominent in the thyroid than in other tissue, and is localized largely to the mitochondria (121).

A histochemical study of nine oxidative enzymes in Hashimoto's thyroiditis, nodular goiter, and exophthalmic goiter was reported by Tremblay and Pearse (320). These consisted of: NAD and NADP diaphorases, succinic dehydrogenase, glucose-6-phosphate dehydrogenase, β-hydroxybutyrate dehydrogenase, NAD- and NADP-linked isocitrate dehydrogenases, and NAD-linked malate and glutamate dehydrogenases. All enzymes were reported to be present in these various types of thyroid tissue without any clear quantitative differences being observed between normal and diseased thyroids. These workers found a high level of enzyme activity in the Askanazy cell, leading them to dispute previous opinions that such cells are degenerate.

C. Effect of drugs and altered physiological state of the thyroid

Lindsay and Jenks (186), in a comprehensive survey, studied the histochemical distribution of twenty intracellular enzymes in the thyroid gland of rats subjected to various experimental conditions, designed to produce different levels of thyroid activity. They demonstrated the following enzymes: Phosphatase (acid phosphatase, alkaline phosphatase); peptidase (leucine aminopeptidase); esterases (As-naphthol esterase, indoxyl esterase); oxidases (monoamine oxidase, cytochrome oxidase, peroxidase); dehydrogenases (succinic, malic, isocitric, lactic, glutamic, β-hydroxybutyric, ethanol, α-glycerophosphate, glucose-6-phosphate, 6-phosphogluconic); diaphorases (diphosphopyridine nucleotide or NAD, triphosphopyridine nucleotide or NADP).

Alterations in enzyme activity were evaluated in normal and hypophysectomized rats, and in rats to which acetylaminofluorene, thyroid hormone, iodide, thiocyanate, perchlorate, or propylthiouracil had been administered. All the enzymes excepting *iso*citric, glutamic, β-hydroxybutyric, and ethanol dehydrogenases were found in the thyroid gland of normal rats. Furthermore, the enzymes were present only within the cytoplasm of thyroid parenchymal cells, with the exception of alkaline phosphatase which was detected in the capillary basement membrane and in the cytoplasm of capillary endothelial cells.

Except for a few differences, the patterns of enzymes in the glands of the hypophysectomized rats and rats fed thyroid extract or iodide were similar, and consisted in a decrease in enzyme activity. It should be noted, however, that

in the thyroids of these animals there was no alteration in diaphorase, cytochrome oxidase or peroxidase, the esterases, phosphatases, or peptidase activity. Enzymatic activity in rats fed 0.03% propylthiouracil, or in cold-exposed or thyrotropin-injected rats was usually greater than that observed in normal rats. The four enzymes that were not demonstrable in normal rats were now evident. The pattern of enzymes in the rats fed thiocyanate or perchlorate bore more resemblance to the normal group than did that of the hyperplastic propylthiouracil-treated group. This might be related to the high iodine content of the rat diet, namely, 12 mg per kg food. In this group of animals, the administration of perchlorate or thiocyanate did produce a definite increase in alkaline phosphatase activity.

In general, it appears that the enzyme systems in the thyroid, as measured histochemically, vary with the functional state of the thyroid (186, 233, 291). Compounds which inhibit the transport of iodide by the thyroid or the iodination reaction in the thyroid do not inhibit these enzyme systems (174, 186, 233). This leads one to conclude that the inhibitory effect of such compounds on the iodide reactions is not contingent on their ability to inhibit the aforementioned enzyme systems.

III. PROTEOLYTIC ENZYMES

The secretion of thyroxine from the thyroid presumably follows its hydrolysis from thyroglobulin by proteolytic enzymes. This is such a unique situation that a review of the proteases and peptidases in thyroid tissue appears indicated.

The presence of a proteolytic substance in the thyroid gland was first demonstrated by the digestion of a gelatin film with follicular colloid, obtained by microdissection from a rat thyroid (69). This proteolytic substance is more active at acid pH, and is stimulated by the administration of a pituitary extract. The proteolysis of thyroglobulin has been accomplished with thyroid extracts from dogs and swine (196, 253). Alpers *et al.* (6) isolated a preparation with proteolytic activity from rat thyroids by simple saline extraction of thyroid slices. This method supposedly extracted largely the follicular colloid and very little intracellular material, suggesting that proteolytic activity was largely extracellular. Hydrolysis of thyroglobulin occurred between pH 2.5 and 5.5, with peak activity at pH 4.9. Fifty-five per cent of the thyroglobulin was hydrolyzed at 48 hours, yielding iodide, monoiodotyrosine, diiodotyrosine, and thyroxine.

Weiss (333) made a fairly thorough study of the nature and localization of the proteinase and peptidase activity of beef thyroid tissue. He believed that proteinase activity resided in the cell and was similar to the proteinase found in the colloid. However, he was unable to reconcile the pH optimum for his proteinase (4.0) with the reported pH of colloid, namely 6.6 (69). Proteinase activity was partitioned among all the cytoplasmic particles, but the highest activity appeared in the nuclei and mitochondria. It required sulfhydryl groups for activity since it was inhibited by *p*-chloromercuribenzoate, Cu^{++}, and iodoacetate, and was stimulated by reducing agents such as ascorbic acid and cysteine. Unfortunately,

the inhibitors were tested at only one concentration and this was rather high, namely 4×10^{-3} M.

Weiss (333) described also a peptidase which hydrolyzed a number of di- and tripeptides at an optimum pH of 7.2 to 8.4, and was located exclusively in the submicroscopic fraction (mitochondrial supernatant) of the cell. He found no metal activation of the peptidase, but the activity was inhibited by ethylenediaminetetraacetic acid. Laver and Trikojus (177) confirmed the studies of Weiss (333), finding an intracellular proteolytic enzyme in hog thyroids, located both in the "microsomes" and in the "microsomal" supernatant. Subsequently Laver and Trikojus (178) separated this proteolytic activity into a peptidase and a proteinase by acetone fractionation. Beckers *et al.* (22) found a peptidase which hydrolyzed iodinated dipeptides (L-leucyl-L-mono- or diiodotyrosine and glycyl-L-mono- or diiodotyrosine). This activity was present and of essentially equal magnitude in thyroid and liver "microsomes," their respective soluble fractions, as well as in human serum. It was inhibited by Hg^{++} (10^{-3} M), but not by ethylenediaminetetraacetic acid (5×10^{-3} M). Loughlin *et al.* (190) have reported the presence of still another peptidase in thyroid tissue, cysteinyltyrosinase, which seems to be metal-dependent (Zn^{++}) (191), and is inhibited by methylthiouracil (190).

The subject of proteinase and peptidase activity in thyroid tissue has recently been discussed by McQuillan *et al.* (195). The pH optimum of their proteinase is 3.5 (hemoglobin substrate) while their two peptidases, acetyl-L-phenylalanyl-L-tyrosinase (APATase) and cysteinyltyrosinase, show maximum activity at pH 4.0 and 5.3, respectively. The proteinase, when freed of peptidase activity, supposedly has no action against simple peptides. They concluded that thyroid acid proteinase is able to degrade thyroglobulin with the release of mono- and diiodotyrosine and thyroxine. They did not rule out the possibility that a trace amount of APATase in the purified proteinase sample was the catalyst responsible for the release of the iodinated amino acids from small peptides, produced as intermediates by the proteinase.

Further work will be necessary to define clearly the locus and the primary action of the proteolytic enzymes in thyroid tissue.

IV. GLYCOPROTEINS

There is considerable evidence that thyrotropin, thyroglobulin, and the serum thyroxine-binding protein are glycoproteins. This interesting problem of protein-carbohydrate complexes has been reviewed by Bettelheim-Jevons (26). The common occurrence of a carbohydrate fragment in thyrotropin, thyroglobulin, and the serum thyroxine-binding protein may be significant with regard to their interrelationships in thyroid activity; however, the relationship remains obscure.

A. Thyrotropin (TSH)

Fels *et al.* (92) partially purified beef TSH. The low value for the Kjeldahl nitrogen, 12.2%, was indicative of a conjugated protein. The active component had a molar tyrosine-tryptophan ratio of two and contained carbohydrate as

indicated by a positive Molisch test. The nature of the carbohydrate moiety was not investigated. Quantitative end-group analysis by the dinitrofluorobenzene method indicated that no free amino end-group was present in the TSH molecule. Fraenkel-Conrat *et al.* (105) reported that TSH was a glycoprotein with a glucosamine content of 2.5%. Carsten and Pierce (40) found 4.0% carbohydrate by the anthrone reaction in a preparation with an activity of 5 USP units/mg. Pierce *et al.* (234), in a recent review of the chemistry and physiology of purified TSH, reported that TSH (30 USP units/mg) contained the following number of sugar residues per mole: glucosamine, 5 or 6; galactosamine, 2 or 3; mannose, 4 or 5; and the methylpentose, fucose, 1. Sialic acid was not detected. They also found that TSH had a high sulfur content, 16 to 18 half-cystine residues per mole. These calculations were based on a molecular weight of 28,000 for TSH.

Geschwind and Li (118) have shown that the treatment of crude thyrotropin preparations with periodate caused loss of most of the biological activity, due supposedly to the oxidation of the glycol groups in the carbohydrate fraction. This treatment also produced inhibition of most of the activity of two other pituitary hormones, namely, follicle-stimulating hormone and interstitial-cell-stimulating hormone. However, Sonenberg and Money (293), utilizing similar conditions, were unable to inactivate TSH with periodic acid. They concluded that the carbohydrate moiety of TSH is not crucial for its biological activity.

B. Thyroglobulin

Thyroglobulin ($S^{\circ}_{20,w}$ = 18.7 S) is the major protein in normal thyroid tissue, comprising about 80% of the total protein. For an excellent review of thyroglobulin and other proteins associated with thyroid hormones, the reader is referred to the paper of Robbins and Rall (250).

The presence of a carbohydrate component in thyroid colloid (150, 194) as well as in the follicular cells (116) has been demonstrated with the periodic acid-Schiff (PAS) technique. Gersh (116) localized the intracellular glycoprotein to either the Golgi apparatus or the "microsomes" or both. Leblond *et al.* (180) showed that once glycogen has been removed from tissue, then the PAS stain is specific for protein-carbohydrate complexes, in which the sugars must have 1,2-glycol groups free for oxidation with periodic acid. The observations reported above with PAS (116, 150, 194) were made without removing glycogen from thyroid tissue and hence are open to some criticism.

Sugars and amino sugars have been found chemically as components of thyroglobulin. Thyroid glands of various species of animals or man have a high content of hexosamine (1 to 2% dry weight) (29). Thyroglobulin was found to contain most of the hexosamine (72%) as well as the hexoses (61%) of the thyroid (29). Thyroglobulin is, therefore, a glycoprotein rich in hexosamine (3.3%) and hexoses (4.5%). Hooghwinkel *et al.* (150) demonstrated electrophoretically, both at pH 5.4 and 8.6, that the hexosamine and the iodine-containing protein of rat thyroid tissue are inseparable, suggesting that the hexosamine is firmly bound to thyroglobulin.

Brand *et al.* (33) reported that hog thyroglobulin contained 2.2% glucosamine.

Mannose and galactose were identified by Lacombe and Michel (175). A careful carbohydrate analysis of various glycoproteins including thyroglobulin was made by Gottschalk and Ada (125). Thyroglobulin was found to contain 1.2% galactose, 2.7% mannose, 0.4% fucose, 4.0% glucosamine, and 2.7% sialic acid, but no galactosamine. Wollman and Warren (354) have determined the concentration of sialic acid in the thyroid of normal and thiouracil-fed rats and suggested that 80% of the sialic acid is associated with thyroglobulin. They found that stimulation by TSH or the administration of thiouracil produced a decrease in the total sialic acid and a doubling of the free sialic acid concentration in the rat thyroid, presumably due to the hydrolysis of thyroglobulin and the release of sialic acid.

C. Mucopolysaccharides and sialomucin

Spicer *et al.* (299) demonstrated histochemically the presence of neutral mucopolysaccharides in the colloid of rat thyroids. They also noted that the colloid and cells of rat thyroid tumors possessed histochemical properties of non-sulfated acid mucopolysaccharides. The basophilia of the latter is due to enzymatically hydrolyzable N-acetylneuraminic acid.

Bollet (30) found that human thyroid tissue contained acid mucopolysaccharide but that this represented only 1% of the total hexosamines. The acid mucopolysaccharides were studied by Bollet and Beierwaltes (31) in a variety of thyroid diseases and no correlation was found between the level of hexosamine and the nature of the thyroid disease.

D. Thyroxine-binding protein

The evidence that the thyroxine-binding protein is a glycoprotein has been circumstantial (26, 250). However, Seal and Doe (272) have recently purified a thyroxine-binding protein from normal human plasma. The 8,000-fold purified protein was homogeneous by several criteria and had an estimated molecular weight of about 50,000. It contained 2 moles each of fucose, hexose, and sialic acid, and three moles of hexosamine. The protein had one binding site for L-thyroxine.

V. CARBOHYDRATE METABOLISM

Over the years it had been noted that thyroid tissue respired *in vitro*. Furthermore, Canzanelli and Rapport (39) reported in 1938 that a crude extract of TSH, added to the incubation medium, produced a 38% increase in the Q_{O_2} of guinea pig thyroid tissue. However, it was Weiss (331) who initiated a study relating respiration and the carbohydrate metabolism of thyroid tissue *in vitro*. He found that thyroid slices of steers respired for 12 hours without added substrate. The respiratory quotient was calculated to be *ca.* 1.0, suggesting that oxidation of carbohydrates had occurred. Intermediates of the Embden-Meyerhof pathway or Krebs cycle did not affect the respiration of thyroid slices. However, homogenates of thyroid tissue, fortified with cytochrome c (3.8×10^{-4} M), NAD (2.5×10^{-4} M) and ATP (7×10^{-4} M), did utilize these intermediates,

especially fructose-1,6-diphosphate. Iodoacetate (1.3×10^{-2} M) and cyanide (4.0×10^{-5} M) inhibited respiration under these conditions.

A. Krebs cycle enzymes

Krebs cycle enzymes have been found in sheep thyroid tissue (79). These include: aconitase, isocitric dehydrogenase, α-ketoglutaric oxidase, succinic dehydrogenase, fumarase, malic dehydrogenase, and possibly the condensing enzyme which catalyzes the reversible formation of citric acid from acetyl-CoA and oxaloacetic acid. The levels of activity of five of these enzymes in the thyroid, per mg K^+, DNA, or RNA, were lower than the levels in sheep liver tissue by a factor of two to five. Attempts were made to localize these enzymes in various cytoplasmic fractions. Only succinic dehydrogenase was confined largely to the mitochondrial fraction. *Iso*citric dehydrogenase was found largely in the 8,000 $\times$ *g* supernatant. Aconitase, fumarase, and malic dehydrogenase were distributed throughout the cytoplasmic fractions. On the basis of these data, Dumont (79) claimed that Krebs cycle enzymes in thyroid tissue are not confined to the mitochondria. This interpretation is open to criticism for two reasons. First, there is no evidence that the various cytoplasmic fractions were cleanly separated. Secondly, Dumont may have been working with fragmented mitochondria.

B. Hexose monophosphate pathway

Glock and McLean (119) found glucose-6-phosphate (16 units) and 6-phosphogluconic (41 units at pH 9.0) dehydrogenases in rabbit thyroid tissue. The levels were recorded as units of enzyme activity/g tissue required to reduce 0.01 μmol NADP/min at 20°. These data plus the preliminary observations of Schussler and Ingbar (268) suggested that a hexose monophosphate pathway existed in thyroid tissue. It appears that the HMP shunt is operative in other endocrine glands such as the testes (27), the ovary (96), the adrenal (162), the functioning β-cells of the islets of Langerhans (95), the parathyroid, and the pituitary (78, 96, 122). Highly suggestive evidence for this pathway in thyroid tissue, assessed by relating the metabolism of 1-C^{14}-glucose and 6-C^{14}-glucose (28), as measured by $C^{14}O_2$ evolution, has derived from the nearly simultaneous observations of Field *et al.* (98) and Dumont (77). These authors (77, 98) noted a greater metabolism of 1-C^{14}-glucose in comparison to 6-C^{14}-glucose by thyroid slices, resulting in a C_1/C_6-CO_2 ratio of greater than unity, varying from 3 to 10. The limitations of this type of analysis and its interpretation as representative of the HMP pathway have recently been reviewed (159, 357).

1. Effect of TSH. Thyrotropin stimulates 1-C^{14}-glucose metabolism preferentially by thyroid slices *in vitro* (98). The effect can be detected as early as 5 minutes after the addition of TSH to the medium (98). Furthermore, the effect of TSH in stimulating the HMP shunt in thyroid tissue was fairly specific since other pituitary hormones were unable to stimulate 1-C^{14}-glucose metabolism by thyroid tissue (98) or their respective end organs (96).

Attempts have been made to determine the mechanism by which TSH stimulates the metabolism of glucose *via* the HMP pathway. Since TSH does not in-

crease the activities of glucose-6-phosphate or 6-phosphogluconate dehydrogenases in thyroid slices (99), it was suggested that TSH stimulated the HMP shunt in thyroid tissue indirectly by stimulating the production of NADP (99). The level of NADP has been found to control the activity of the HMP shunt in other tissues (20, 35, 37, 164, 193, 334).

The speculation that TSH stimulates glucose metabolism by thyroid slices by increasing the concentration of NADP in this tissue (99), was confirmed in a preliminary report by Pastan *et al.* (229) and by more elaborate studies by Field *et al.* (97) and Dumont (80). Pastan *et al.* (229) measured oxidized and reduced pyridine nucleotides in dog thyroid slices before and after incubation with TSH. The addition of TSH produced an increase in NADP from a control level of 7 mμmol/g wet weight to 30 mμmol/g wet weight. This change was not associated with any alteration in the concentration of NADPH, but was associated with a decrease in the concentration of NAD from 88 to 62 mμmol/g wet weight. Hence, it was postulated that the increase in NADP resulted from the conversion of NAD to NADP (229) *via* a kinase in thyroid tissue similar to that found in other mammalian tissue (329). Unfortunately, the effect of added NAD on the metabolism of 1-C^{14}-glucose was not tested (97).

Further evidence that the concentration of NADP may be rate-limiting in the stimulation of the HMP pathway in thyroid tissue has been presented (80, 97). Nicotinamide-adenine dinucleotide phosphate (NADP), but not NADPH, was found to stimulate 1-C^{14}-glucose metabolism by calf thyroid homogenates (97). However, the level of added NADP which was effective (130 mμmol/150 mg of thyroid tissue) was roughly one hundred times the reported level of NADP in thyroid tissue (*ca.* 10 mμmol/g wet weight). This calculation is based on values for pyridine nucleotides in thyroid tissue obtained from dogs (229), guinea pigs (120), sheep (187), and ox (131). The values are similar, with reported levels of total pyridine nucleotides averaging about 100 to 200 mμmol/g wet weight. The true levels for pyridine nucleotides in tissue remain to be determined in view of the recent report of Lowry *et al.* (192).

Thyrotropin also stimulates the metabolism by thyroid slices of substrates other than glucose which require NADP as a cofactor, such as 1-C^{14}-gluconate, 1-C^{14}-gluconolactone, 1-C^{14}-acetate, and 1-C^{14}-pyruvate (97). The stimulation of the former two substrates was minimal, whereas that for the latter two was equal to that for 1-C^{14}-glucose.

2. Effect of iodotyrosines. Either monoiodotyrosine (MIT) or diiodotyrosine (DIT) (10^{-3} to 10^{-4} M), is able to stimulate 1-C^{14}-glucose metabolism by thyroid slices (80, 97). A possible explanation for this stimulation stems from the studies of Stanbury (301, 304) who has described a microsomal enzyme in thyroid tissue which deiodinates MIT and DIT and specifically requires NADPH. This reaction would probably lead to the generation of NADP. Neither tyrosine nor iodide simulated the effect of the iodotyrosines. The failure to demonstrate a stimulatory effect with iodide may be due to the concentration used (*ca.* 3.9×10^{-3} M), since Green and Ingbar (128), in a preliminary report, noted a several-fold increase in 1-C^{14}-glucose metabolism by sheep thyroid slices with iodide

levels up to *ca.* 10^{-4} M. Higher levels of iodide, up to 5×10^{-2} M, were ineffective or inhibitory (128).

3. Effect of artificial electron acceptors. Methylene blue (7×10^{-4} M) or Synkavite (2-methyl-1,4-naphthohydroquinol diphosphoric acid ester) (*ca.* 10^{-5} M), artificial electron acceptors, were also found to stimulate 1-C^{14}-glucose metabolism in the thyroid (80) as they do in other tissue (35, 37). The stimulatory effect of these compounds is presumably due to the oxidation of NADPH.

4. Effect of acetylcholine or epinephrine. Several other compounds, such as acetylcholine and epinephrine, have been reported to simulate the effects of TSH on the metabolism of glucose by thyroid tissue. Thyrotropin and acetylcholine have been found to stimulate the release of radioactive iodine from the thyroid *in vivo* (292), and both of these unrelated compounds stimulate glucose uptake and glucose metabolism by thyroid slices *in vitro* (230). Stimulation (200% to 100%) was noted with concentrations of acetylcholine which varied from 2.8×10^{-4} M to as little as 2.8×10^{-7} M. Eserine (physostigmine) (3.6×10^{-4} M) was required when low levels of acetylcholine were used. Although there are similarities between the actions of TSH and acetylcholine in these studies (230), there are four differences: (1) TSH stimulates the metabolism of 1-C^{14}-glucose more than that of 6-C^{14}-glucose, whereas acetylcholine stimulates both equally well; (2) atropine (2.2×10^{-4} M) abolishes the acetylcholine (2.8×10^{-5} M) stimulation of glucose metabolism, but not the TSH effect; (3) iodide (2×10^{-4} M) partially inhibits the acetylcholine stimulation but not the TSH stimulation, and (4) acetylcholine (2.8×10^{-5} M), but not TSH, was found to stimulate glucose metabolism to a slight degree (10 to 30%) in other tissues such as brain, pancreas, epididymal fat, testes, and liver.

Epinephrine has also been found to stimulate the release of radioactive iodine from the thyroid *in vivo* (292) and to stimulate 1-C^{14}-glucose metabolism preferentially by mammalian thyroid slices and homogenates (231). A lag in the stimulatory effect was noted for epinephrine, but not for adrenochrome, its oxidation product. This observation plus the fact that the former was oxidized to the latter in this system suggested that adrenochrome was the active compound. Furthermore, the concentration of adrenochrome (*ca.* 1.2×10^{-4} M) necessary to stimulate glucose metabolism was somewhat less than required for epinephrine (7.5×10^{-4} M). Adrenochrome has not been found as a product of epinephrine *in vivo* (15), but it appears to serve as an electron acceptor from NADPH *in vitro* (126). Since epinephrine stimulated NADPH oxidation by thyroid cytoplasmic particles, its stimulation of carbohydrate metabolism is probably secondary to the increased availability of NADP resulting from the oxidation of NADPH (231).

Another system relating carbohydrate metabolism and epinephrine *in vitro* has been reviewed by Sutherland and Rall (307). Epinephrine at concentrations of about 10^{-6} M has been found to stimulate, in various tissues, the formation of cyclic adenosine-3′,5′-phosphate, which leads to the activation of phosphorylase and the subsequent production of NADPH. The preliminary studies by Klainer

et al. (166) indicate that thyroid tissue contains small but significant levels of adenyl cyclase activity. Furthermore, these authors (166) reported that the formation of cyclic adenosine-3′,5′-phosphate by particulate preparations from sheep thyroids is stimulated by TSH.

C. Role of carbohydrate metabolism in thyroid function

The role of the glycolytic and HMP pathways in thyroid tissue remains to be determined. The data of Schussler and Ingbar (269) suggest that the HMP shunt may be involved in the iodination reaction in the thyroid. However, propylthiouracil, thiouracil, or thiocyanate, 2×10^{-4} M to 1.3×10^{-2} M, and sodium iodide at 3.9×10^{-3} M, compounds which inhibit iodination in thyroid tissue, had no effect on glucose metabolism by calf thyroid slices (99) or homogenates (331). Nevertheless, recently Mulvey *et al.* (218) have demonstrated that propylthiouracil (10^{-3} M) does inhibit the metabolism of both 1-C^{14}-glucose and 6-C^{14}-glucose by rat thyroid slices. However, propylthiouracil at a concentration of 10^{-8} M stimulated the metabolism of 6-C^{14}-glucose (218) as well as oxygen uptake and radiodiiodotyrosine formation (219). This low level of propylthiouracil still inhibited the metabolism of 1-C^{14}-glucose (218). It is unfortunate that these authors did not present observations on the effects of propylthiouracil at concentrations other than those at 10^{-3} or 10^{-8} M. The obvious discrepancies between their data and those of Field *et al.* (99) remain to be resolved.

The data of Freinkel (111) suggest that carbohydrate metabolism may be involved in lipogenesis in the thyroid. Hall (135) has stated, on the basis of preliminary observations, that the HMP pathway may be important in purine synthesis in thyroid tissue. Precedence for the role of the HMP shunt and NADPH in synthetic reactions has been established for cholesterol and fatty acid synthesis (285) as well as protein synthesis (336) in rat liver and dog kidney. These data are in agreement with the suggestion (158, 171) that the HMP shunt may function to provide NADPH for directing specific reductive syntheses in various tissues.

VI. IODOPROTEINS—COMPOSITION AND BIOSYNTHESIS

The normal thyroid gland has as a unique function that of synthesizing thyroglobulin (mol. wt. *ca.* 650,000), its major protein as well as its major iodine-containing protein. The preparation, homogeneity, and chemical composition of this protein, and the nature and importance of other iodoproteins in thyroid tissue have been reviewed recently by Robbins and Rall (250) and Rall *et al.* (241). A peculiar feature of this globulin is its high percentage of arginine (12.4%) (72), and the stability of some of its arginine-containing peptides toward acid hydrolysis (256). The tyrosine content is not unusually high, 3.12% (72). Even though the iodine content of thyroglobulin varies from 0.1 to 1.23%, the ratio of iodothyronines to total iodine content is usually constant (254).

Enlarged, goitrous thyroids of pigs and calves have a decreased concentration of cystine in thyroglobulin (72). However, this was not found to be true for the thyroglobulin derived from human goitrous thyroids (41). Furthermore, there

was no alteration in the cystine or tyrosine content of thyroglobulin derived from patients with adenomatous and exophthalmic goiters (41).

Bovine fetal thyroglobulin differs only slightly from the adult protein in that it is slightly less soluble in ammonium sulfate (124). Thyroglobulin from dogs or guinea pigs which were treated with 6-propylthiouracil shows an increased solubility in ammonium sulfate (255) and a decreased electrophoretic migration in barbital (Veronal) buffer at pH 8.0 (62). Stanley (306) reported differences in the salting-out of iodine-containing proteins from normal and neoplastic thyroid tissue. Easty *et al.* (81) found a difference in the electrophoretic mobility of thyroglobulin isolated from normal and cancerous thyroid tissue of one human subject, yet no difference in antigenicity as determined by the method of Ouchterlony. However, immunological differences have been noted for thyroid extracts from different mammalian species (257) and for normal and neoplastic thyroid tissue in the same patient (341). These extracts were crude and the true nature of the antigens remains to be determined.

Recently thyroid iodoproteins other than thyroglobulin have been characterized. Shulman *et al.* (278) have isolated an iodinated protein from hog thyroid which they have designated as "thyralbumin." It is characterized by a low sedimentation constant ($S^{\circ}_{20,w} = 4.2$ S) and a greater solubility in ammonium sulfate (2.5 to 3.0 M) than thyroglobulin. Serological studies indicated considerable antigenic similarity to serum albumin; however, physico-chemical data prevented a simple identification with serum albumin. A similar (or identical) soluble iodinated protein has been found in a transplantable thyroid tumor (251), in normal sheep thyroids (1 to 6% of the total iodine), in normal and abnormal human thyroid tissue (21, 61, 252), and in rats which had been on an iodine-deficient diet or to which propylthiouracil had been administered (56). A second type of iodinated protein, also different from thyroglobulin, and localized in cytoplasmic particulate fractions of thyroid tissue, has been identified *in vitro* and *in vivo* (54, 55, 56, 61, 251, 252, 289). As yet, there is no evidence that this particulate iodinated protein is a precursor of the soluble iodinated proteins.

It has been assumed that the iodination reaction and the synthesis of thyroglobulin are either related or at least occur *pari passu*. Nevertheless, the observations on one patient clearly demonstrate that the usual iodinated amino acids can occur in the thyroid of man without the presence of electrophoretically detectable amounts of thyroglobulin (61). Conversely, Feldman *et al.* (91) have found that a protein immunologically similar to thyroglobulin is present in the thyroid of the 17-day old rat fetus before I^{131} is concentrated. The fact that these thyroid cells contain material immunologically similar to thyroglobulin, as well as the organelles associated with protein synthesis, at a time before measurable quantities of I^{131} are concentrated in the thyroid, suggests that thyroglobulin synthesis may precede iodination, or at least that they do not occur simultaneously (91).

It has also been assumed that the iodination of tyrosyl radicals occurs directly on preformed thyroglobulin. This assumption is based largely on the work of Tong *et al.* (318) who found only a small fraction (3%) of the total I^{131} in the

thyroid gland of the rat to be free, *i.e.*, butanol-extractable and not peptide-linked. The values for free diiodotyrosine and free thyroxine were 0.5% of the total iodine in the thyroid; no free monoiodotyrosine was detected. Recently, Pitt-Rivers and Cavalieri (238) have found free monoiodotyrosine in the thyroid, as 0.01% of the total radioactive iodine. The argument favoring iodination of preformed thyroglobulin is not completely valid, since iodination of free tyrosine followed by its incorporation into protein could occur so rapidly as to preclude significant levels of free iodinated amino acids. It is known that a protein can be synthesized rapidly from amino acids. Ferritin is synthesized in minutes by the rat *in vivo* (189).

To date, there are no comprehensive studies on thyroglobulin synthesis in the thyroid. Winnick *et al.* (337) injected 5 mg of DL-tyrosine (C^{14} labeled in the β position) intravenously into rats and found 1.3% of the administered dose per gram of thyroid protein after 6 hours (concentration: 3.7×10^{-4} M). Unfortunately, there were no simultaneous studies of I^{131}-incorporation. Recently Nadler *et al.* (221) have injected tritium-labeled methionine, glycine, or leucine into rats and have followed the concentration of the radioactivity in the thyroid by radioautography. Within 30 minutes of the injection, the radioactivity was present in the epithelium of every follicle, while at 35 hours and later it appeared in the colloid. This sequence and pattern of the distribution of tritium resemble the results of their previous studies using I^{131} (181). Unfortunately, these results still do not help in relating the iodination reaction to thyroglobulin synthesis. Furthermore, these studies (221) do not tell specifically whether the tritium-labeled amino acid is incorporated into thyroglobulin or some other thyroid protein. Nevertheless, these authors (221) concluded that the leucine-containing protein, synthesized by the cells, must be the precursor of thyroglobulin. It is, supposedly, then secreted into the colloid where it is iodinated.

Recently, Herzfeld and Soodak (143) have found amino acid-activating enzymes, as measured by radioactive pyrophosphate exchange in the "pH 5 fraction" of thyroid homogenates, for both tyrosine and monoiodotyrosine. A similar enzyme for monoiodotyrosine was not found in liver or kidney tissue. Further investigations of this nature should be helpful in evaluating the problem of thyroglobulin synthesis.

Brand *et al.* (33) calculated that there were about two molecules of thyroxine/mole of thyroglobulin. The thyroid is, therefore, unique if it does synthesize a protein molecule containing about 5,000 amino acids every time two molecules of thyroxine are stored. Further studies by Spiro (300) concerning the fingerprinting of peptide chains in the thyroglobulin molecule should be very helpful in determining the structure of this protein and the location of the thyroxine moieties.

VII. NUCLEOTIDES AND NUCLEIC ACIDS

A. Nucleotides

Bessey *et al.* (25) have measured fluorimetrically the flavin nucleotides in the tissues of the rat. The rat thyroid was found to contain: total riboflavin, 4.56

μg/g wet weight; FAD, *ca.* 3 mμmol/g, and both FMN and free riboflavin, *ca.* 3 mμmol/g. These values were much lower than those for liver, kidney, and heart (factor of 9 to 5) and adrenal (factor of 5). They were slightly lower than those of pancreas and ovary (by 2) but were similar to those of pituitary and testis. Suzuki and Nagashima (310) have found similar values for the pig thyroid: total riboflavin, 7.06 μg/g wet weight; FAD, *ca.* 5 mμmol/g; FMN, *ca.* 5 mμmol/g; and riboflavin, 2 mμmol/g.

Recently the acid-soluble nucleotides have been examined in thyroid tissue (130, 131, 187). Beef and sheep thyroid contain about 125 μmol of total nucleotides/100 g of thyroid tissue, a figure lower than that for other mammalian tissue. The nucleotides were partitioned as follows on a percentage molar basis: adenylic, 45%; uridylic, 27%; guanylic, 12%; cytidylic, traces; NAD plus NADP, 8%; inosinic and others were 8%. In the case of the rabbit, values were given for the distribution of the free adenine nucleotides: ATP, 67%; ADP, 21%; and AMP, 12%. Among the conjugated nucleotides, these workers (131, 187) found: uridine diphosphate-N-acetylglucosamine, uridine diphosphate-N-acetylgalactosamine, uridine diphosphate galactose, and guanosine diphosphate mannose. The uridine diphosphate acetylhexosamine content was found to be 0.25 μmol/g of fresh tissue, or 20% of the total nucleotides of the thyroid. Some of these conjugated nucleotides are the probable intermediates for the biosynthesis of the carbohydrate moiety of thyroglobulin.

B. Nucleic acids

Fiala *et al.* (93) and Matovinovic and Vickery (208) have measured the RNA and DNA of mammalian thyroid tissue. The total DNA content of the rat thyroid (93) was found to be 166×10^{-6} g; the total RNA was 122×10^{-6} g. Similar values were found for the guinea pig (208). The total DNA content of the guinea pig thyroid was 225×10^{-6} g; the total RNA was 93×10^{-6} g. In hypophysectomized rats, the administration of pituitary hormones such as thyrotropin, follicle-stimulating hormone, or adrenocorticotropin increased the levels of cytoplasmic RNA in the thyroid, ovary, and adrenal, respectively, without altering the nuclear DNA (93). The administration of TSH or thiouracil to the normal guinea pig also increased the concentration of RNA in the thyroid cell (208).

VIII. LIPID METABOLISM

Although studies on the lipid metabolism of the thyroid are in their infancy, the progress made is sufficient to warrant review and an evaluation of the role that these compounds may play in thyroid function. Most of the studies to be discussed deal with the incorporation of radioactive orthophosphate into the phospholipids of thyroid tissue. Phospholipids are components of all the membranous structures of the cell, such as the cell membrane, the lipoprotein portion of the endoplasmic reticulum, the mitochondrial membranes, and cristae. They have been implicated in ion transport (145, 147) and in the transport of secretory products out of cells (144, 146, 148).

A. Composition of phospholipids

Initially, it seems appropriate to describe the chemical nature of phosphorus compounds and the phospholipids in thyroid tissue. In fresh sheep thyroids, the water-soluble phosphorus is 201.5 ± 54 μg P/g wet weight, and this constitutes 18.2 ± 5.4% of the total thyroid phosphorus (110). The phosphorus in lipid extracts averaged 191.5 ± 30.1 μg/g wet weight and constituted 17.3 ± 5.8% of the total thyroid phosphorus. Freinkel (108, 110) found phosphatidylcholine (*ca.* 52% of total phospholipids), phosphatidylethanolamine (25%), phosphatidylserine (11%), phosphatidic acid (3%), and phosphoinositide (9%) in $CHCl_3$-extracts of freshly excised sheep thyroids. He also found that no less than 15 to 20% of the organic acid-soluble phosphorus consisted of the phosphomonoesters, phosphorylcholine (PC) and phosphorylethanolamine (PE), and the phosphodiesters, glycerophosphorylcholine (GPC) and glycerophosphorylethanolamine (GPE). Studies utilizing radioactive orthophosphate (P_i^{32}) and 1-C^{14}-glycerol suggest that the phosphorylated bases (PC and PE) are precursors of the phosphoglycerides, whereas the phosphodiesters (GPC and GPE) are breakdown products of the phosphoglycerides (109). Recently Dawson (51) has demonstrated a lipase in thyroid tissue, phospholipase B, which forms GPC and GPE by splitting fatty acids from lysolecithin or lysophosphatidylethanolamine.

B. Effect of TSH on phospholipids

Borell and Holmgren (32) reported that the administration of TSH to guinea pigs produced an increase in the uptake of radioactive orthophosphate by the thyroid, before there was any evidence of histologic change. These studies were confirmed by Money and Rawson (214) who noted an increased P_i^{32}-uptake by the thyroid of chicks following the administration of TSH or thiouracil, as well as by that of chicks which had been on an iodine-deficient diet. Studies *in vitro* have extended the observations *in vivo* concerning the incorporation of P_i^{32} into thyroid tissue, in particular into the phospholipids (101, 108, 109, 110, 111, 217, 271, 328). Schwartz and Morton (271) reported that the ability of beef thyroid slices to incorporate P_i^{32} into organic phosphorus compounds was comparable to that of beef kidney and liver tissue. Absolute amounts of organic phosphorus compounds synthesized were not measured, but it appeared that the precursors of both protein and lipid phosphorus compounds were in the organic acid-soluble pool. Homogenization (271) or freezing and thawing of thyroid tissue (101) inhibited the incorporation of P_i^{32}. However, Vilkki (328) found that glycerophosphate-P^{32} was actively incorporated into the phosphatidic acid and phosphatidylinositol of thyroid homogenates. Coenzyme A, cytidine triphosphate, and adenosine triphosphate appeared to stimulate glycerophosphate incorporation.

Morton and Schwartz (217) found that TSH caused an increase of 186% in the incorporation of P_i^{32} into the phosphatides of beef thyroid slices. It was subsequently reported that the action of TSH in stimulating P_i^{32}-incorporation into thyroid slices was confined largely to the phospholipids, in particular the

alkali-labile phospholipids (102). In the presence of TSH, phosphoinositide became more heavily labeled than any other phosphoglyceride (109). There was some increase in the labeling of phosphatidylethanolamine and phosphatidylserine, but no increase in the labeling of phosphatidylcholine, a compound which constitutes about 50% of the total thyroidal phosphoglycerides (109). Other pituitary hormones failed to mimic these effects of TSH in thyroid tissue (102, 109).

Extending these metabolic studies, Freinkel (111) observed that TSH augmented the oxidation of glucose uniformly labeled with C^{14} (U-C^{14}-glucose) by sheep thyroid slices and increased the assimilation of U-C^{14}-glucose and 2-C^{14}- or U-C^{14}- inositol into the lipids of thyroid tissue. In the experiments with inositol, only the phosphoinositide was labeled with C^{14}. Furthermore, the effect of TSH in stimulating the incorporation of C^{14}-glucose and C^{14}-fatty acids, such as palmitate, into phospholipids at the expense of neutral fats suggested that TSH was affecting an important biosynthetic pathway in thyroid tissue (115).

C. Inositol

Freinkel *et al.* (112) and Dawson and Freinkel (52) have measured the level of free inositol in the thyroid of a variety of species and in the various tissues of guinea pigs by a microbiological assay. The average inositol level of 20 μmol/g wet weight in sheep thyroid tissue can be calculated to be 1.7% of the dry weight of ovine thyroid. The free inositol was confined largely to the supernatant fraction after centrifugation at 77,000 to 105,000 $\times$ *g* for one hour. Of all the tissues of rat, rabbit, or guinea pig, the free inositol concentration was greatest in some secretory glands, especially in the thyroid and pituitary (52). Unlike other hexoses, inositol was concentrated in the rat pituitary and thyroid by a factor of 80 over the concentration in plasma. The role of free inositol in tissue remains obscure, but Freinkel *et al.* (112) have postulated that it might be implicated in the formation of intrathyroidal mucopolysaccharides, since inositol is converted to glucuronic acid (43). It may also be involved in a secretory mechanism, for the incorporation of inositol into phospholipids increases during the secretory activity of endocrine glands (109, 144, 148).

D. Effect of drugs on lipid metabolism

How can one relate the studies on lipid metabolism with thyroid function? It has been reported that iodide (0.5 to 2.5 $\times$ 10^{-4} M) (271), thiocyanate (10^{-2} M) (168, 271), and thiouracil (1.5 $\times$ 10^{-3} M to 10^{-2} M) (168, 271) do not inhibit the incorporation of P_i^{32} into the phospholipids of beef thyroid slices. Hence, it appears that these compounds do not inhibit iodine metabolism by virtue of an effect on the incorporation of P_i^{32} into the phospholipids of thyroid tissue. However, a common denominator has been found which relates the concentration of iodide by the thyroid and the incorporation of P_i^{32} by thyroid tissue. Recently, it has been shown (328) that P_i^{32}-incorporation into the phospholipids of thyroid slices is inhibited by digitoxin (10^{-5} M) and is restored by K^+ (see below). Wolff and Maurey (346) had previously demonstrated that cardiac glycosides

(2×10^{-7} M) block the iodide-concentrating mechanism in thyroid tissue and that this inhibitory effect is overcome by K^+ (343).

It is premature, at present, to define the role of lipid metabolism in thyroid function.

IX. IODIDE-CONCENTRATING MECHANISM

The concentration or "trapping" of iodide by the thyroid is not specific for this organ (see below). The extensive literature concerning this interesting biologic phenomenon has been reviewed (24, 137, 247, 323). The recent paper by Halmi (137) will serve as a reference for the extensive studies which he has carried out in this area.

Techniques *in vitro* and *in vivo* have revealed that the concentration of iodide by the thyroid can be dissociated from the organic binding of iodine. Schachner *et al.* (263) and Franklin *et al.* (107) found that the concentration of iodide by sheep and beef thyroid slices was not altered by sulfanilamide, thiocarbamides, *p*-aminobenzoic, or *p*-aminophenylacetic acid (all 10^{-3} M), compounds which inhibit the organic binding of iodide. Iodide concentrated under the influence of the thiocarbamide compounds *in vivo* is dialyzable, ultrafiltrable, non-precipitable with protein, and potentiometrically as well as chromatographically similar to ionic iodide (312, 325).

The inorganic iodide concentration of the thyroid of rats, rabbits, dogs, and sheep comprises only about 1% of the total iodine of the gland, its concentration varying from 0.6 to 2 mg per 100 g (5×10^{-5} M to 1.6×10^{-4} M) (314). The thyroid/serum (T/S) ratio for I^{131} is 25/1 in the rat (325) and man (305), and 250/1 in the mouse (353). This ratio may be increased by the prolonged administration of antithyroid compounds (325). It is depressed by hypophysectomy, by increasing plasma levels of iodide, or by the administration of thyroxine (137).

Iodide ion is concentrated not only in thyroid tissue, but also in salivary glands and saliva, in mammary tissue and milk, in the secretions of the stomach and small intestine, in the placenta, and skin, as well as in various brown algae. In spite of similarities between the thyroidal "trapping" of iodide and that of these other tissues, there are definite differences which have been reviewed thoroughly by Halmi (137) and Brown-Grant (36).

Stanbury and Chapman (303) have recently reported studies *in vivo* and *in vitro* on a 15-year-old hypothyroid male whose thyroid, salivary glands, and gastric mucosa were unable to concentrate iodide, *i.e.*, exhibited a generalized defect in the iodide-concentrating mechanism. Although the mechanism for this abnormal defect was not elucidated, the consequences of the defective thyroidal pump, namely, a goiter and hypothyroidism, were overcome by the administration of 3.0 mg of potassium iodide per day for one month.

Two models have been postulated to account for the mechanism whereby the thyroid accumulates ionic iodide (137): 1) adsorption of iodide to some thyroid constituent in simple anionic electrostatic binding, or 2) complexing of iodide with a carrier in the cell membrane and its subsequent release into the sites of

high concentration where, in the absence of binding, it exists as free iodide. The latter concept would be consistent with the possibility that iodide is actively transported into the thyroid cell. The complexity of this problem is compounded by the division of the thyroid into two main components, the follicular cell and the follicular colloid. Though the cell would seem to be the site of this energy-dependent process, Pitt-Rivers and Trotter (239), and Doniach and Logothetopoulos (74) have implied from their radioautographic studies that the concentration of iodide occurs chiefly in the follicular colloid. In each of these studies, the prolonged administration of antithyroid drugs prior to the observations leads one to wonder about the validity of the conclusions. Recently, Tong *et al.* (317) have presented preliminary evidence that isolated sheep thyroid cells can concentrate I^{131}. If verified, this observation would clearly establish the follicular cell as the site of the iodide concentrating mechanism.

A. Active transport

Wolff (343) and Halmi (137) have reviewed the literature concerning the possibility that iodide is actively transported into the thyroid, and found the following as evidence in support of such a concept. A bound form of iodide, not in equilibrium with extracellular iodide (312, 325), has not been detected in thyroid tissue except in the studies of Wyngaarden *et al.* (359, 360). Anaerobiosis (263), low temperature (287), and agents such as 2,4-dinitrophenol, which uncouple oxidative phosphorylation, inhibit iodide-concentration. This suggests that phosphate-bond energy is required (113, 114, 263, 287, 348). Thiocyanate (10^{-4} M), an inhibitor of iodide transport, has been found to decrease (20%) the incorporation of P_i^{32} into the acid-soluble organic phosphorus fraction of beef thyroid slices, with little or no effect on O_2-uptake (157). Since P_i^{32}/O_2 ratios were decreased, it was postulated that SCN^- also inhibits oxidative phosphorylation, but the slight effect of SCN^- on the P_i^{32}/O_2 ratio leads one to question this interpretation. The iodide-concentrating mechanism exhibits saturation at extracellular iodide concentrations of approximately 5×10^{-4} M (136, 325). Monovalent anions such as SCN^- or ClO_4^- can compete with I^- for the iodide-concentrating mechanism (351, 362). The fact that SCN^- inhibits the concentration of I^- and is not concentrated by the thyroid, led Wollman *et al.* (352) also to favor active transport.

B. Effect of cardiac glycosides

There have been several interesting studies recently which have probed into possible biochemical mechanisms for the iodide-concentrating mechanism in thyroid tissue (328, 343, 346, 347). Wolff and Maurey (346) and Wolff (343) found that the iodide-concentrating capacity of thyroid slices from sheep, beef, guinea pig, and man was depressed by various cardiac glycosides and their aglycones at 10^{-7} M to 10^{-6} M, concentrations which inhibit the transport of K^+ into erythrocytes (156). It was subsequently shown that scilliroside was the most potent of these compounds. At a concentration of 3×10^{-8} M, it caused a 50% inhibition (347). The iodide-concentrating capacity of slices of a rat thyroid

tumor, the submaxillary gland of the mouse, and of brown algae (*Fucus vesiculosus*) was much less sensitive to the inhibitory action of the glycosides (8×10^{-4} to 3.5×10^{-3} M) (343). Increased extracellular concentrations of potassium, rubidium, and cesium ions (3×10^{-2} M) overcame the inhibitory effect of scilliroside (10^{-5} M) in a rat thyroid tumor (347). These cations also overcame the effect of ouabain (6.4×10^{-7} M), but not of 2,4-dinitrophenol (DNP) or ClO_4^- (3×10^{-4} M) in sheep thyroid slices (343). Furthermore, K^+ is able to reverse the depression in iodide-concentrating ability resulting from the leaching of thyroid slices in a K^+-free medium. From these data, it has been concluded that K^+ is required for the maintenance of a concentration gradient for iodide in thyroid tissue (343, 347).

Subsequently, Vilkki (328) found that P_i^{32}-incorporation into thyroid slices is inhibited by digitoxin (10^{-5} M), and that the inhibition is overcome by K^+. If the effect of the cardiac glycosides is truly one of an inhibition of P_i^{32}-turnover and is not on the penetration of P_i^{32} into thyroid tissue, then one might suggest that these compounds inhibit the synthesis of an "iodide-carrier" such as a phosphatide. The significance of the effects of the cardiac glycosides on P_i^{32}-incorporation into thyroid tissue requires further analysis since Nicholls *et al.* (222) have reported that ouabain may have a biphasic effect. Unlike previous studies which revealed that the cardiac glycosides inhibit P_i^{32}-incorporation into phospholipids, these authors found that ouabain stimulated P_i^{32}-incorporation when studied at short intervals.

X. IODINATION

A. Site

The question of whether the iodination of tyrosyl groups in thyroid tissue occurs in the cell or in the colloid remains an enigma. However, the present reviewers believe that the weight of evidence favors the concept that iodination occurs intracellularly.

It appears evident that iodide-uptake and probably its binding precede follicle formation in a variety of species (see Table 1). An initial uptake of I^{131} was found in sheep fetuses on the fiftieth day of gestation, whereas the formation of thyroid follicles was first noted histologically on the fifty-second day (19).

TABLE 1

Onset of iodide-uptake and follicle formation in the thyroid of various species

Species	Ref.	Gestation (Days)	I^--Uptake (Days)	Follicles (Days)
Chick embryo	(356)	—	5 to 7	10
Mouse	(326)	21	16	17
Rat	(123)	21	18	18
Pig	(243)	114	46 to 50	60
Sheep	(19)	145	50	52
Man	(42)	270	101	112
Calf	(170)	285	60	75 to 88

Thyroxine- and diiodotyrosine-like iodine were detected chemically in the thyroid of 52-day pig fetuses at a time when their thyroids contained neither follicles nor colloid (243). Koneff *et al.* (170) reported similar findings in fetal calf thyroids. Chapman *et al.* (42) correlated the uptake of I^{131} by the human fetal thyroid with follicle formation. Though they stated that these two correlate, it would appear from their data that uptake preceded follicle formation. Wollman and Zwilling (356), Trunnell and Wade (321), and Dennis (67) found that the chick embryo was able to concentrate iodide by the fifth to seventh day and to make monoiodotyrosine by eight and one-half days, both prior to the development of follicles. On the ninth day, there was periodic acid-Schiff-positive material (67, 356) and histochemical evidence of disulfide and sulfhydryl groups (67) in the cells. The presence of thyroxine was correlated with the appearance of the first colloid droplets on the tenth day (67).

Several investigators have indicated that iodination occurs in the colloid (220, 355). Wollman and Wodinsky (355) favored this concept since they found, by autoradiography, that protein-bound iodine occurred largely in the colloid within eleven seconds following the injection of I^{131} into mice. The objection to these observations would appear to be the hyperactivity of the thyroid tissue at the time of study. It has been shown that I^{131} appears initially in the cells of animals in which the metabolic activity of the thyroid has been lowered by a high-iodine diet or hypophysectomy (181, 220), or the administration of DL-thyroxine (73).

The difficulty in establishing whether iodination takes place in the cell, in the colloid, or in a structural milieu where both are required is due to the inability to separate clearly one structure from the other. Nevertheless, the observations on fetal tissue (*vide supra*), which is largely cellular at a specific time, suggest that iodination takes place in the cell.

Further evidence for this thesis has been obtained from the observations of Pulvertaft *et al.* (240), Pastan (228), and Tong *et al.* (317) on isolated single cells obtained by the trypsinization of human (240), calf (228), and sheep (317) thyroid tissue. Isolated calf thyroid cells, incubated in the presence of buffer, pH 7.4, and glucose, were metabolically active as evidenced by their ability to oxidize C^{14}-glucose and to liberate CO_2 (C^1O_2/C^6O_2 ratio = 17/1) and to incorporate P_i^{32} into phospholipids (228). The calf cells, unlike sheep cells, were unable to maintain a concentration gradient for I^{131}. The isolated calf and human thyroid cells were able, however, to incorporate I^{131} into soluble and particulate protein primarily in the form of peptide-linked monoiodotyrosine (228, 240). The sheep cells apparently also produce peptide-linked diiodotyrosine, triiodothyronine, and thyroxine (317). Electrophoresis of the iodinated soluble proteins revealed more than one peak migrating toward the anode, none of which coincided with that of thyroglobulin (228). The iodination was inhibited by heating the cell system or by the addition of propylthiouracil, thiocyanate, or glutathione, all 10^{-3} M, but not perchlorate, 10^{-3} M. Catalase, 1000 units/3 ml, was also inhibitory (228). The addition of free tyrosine to the incubation system led to the formation of free monoiodotyrosine. I^{131}-labeled monoiodotyrosine was not incorporated into thyroid protein nor was it deiodinated by the cells;

hence, these cells had lost the ability or the cofactor necessary for deiodination (228).

B. Iodination of tyrosine in vivo

It has been clearly shown in the chick embryo (321) and in man (365), that there is a sequential development of thyroid function for concentrating iodide and forming iodinated tyrosines and thyronines. The thyroid of the chick embryo is able to concentrate iodide at seven and one-half days, to synthesize monoiodotyrosine at eight and one-half days, diiodotyrosine at nine and one-quarter days, and thyroxine at nine and three-quarter days (321). The normal human thyroid traps iodide at twelve to fourteen weeks, and by nineteen weeks forms monoiodotyrosine, diiodotyrosine, and thyroxine (365).

There has also been clearly demonstrated in man a series of defects involving most of these steps: 1) Iodide trapped by the thyroid but no iodination of tyrosyl groups. 2) Iodide trapped, but only partial iodination of tyrosyl groups. 3) Complete iodination of tyrosyl to iodotyrosines, but no iodothyronines formed. The evidence for these defects has been reviewed in detail by Stanbury (302) and will not be recounted here. In spite of the extensive literature concerning these abnormalities, the cause remains unknown.

C. Iodination in vitro

Iodination, *in vitro*, has been studied under a wide variety of experimental conditions: thyroid slices, thyroid homogenates, cytoplasmic particulate fractions, and partially purified enzyme systems. In these systems, the acceptor of the "active iodide" also varies from tyrosyl present in the protein in the preparation to free tyrosine. Furthermore, iodination has been studied in the presence or absence of a peroxide-generating system. An analysis of each of these systems will be made.

It would appear that high-energy phosphate bonds are not required for iodination *in vitro*, since ATP is not required and frozen thyroid tissue is able to iodinate nearly as well as fresh tissue. There is some evidence that oxidative phosphorylation and iodination may be related in thyroid slices, since 2,4-dinitrophenol (10^{-5} M) was shown to inhibit iodination and phosphorylation to a similar extent (308). Nevertheless, the evidence that iodination and oxidative phosphorylation are related is equivocal at best.

Thyroid slices. Fresh thyroid slices can incorporate I^{131} into protein-bound mono- and diiodotyrosine (216), whereas frozen slices form only protein-bound monoiodotyrosine (211). Evidence for the role of an oxidative enzyme in this reaction was based initially on the inhibition of iodination by anaerobiosis and by relatively large concentrations of compounds known to inhibit cytochrome oxidase, such as azide (5×10^{-3} M), sulfide (3×10^{-3} M), and cyanide (10^{-2} M) (262). Carbon monoxide also inhibited iodination, and this effect was partially reversible in the presence of strong light, again suggesting a possible role for the cytochrome enzymes. However, subsequent studies revealed that the cytochrome system is probably not involved (59, 184, 197, 273).

Thyroid homogenates. Cell-free homogenates of thyroid tissue (88, 89, 332) can

form protein-bound iodine from I^{131}. Unlike fresh thyroid slices, the homogenate is able to make mainly protein-bound monoiodotyrosine (313). This is true even when iodination is stimulated by various factors. The poor degree of iodination in thyroid homogenates is probably due to the presence of high concentrations of reducing agents, such as ascorbic acid (167, 311) or glutathione (269), and of catalase (57, 310). Zeiger *et al.* (367) have determined that the concentration of ascorbic acid and glutathione in sheep and rat thyroid tissue is of the order of 10^{-3} M and that both are largely present in the reduced form. The concentration of catalase was reported to be 0.255 μg/g wet weight (*ca.* 10^{-9} M) in beef thyroid tissue (310). The stimulation of iodination in thyroid homogenates by Cu^{++} (88) and by cytochrome c (316) is probably due to the oxidation of ascorbic acid and glutathione by Cu^{++}, and of glutathione by cytochrome c.

Thyroid cytoplasmic particles. The iodinating activity in the cytoplasmic particles resides largely in the "mitochondrial-microsomal" fraction (313). Despite extensive studies with these thyroid particles, no one has clearly demonstrated whether iodination takes place chiefly in the "mitochondria," the "microsomes," or both. The cytoplasmic particles also appear to incorporate I^{131} largely into protein-bound monoiodotyrosine (313). Furthermore, the protein to which monoiodotyrosine is peptide-linked does not appear to be thyroglobulin (54). Recently Igo and Mackler (154) have described a particulate enzyme system for iodination *in vitro* isolated from beef thyroid mitochondria treated with deoxycholate and trypsin. The system utilized iodide in the presence of tyrosine and a peroxide-generating system, and catalyzed the disappearance at 38°, pH 7.0, of a fantastic amount of iodide, namely, 20 to 30 μmol iodide per hour per mg of enzyme protein. The reaction products were said to be monoiodotyrosine, diiodotyrosine, triiodothyronine, and thyroxine. Furthermore, the system was capable of catalyzing the stepwise formation of diiodotyrosine from added monoiodotyrosine, and of thyroxine from added diiodotyrosine. The system was inhibited by heating at 75° for 5 minutes, or by thiocyanate, cyanide, or propylthiouracil, all at 10^{-3} M.

The cytoplasmic particles also form lipid-bound iodine. The latter has been shown to be I^{131}-labeled "nonphosphatide," phosphatidylserine and sphingomyelin (311).

D. Stimulation of iodination in vitro

Flavin mononucleotide (FMN), 5×10^{-5} M, increases the formation of protein-bound I^{131} by sheep thyroid particulate fractions (319), but not by the whole homogenate (316). However, recently Schussler and Ingbar (269) have reported a stimulatory effect of FMN in thyroid homogenates. A high pH optimum, of 9 to 10, was found for iodide-utilization by both the flavin-supplemented and unsupplemented particulate fraction (319). Flavin mononucleotide could reverse the inhibitory action of anaerobiosis (319). The stimulatory effect of FMN was inhibited by thiouracil, thiocyanate, and cyanide, but not by perchlorate, malonate, monofluoroacetate, or antimycin A (all 10^{-3} M). It was stated that catalase (0.5 mg/2.0 ml) did not inhibit the stimulation by FMN (319), but a

careful analysis of the data reveals that it did. Subsequent studies revealed that the stimulation of iodination by FMN (294) and methylene blue (57) was markedly reduced or completely eliminated when the system was incubated in the dark, suggesting that the effect of these compounds was due to a photochemical reaction which resulted in the generation of peroxide. A similar explanation probably accounts for the stimulation of iodination which was found with free riboflavin (319), which is not a co-enzyme.

An analysis has been made of the endogenous flavins of various cytoplasmic fractions of thyroid tissue (310). The "mitochondrial-microsomal" fraction has the lowest content, 6.7 % of the total. Data concerning inhibitors of endogenous flavin nucleotides in the various iodination systems *in vitro* are confusing. Suzuki *et al.* (310) found that quinacrine (Atabrine) (10^{-3} M) was not inhibitory, but that acriflavin (10^{-3} to 10^{-4} M) and chlorpromazine (10^{-3} M) did inhibit iodination in a thyroid particulate fraction, with and without the presence of a peroxide-generating system. However, DeGroot and Davis (57) reported that acriflavin stimulated iodination.

Pyridine nucleotides. Recently it has been demonstrated in sheep thyroid cytoplasmic particles (57) and in sheep thyroid homogenates (269) that protein-bound I^{131} formation is stimulated slightly by oxidized pyridine nucleotides, but much more so by low concentrations (7.5×10^{-6} to 10^{-4} M) of reduced pyridine nucleotides. High concentrations (10^{-3} M) of the latter were inhibitory. The reason for the stimulatory effect of oxidized pyridine nucleotides is not clear, since they are not reduced by thyroid particles in the absence of added substrates (57). The presence of a transhydrogenase from NADPH to NAD (57) in thyroid particulate preparations does not seem to explain the differences in the stimulatory effects of the oxidized and reduced pyridine nucleotides. The specificity of this stimulation for pyridine nucleotides remains to be assessed, since it has been found that low concentrations of cysteine and glutathione (*ca.* 10^{-5} M) also stimulate iodination in similar systems and to a similar degree (296). It is possible that NADPH stimulates iodination by reducing oxidized glutathione to yield low concentrations of reduced glutathione by way of the glutathione reductase enzyme which has been found in thyroid tissue (209). The iodination in thyroid homogenates and its relationship to the intermediary metabolism of the thyroid have been extensively reviewed recently by Schussler and Ingbar (269) who postulated that the iodination reaction *in vitro* is governed by glucose metabolism *via* reactions coupled to pyridine nucleotide-linked dehydrogenations. This is a likely possibility, but remains to be proven. One must bear in mind that most iodination reactions *in vivo* and *in vitro* occur at the millimicromolar level, while the metabolism of carbohydrates probably takes place at a micromolar level.

Summary. It is difficult to untangle the maze of data published on the iodination reactions *in vitro*, since the systems utilized for iodination vary. Some are concerned with the formation of protein-bound I^{131}, and others with the iodination of free tyrosine in the presence of a peroxide-generating system. This complexity is compounded by the lack of precise knowledge as to the biological acceptor for the "active iodide," *i.e.*, free or protein-bound tyrosine. Furthermore, the

impurity of the enzyme systems makes it impossible to evaluate clearly the role of inhibitors and stimulators. A dual effect for NADPH on iodination has already been demonstrated. Although it stimulates iodination in a crude thyroid particulate system (57), it inhibits in a partially purified, solubilized iodinating system (58). Purification of the enzyme systems should resolve such discrepancies.

E. Iodide-oxidizing enzyme

Thyroid peroxidase. To date, the evidence favors the view that there is an iodide-oxidizing enzyme system in thyroid tissue and that it is a peroxidase. The literature on this subject up to about 1954 was reviewed by Astwood (12). A peroxidase in thyroid tissue has also been implicated in the coupling of diiodotyrosine to yield thyroxine (188).

Renewed interest concerning an iodide peroxidase has been stimulated by the observations of Serif and Kirkwood (273) and Alexander (2). Iodide incorporation into free tyrosine or protein by thyroid preparations *in vitro* is stimulated by peroxide and inhibited by azide, cyanide, thiouracil, *p*-aminobenzoate, 3-amino-1,2,4-triazole (all 2×10^{-3} M) and catalase (0.66 mg/ml) (2, 3, 4). Hydrogen peroxide can be added directly (273), generated enzymatically (2), or furnished by the auto-oxidation of substances such as flavin nucleotides (3, 319). Such systems not only iodinate free tyrosine, but have been shown to iodinate thyroglobulin (169).

The existence of a thyroid peroxidase appears to be well documented (58, 59, 60, 151, 167, 169, 311, 342). Recently some progress concerning the nature of this peroxidase has been made. An enzyme system functioning as an iodide peroxidase and tyrosine iodinase has been solubilized by brief chymotrypsin digestion of sheep thyroid "mitochondria" and "microsomes," and subsequent treatment with *n*-butyl alcohol and acetone (58, 59). A similar soluble enzyme was prepared, using the same technique, from rat spleen and salivary tissue. No detailed spectroscopic measurements were made of this enzyme, but it was reported not to contain porphyrin (59). Alexander and Corcoran (5) have been able to dissociate reversibly the thyroid, but not the salivary, iodide peroxidase into an apoenzyme and a prosthetic group by passing a crude extract of thyroid tissue through a cation-exchange column. The activity of the apoenzyme can be completely restored by the addition of hematin or methemoglobin. It was concluded that the iodide peroxidase in thyroid extracts is a heme protein, the prosthetic group of which is probably ferriprotoporphyrin IX. Unfortunately, spectroscopic measurements were not made on these fractions.

Detailed analyses of two types of abnormal thyroid tissue should be helpful in determining the nature of the iodide-oxidizing enzyme. Wolff *et al.* (348) have studied an interesting transplantable rat thyroid tumor which mainly concentrates iodide but is unable to form organically bound iodine (1%). The defect in hormone synthesis appears to lie within the iodinating system, since a small amount of unlabeled protein, with a sedimentation coefficient like that of thyroglobulin, was present in the tumor. Haddad and Sidbury (133) have described

a somewhat analogous situation in the thyroid of a fifteen and one-half year old goitrous cretin. The addition of a peroxide-generating system to a cytoplasmic particulate fraction of this thyroid *in vitro* failed to stimulate the formation of protein-bound I^{131}.

Chloroperoxidase. An interesting correlation with the above stems from the work of Shaw and Hager (274, 275) who have isolated a chloroperoxidase, from the mold *Caldariomyces fumago*, which chlorinates, brominates, and iodinates (82). This is the only non-thyroidal biological halogenating system which has been subjected to enzymatic analysis. The chloroperoxidase preparation, which oxidized chloride, iodide, and bromide ions with the formation of the corresponding carbon-halogen bond when supplied with a suitable acceptor molecule, is inhibited by thiocarbamides (83, 274). This system simulates the thyroid in that it is able to halogenate aromatic compounds. However, the reviewers have been unable to stimulate iodination in thyroid tissue with this chloroperoxidase (kindly supplied by Dr. L. Hager).

F. Iodinating intermediate

It is generally agreed that iodide concentrated by thyroid tissue is oxidized to a higher valence state, such as I^+ (iodinium ion), an electrophilic agent, which then displaces hydrogen from tyrosyl residues. It has been theorized that I_2 might be the active iodinating species (87, 359), but as yet such a species has not been isolated from thyroid tissue. Furthermore, current biochemical concepts would argue against free I_2 as an iodinating intermediate.

A stable sulfenyl iodide group was found in iodinated tobacco mosaic virus by Fraenkel-Conrat (104) and in β-lactoglobulin and ovalbumin by Cunningham and Nuenke (46, 48). Subsequent observations (47) revealed that several thiocarbamides and thiocyanate ion caused a rapid disappearance of the sulfenyl iodide group, *i.e.*, a disappearance of absorbancy at 355 mμ in iodinated β-lactoglobulin. Cunningham and Nuenke (47) calculated an equilibrium constant for the reaction:

$$\text{P—SI} + \text{SCN}^- \rightleftharpoons \text{P—SSCN} + \text{I}^-$$
$$K = [\text{P—SSCN}]\,[\text{I}^-]/[\text{P—SI}]\,[\text{SCN}^-] = 10.5$$

These interesting data suggested to the reviewers that a sulfenyl iodide species might serve as an iodinating intermediate in thyroid tissue. This concept received further support when it was found that certain proteins were iodinated (protein-bound monoiodotyrosine) in the presence of light, FMN, I^{131}, and buffer, pH 7.4 (295). Of a series of nine crystalline proteins tested, only bovine serum albumin, insulin, and lysozyme were found to react. It should be noted that these three proteins contain a relatively large number of disulfide bonds. Although evidence for a sulfenyl iodide group in thyroid tissue has not been found, it has been reported that the isolation procedures currently employed in the analysis of the distribution of organically bound iodine in thyroid tissue would convert sulfenyl iodide to iodide (47).

XI. MECHANISM OF ACTION OF ANTITHYROID COMPOUNDS

An attempt will be made to discuss the effects of several antithyroid compounds, which may act either by: (a) a direct effect on the biochemistry of the thyroid, or (b) other nonspecific peripheral effects.

"Most drugs undergo chemical transformation in the body. As a consequence of these changes, the resulting metabolites may differ in their pharmacological properties from those of the parent compound, being equally, less or more active. Knowledge concerning the biochemical changes that a pharmacological agent undergoes *in vivo* and the factors influencing these changes may be of considerable importance in the understanding of drug action and the elucidation of new biochemical phenomena." This is a quotation from Axelrod (16) which will serve as a theme for the subsequent discussion. Only those antithyroid compounds which conform to this thesis will be presented. This of necessity will eliminate from the discussion a large number of compounds which inhibit thyroid function, but are included in isolated reports from which it is difficult to evaluate critically their mechanism of action. It is also hazardous at times to correlate effects of compounds *in vivo* and *in vitro* because of the possibility of chemical transformations in either system. Nevertheless, attempts will be made to do so when the data are available.

The first group of compounds to be discussed will be the thiocarbamides. Various aspects of these drugs have been reviewed previously (11, 12, 236, 324).

A. Thiocarbamides

The thiocarbamides, with thiourea as a prototype, were introduced through the extensive efforts of MacKenzie and MacKenzie (199) and Astwood *et al.* (13). It was shown that these compounds were goitrogenic in rats and that their goitrogenic activity required the presence of an intact pituitary. The feeding of thiourea depressed the oxygen consumption of the intact animal but did not interfere with the calorigenic action of administered thyroid hormone, leading to the correct assumption that these drugs depress the production of thyroid hormone. This inhibition was also demonstrated by studies *in vitro* with thyroid slices (107).

It will be the purpose of this report to assess the manner by which the thiocarbamides interfere with the iodination of tyrosyl groups. There is evidence that these compounds also inhibit the supposed coupling of diiodotyrosine or its derivatives to form thyroxine, *in vitro* (235) and *in vivo* (246, 288). This aspect will not be discussed.

1. Direct chemical effect. The reducing power of the thiocarbamides for, or their preferential reactivity with iodine was suggested by Pitt-Rivers (236) as an explanation for their mechanism of action. Subsequently this seemed unlikely since Miller *et al.* (213) found that weakly active antithyroid compounds, such as 6-amino-2-thiouracil and 5-carbethoxy-2-thiouracil, were as reactive toward iodine as is thiouracil. It is conceivable that the supposed inactivity of such compounds *in vivo* is due to their rapid degradation by enzyme systems in the body, their limited accessibility to the thyroid, or both. This is probably true also for

cysteine, which is not goitrogenic yet has a reduction potential (Eo'), −0.15, equal to that of thiourea, −0.21. Furthermore, cysteine stimulates iodination *in vitro* at low concentrations (10^{-5} M) (296), whereas thiourea at this level inhibits iodination (205, 294). This is further evidence against the theory that sulfhydryl compounds inhibit iodination in the thyroid by the simple reduction of iodine to iodide. Recently, Pitt-Rivers (237) has altered her original suggestion (236) that the thiocarbamides inhibit iodination by reducing iodine to iodide, and stated that this explanation can be no more than a partial one.

2. Inhibition of metabolic reactions in the thyroid. Inhibition of metabolic processes in the thyroid by thiocarbamides would seem unlikely as a mechanism of their action, since the administration of these compounds to animals leads to growth of the thyroid, hypertrophy as well as hyperplasia. Furthermore, there is little evidence that they inhibit any of the metabolic processes to be discussed.

Respiration. These compounds have been known not to inhibit the respiration of thyroid slices (184). Subsequent studies have demonstrated that thiouracil, 2×10^{-2} M, inhibits neither cytochrome oxidase nor succinoxidase activity of the rat thyroid *in vitro* (197).

Carbohydrate metabolism. Most of the evidence to date suggests that the thiocarbamides do not alter carbohydrate metabolism in thyroid tissue. The metabolism of 1-C^{14}-glucose by rabbit thyroid slices *in vitro* was not affected by the previous administration of propylthiouracil to the animals for two months (99). Furthermore, propylthiouracil (*ca.* 10^{-3} M) did not affect the metabolism of 1-C^{14}-glucose by calf thyroid slices (99) nor the activity of the six Krebs cycle enzymes of sheep thyroid tissue which were tested (79). These results conflict with the observations of Mulvey *et al.* (218) who found that propylthiouracil (10^{-3} M) did inhibit the metabolism of 1-C^{14}- and 6-C^{14}-glucose by rat thyroid slices.

Nucleic acids. Thiouracil did not interfere with the synthesis of (208), nor was it incorporated into the nucleic acids (203) of thyroid tissue, even though it has been shown to be incorporated into nucleic acids of some tissues. Furthermore, thiourea, a strong antithyroid compound, is not a pyrimidine and could not conceivably be incorporated into nucleic acids.

Lipid metabolism. Neither thiouracil (1.5×10^{-3} M to 10^{-2} M) (168, 271) nor thiocyanate (10^{-2} M) (168) inhibited the incorporation of P_i^{32} into the phospholipids of beef thyroid slices (271). Although methylthiouracil (10^{-3} M) was found to decrease the P_i^{32}-uptake into the organic acid-soluble pool of beef thyroid slices (308), the effect was slight (20%).

Protein metabolism. Administration of the thiocarbamides *in vivo* produces a thyroglobulin with an altered solubility (62) and iodine content (309), yet there is no available evidence to suggest that these compounds inhibit the synthesis of thyroglobulin.

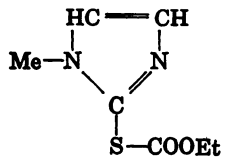
3. Enzyme inhibition. Although xanthine oxidase was at one time thought to be involved in iodination and is inhibited by thiourea (163), it has not been found in thyroid tissue (264). The evidence to date favors the concept that a peroxidase is involved in the iodination reaction (section X, E). Astwood (12)

reviewed the action of the antithyroid drugs in 1954 and presented evidence favoring the hypothesis that the thiocarbamides act by inhibiting a peroxidase. As stated in 1954, this possibility is likely, but unproved.

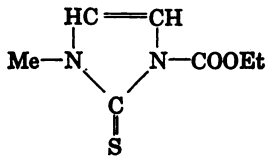
The relationship of several other types of antithyroid compounds to peroxidase activity appears worthy of note.

3-Amino-1,2,4-triazole. Heim *et al.* (141, 142) found one hour after the intravenous injection of 3-amino-1,2,4-triazole (1 g/kg) to rats that there was a marked inhibition of liver and kidney catalase, a lesser inhibition of liver peroxidase, and no inhibition of erythrocyte catalase activity. In view of the action of aminotriazole against tissue peroxidase, Alexander (1) assessed its antithyroid action in rats and found that it was goitrogenic (555 μmol administered orally/100 g food), and that it inhibited the formation of organically bound iodine in the thyroid. Although a large single dose of aminotriazole (695 μmol) to rats decreased the catalase activity of thyroid tissue by 44%, the dose of aminotriazole needed to inhibit iodination (13.9 μmol) had no effect on catalase activity.

Neo-mercazole. Neo-mercazole was first thought to be 2-ethoxycarbonylthio-1-methylglyoxaline (I), but is now known through the work of Lawson and Morley (179) and Baker (17) to have the thione configuration, 1-methyl-3-carbethoxy-2-thioglyoxalone (II).



(I)



(II)

In a recent study, Rimington (248) determined that the thione rather than the thiol configuration is probably responsible for the antithyroid action of a series of compounds chemically similar to neo-mercazole. He found no relationship between the antithyroid action of these compounds *in vivo* and their ability to interfere with a peroxidase/hydrogen peroxide/pyrogallol system *in vitro*, using a highly purified horse-radish peroxidase and measuring the purpurogallin formed.

Nonthyroidal peroxidases. Several nonthyroidal peroxidase systems are worthy of note. Sörbo and Ljunggren (298) have shown that crystalline myeloperoxidase, but not plant peroxidases, can catalyze the oxidation of thiourea, thiouracil, and thiocyanate in the presence of hydrogen peroxide. The system did not oxidize cysteine, thioglycolic acid, or mercaptoethanol, which are true thiols. It was, therefore, suggested that the antithyroid compounds inhibit iodination by competing with thyroxine precursors for hydrogen peroxide (298). The chloroperoxidase of Hager *et al.* (274) is inhibited by thiocarbamides (10^{-4} M). These workers (274) postulated that the mechanism for the inhibition of the halogenation of monochlorodimedone by thiouracil involves its action as a competitive substrate, the thiouracil competing with the substrate acceptor molecule for the

oxidized halogen ion-enzyme complex (83). In this system, thiouracil is oxidized only in the presence of a halide, hence it was postulated that the oxidation may proceed *via* a uracil sulfenyl halide intermediate (83).

4. Metabolism of thiourea by the thyroid. In an attempt to elucidate the mechanism of action of the thiocarbamide-type compounds, it was decided to pursue systematically the initial observations of Schulman (266, 267) concerning the uptake and metabolism of thiourea by thyroid tissue. The discussion will be centered chiefly about thiourea, which contains the thioureylene grouping (—NH·CS·NH—), previously suggested as essential for antithyroid activity (11). However, thioacetamide (CH_3CSNH_2) (202) and goitrin (5-vinyl-2-thiooxazolidone, which contains the group —NH—CS—O—) (129) have been found to inhibit iodination in the thyroid; hence, it appears that only the thiocarbamide grouping is essential for activity. Studies have been carried out with three compounds, thiourea, thiouracil, and thioacetamide, with similar results.

a. In vivo. Initially, the metabolism of S^{35}-labeled thiourea was studied in animals (200). The results of these experiments led to the conclusions that: 1) Thiourea reaches its site of action in the thyroid within minutes after its intraperitoneal injection into rats. 2) There is no concentration gradient for thiourea between the thyroid and serum (0.4). 3) Thiourea is metabolized by the thyroid. The major sulfur products are sulfate, thiosulfate, and protein-bound sulfur (PB—S). 4) The minimal effective concentration of thiourea within the thyroid which inhibits iodination is about 2.5×10^{-5} M.

It would appear that the rapidity with which the thiocarbamide compounds are metabolized by the thyroid is a major factor in determining the duration of their inhibitory activity. The duration of action of equimolar doses of the thiocarbamides is thiouracil > thiourea > thioacetamide. This corresponds to the rate by which they are metabolized by the rat thyroid: thioacetamide, 1 μmol/g per hr (202); thiourea, 0.25 μmol/g per hr (200); and thiouracil, 0.080 μmol/g per hr (203). It is pertinent to relate the quantity of organic iodine formed by the thyroid to the quantity of these compounds metabolized by the thyroid. Nadler and Leblond (220) have calculated that the thyroid of the rat is able to change iodide to organic form at the rate of 0.081 μg/hr. This is equivalent to *ca.* 1 mμmol/hr, and is of the same order of magnitude as that of the thiocarbamides metabolized (0.8 to 10 mμmol/10 mg thyroid per hr). This may be more evidence for a common oxidizing enzyme system in the thyroid for both iodide and the thiocarbamides.

b. In vitro. Subsequently, a system (207) was found which simulated *in vitro* the metabolism of thiourea *in vivo*. The optimal requirements for the desulfuration of thiourea *in vitro* are: a cytoplasmic particulate fraction of thyroid tissue[2];

[2] Preliminary studies in our laboratory have led to the solubilization of this enzyme system. An acetone powder of sheep thyroid "microsomes" is sonicated and centrifuged. The resultant supernatant is clear yellow and contains 70% of the "microsomal" protein. The enzyme activity resists sedimentation at 105,000 × *g* for two hours, but 50% of it sediments after four hours. Spectroscopic analysis of the clear yellow solution reveals a Soret band at 412 mμ. Upon reduction of the solution with dithionite, the Soret band shifts

1.0 mg protein; KSCN, 10^{-3} M; ascorbic acid, 10^{-3} M; thiourea, 5×10^{-5} M; tris buffer, 1.5×10^{-1} M, pH 7.4, and water to a final volume of 1.0 ml. The optimal incubation period is one hour at 37°C. Similar cytoplasmic particulate preparations of liver and kidney tissue are inactive. Thiocyanate ion is a specific requirement, but ascorbic acid can be replaced, in part, by other reducing agents such as NADH.

Inhibitors of the desulfuration of thiourea *in vitro* are noted in Table 2. Three features of these inhibitors are worthy of mention. First, of the halides, only iodide is inhibitory. Second, this is the same pattern of inhibition observed in the iodination of tyrosyl groups by thyroid cytoplasmic particulate fractions (319). Third, the most potent inhibitors are sulfite, cyanide, and thiols.

The sulfur metabolites of this system *in vitro* are sulfate, thiosulfate, and protein-bound sulfur. Recently, the reviewers have been concerned chiefly with the nature and the linkage of the sulfur of thiourea which is transferred to the thyroid protein (204, 207). By using C^{14}-labeled thiourea, it was determined initially that none of the ureido portion of the molecule becomes protein-bound. Hence, only the sulfur portion of thiourea becomes bound to protein during the course of the reaction. C^{14}-urea was identified as one of three C^{14}-labeled products formed during the incubation. The sulfur is firmly bound to protein and resists dialysis and treatment with 1 N HCl at room temperature for two hours. However, about 80% of it can be displaced by nucleophilic anions (0.01 M) such as sulfite or cyanide at pH 7.4, by 0.1 N NaOH, or by thiols (0.01 M), such as cysteine, or reduced glutathione. Incubation with sulfite, cyanide, or thiols led to the formation of thiosulfate, thiocyanate, or hydrogen sulfide, respectively. These properties are similar to those of the organic polysulfide reported by Hylin and Wood (153) to result from the transsulfuration reaction involving β-mercaptopyruvate.

The potent inhibitors of this *in vitro* system, namely, sulfite and thiols, are nucleophilic anions which cleave disulfide bonds:

$$RSSR' + SO_3^{=} \rightleftharpoons RS^- + R'SSO_3^-$$
$$RSSR' + R''S^- \rightleftharpoons RS^- + R'SSR''$$

Preincubation of the particulate fraction with sulfite, cysteine, or thioglycolic acid (all 0.01 M) at 4° for 15 minutes, pH 7.4, followed by dialysis overnight at 4° against distilled water, leads to a 67 to 80% inhibition of the subsequent desulfuration of thiourea. Preincubation with sulfhydryl group inhibitors, such as iodoacetamide, *p*-chloromercuribenzoate, or methyl mercuric nitrate (all 0.01

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to 423 mμ and an α and β peak develop at 530 and 560 mμ. The addition of alkaline-pyridine to the original yellow solution yields a hemochromogen with peaks at 418, 523, and 555 mμ. The spectral pattern of this hemochromogen is consistent with one of three possibilities: (1) a peroxidase; (2) cytochrome b, or (3) hemoglobin. The presence of thiocyanate ion (10^{-3} M) or iodide (10^{-4} M) throughout the extraction procedure was essential to retain enzymatic activity. This enzyme system also contains activity for the oxidation of thiocyanate and the iodination of organic molecules.

TABLE 2

Inhibition of the desulfuration of thiourea (50 mμmol) by thyroid cytoplasmic particulate fractions (207)

Inhibitors	Concentration (M)	Inhibition %
Anaerobiosis	—	80
Iodide	10^{-3}	78
Azide	10^{-3}	84
Cyanide	10^{-4}	91
Sulfide	5×10^{-4}	82
Sulfite	5×10^{-5}	90
Thiosulfate	10^{-4}	60
Thiols	10^{-4}	80
Aromatic antithyroid drugs	4×10^{-3}	50

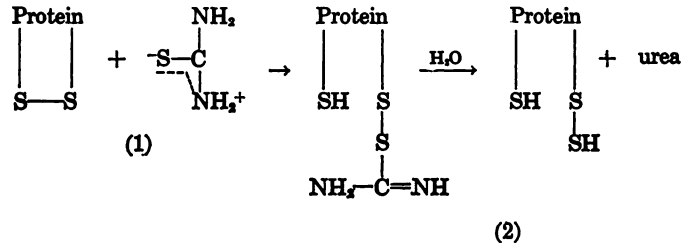
M), produces no inhibition. Hence, a free sulfhydryl group is not involved in the reaction.

Since the reaction of sulfite with proteins is fairly specific (23), further studies with this anion were carried out. Incubation of the thyroid cytoplasmic particulate fraction with S^{35}-labeled sulfite ($S^{35}O_3^=$) yields protein-bound radioactivity (PB—$S^{35}O_3^-$). This reaction is non-enzymatic and occurs rapidly. The amount of sulfite bound to protein is of the same order of magnitude as that of the thiourea sulfur which becomes bound to protein. The sulfite attached to protein resists dialysis and treatment with 1 N HCl at room temperature, but 80% of it can be displaced from the protein by precisely the same reagents that remove the sulfur from the PB—S. Hence, sulfite and the sulfur of thiourea seem to be attached to thyroid protein in a similar fashion. Further and fairly conclusive evidence for this latter point was obtained from inhibition studies which clearly revealed that sulfite is a competitive inhibitor of the desulfuration of thiourea (204).

From the experiments with sulfite and the preincubation studies with nucleophilic anions, it may be inferred that an intact disulfide bond in thyroid tissue is essential for the desulfuration reaction. Furthermore, this disulfide bond serves as a direct acceptor for the sulfur of thiourea. The desulfuration of thiourea may, therefore, be considered to occur *via* a transsulfuration reaction. Two other transsulfuration reactions are worthy of note. One produces thiocyanate from cyanide and thiosulfate and is catalyzed by the rhodanese enzyme which has been reported by Sörbo (297) to have a disulfide bond as its active site. However, Green and Westley (127) have recently questioned this interpretation. The other produces pyruvate, sulfur, and protein-bound sulfur from β-mercaptopyruvate (94, 212) and is catalyzed by a liver enzyme which has been reported to require a sulfhydryl group (152) or a thiol-disulfide-copper system (172) for activity.

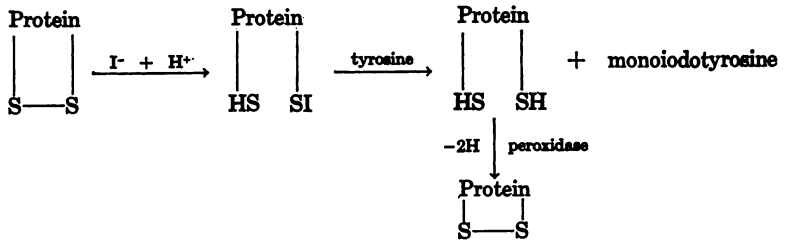
c. Cleavage of disulfide bonds in thyroid by thiourea. These data lead one to conclude that the thiourea transsulfuration reaction probably involves a thiol-disulfide interaction between thiourea and the thyroid protein, which results in the formation of a mixed disulfide (reaction 1), which upon hydrolysis yields a

thiosulfenic acid (reaction 2) and urea:



This formulation is supported by chemical studies in which thiourea was found to cleave the disulfide bond of cystine to yield the mixed disulfide S-(guanyl thio)-L-cysteine (315). Evidence has been accumulating rapidly for the importance of the role which thiol-disulfide interactions may play in biological systems. Recently, Schwartz *et al.* (270) have suggested that such an interaction may be involved in the mechanism of action of vasopressin, a disulfide hormone.

At this juncture, one might ask whether this disulfide bond in thyroid tissue is a structural or a functional site. Parallel observations in the reviewers' laboratory on the iodination reaction *in vitro* have suggested that an intact disulfide bond in thyroid particulates is also essential for protein-bound monoiodotyrosine formation (Table 3). Our working hypothesis has been that a sulfenyl iodide intermediate, resulting from cleavage of a disulfide bond by iodide, may be the active iodinating species in thyroid tissue. According to this formulation, a peroxidase in thyroid tissue would function in the re-oxidation of the dithiol formed after the transfer of the "active iodide" to tyrosine:



Therefore, competition for the disulfide bond in thyroid tissue may explain the inhibitory action of the thiocarbamides. Support for this formulation derives from recent studies which have revealed that 1-methyl-2-mercaptoimidazole, a thiocarbamide, is a competitive inhibitor in a solubilized iodinating enzyme system (59) suggesting that this compound and iodide compete for a common site in the thyroid.

5. Extrathyroidal desulfuration of thiocarbamides. It seems pertinent to digress and to note an interesting consequence of the extrathyroidal desulfuration of the thiocarbamides. Fox (103) reported in 1932 that approximately 30% of white North Americans were unable to detect the bitter taste of phenylthiocarbamide

TABLE 3

Desulfuration of thiourea and iodination by a thyroid cytoplasmic particulate fraction; inhibition by preincubation

	Inhibitor M	Thiourea Desulfuration % Inhibition	Iodination % Inhibition
Sulfite	0.01	73.0	84.0
Cysteine	0.01	73.0	77.0
Thioglycolic acid	0.01	59.0	70.0

(PTC). This non-tasting trait is inherited as probably a Mendelian, autosomal recessive trait (50). The non-tasting of PTC (140, 165), thiourea and propylthiouracil (139) and goitrin (276) has been shown to be more common in adult euthyroid patients with goiters and among congenital athyreotic cretins and their families than among the general population. Shepard (277) found that human saliva and particulate preparations of mammalian taste buds were able to desulfurate thiourea in a manner similar to that for thyroid tissue (207). The complete details of the salivary desulfurase system are not available, but one feature which differentiates it from the thyroid system (207) is the high pH optimum, 10.0. These studies led Shepard (277) to attempt to differentiate tasters and non-tasters by the ability of their saliva to desulfurate thiourea or by the ability of their thyroids to be inhibited by the thiocarbamides. To date, he has found no direct correlation. However, recently Fischer *et al.* (100) have noted that the saliva of non-tasters has an increased ability to alter thiocarbamides, as measured spectrophotometrically.

6. Toxicity of thiocarbamides. The toxicity of these compounds appears to be related to the degree of desulfuration, *i.e.*, the hydrogen sulfide generated by degradation of the thiocarbonyl group. Thiourea is not very toxic to young rats, and Schulman (267) has reported that only 12% of a dose of 13 μmol of thiourea is desulfurated by the animal in 24 hours. Diphenylthiourea is much less toxic to rats and rabbits than phenylthiourea (11, 335), and diphenylthiourea is not desulfurated *in vivo* (290). However, 62% of the labeled sulfur of phenylthiourea appears as total sulfate in the excreta (265). Solutions of pure H_2S, given intravenously, are highly toxic to rats, the LD50 being about 0.27 to 0.55 mg/kg (335). Calculations revealed that one LD50 of phenylthiourea (40 mg/kg) could release 0.7 mg of H_2S/kg of body weight (335).

Another pathway for the detoxification of thiouracil has been reported. Sarcione and Sokal (260) found that rats, injected with 50 μmol of S^{35}-thiouracil, excreted 76% of the radioactivity in the urine in 24 hours. 2-Methylthiouracil accounted for 8.3% of the excreted radioactivity. It was postulated that methylation of the thiol group might serve as a mechanism for detoxifying thiouracil. Furthermore, it was suggested that this process might deplete the active methyl pool in the body, necessary for the formation of leukocytes, and might be responsible for the leukopenia observed during chronic thiouracil therapy. This possibility would seem to be unlikely.

B. Aromatic compounds

Experiments *in vivo* (199) and *in vitro* (106, 263) with the sulfonamides have shown that these drugs inhibit the iodination of tyrosyl groups, but not the concentration of iodide by the thyroid. Although this simulates the effects of the thiocarbamides, the data of Mackenzie (198) suggest that the two types of compounds may affect the thyroid differently. The administration of iodide to rats inhibited moderately or slightly the goitrogenic and hyperplastic effects of thiouracil (0.05%) but not those of sulfaguanidine (0.75%). As a matter of fact, some doses of iodide (50 mg NaI/100 g diet) actually potentiated the goitrogenic effect of sulfaguanidine.

The detailed investigations of Astwood (11) led him to the following generalizations concerning the structure responsible for the action of some of the aromatic antithyroid compounds. The aniline group is common to all. It is apparently necessary that the amino group be free, for activity is lost when this group is substituted, as in *p*-acetamidobenzoic acid and *p*-dimethylaminobenzaldehyde. Aminobenzoic acid was most active when the carboxyl group was *para* to the amino group. Potency was increased when one of the hydrogen atoms of the sulfamide nitrogen of sulfanilamide was replaced by a guanyl-, thiazole-, pyridine-, or pyrimidine grouping, sulfadiazine being the most active.

Fawcett and Kirkwood (87) presented evidence that all aromatic compounds with an electron-donating group on the ring are capable of inhibiting the synthesis of thyroid hormone. Furthermore, these authors postulated that certain aromatic compounds, such as sulfanilamide and the polyhydric phenols inhibit thyroid function by forming molecular compounds with the "active iodide" in thyroid tissue. Inhibition of peroxidase has also been suggested as the basis for the action of the polyhydric phenols (10, 258). Still another possible explanation for the action of the sulfonamides may be suggested. It has been reported that sulfanilamide, as well as *p*-aminobenzoic acid, forms adducts with NAD (132). Evidence discussed above suggests that iodination in thyroid tissue *in vitro* is stimulated by NAD (57, 269). Hence, the inhibitory action of the aromatic compounds may involve the formation of such adducts in thyroid tissue.

C. Hofmeister series of anions

Unlike the thiocarbamides, which inhibit iodination, the anions to be discussed interfere primarily with iodide transport. Some interesting observations concerning these compounds will be presented.

1. Thiocyanate. Thiocyanate is a unique anion which not only inhibits the transport of iodide into the thyroid, but in large doses has been found to inhibit iodination in rats and in surviving thyroid slices (345).

There is no concentration gradient for thiocyanate ion between the thyroid and serum (358). However, thiocyanate ion is metabolized by the thyroid at a maximum rate of about 65 mμmol/g thyroid per hr following the intraperitoneal administration of 20 μmol of S^{35}-thiocyanate to rats (201). Sulfate is the major sulfur product (69% of total radioactivity in thyroid at the end of five hours). Propylthiouracil, which inhibits the formation of organically-bound iodine in the

thyroid, also inhibits the metabolism of thiocyanate (358). Furthermore, the metabolism of thiocyanate in the thyroid is completely inhibited by thiourea, sodium iodide, or sulfadiazine, but not by perchlorate (201). The administration of thyrotropin to guinea pigs increases the intrathyroidal metabolism of thiocyanate (259). Hence, it appears that a common oxidative mechanism is involved in the iodination of tyrosyl groups and in the metabolism of thiocyanate in the thyroid.

Thiocyanate is a ubiquitous anion in the body. The serum level of thiocyanate in the normal rat has been reported to be about 55 mμmol/ml (149). Following the intraperitoneal administration of 20 μmol of thiocyanate to rats, there is a maximal inhibition of the concentration of iodide by the thyroid at a time when the serum level of thiocyanate ranges from 180 to 286 mμmol/ml and the thyroid level is 70 mμmol/g wet weight. Hence, it would appear that there is a narrow range between the level of thiocyanate normally found in the serum of the rat and the level at which there is an inhibition of the concentration of iodide by the thyroid. The role of thiocyanate ion in the homeostasis of the thyroid remains a provocative question.

Recently a system has been found which simulates *in vitro* the metabolism of thiocyanate *in vivo* (206). Inorganic sulfate is the major sulfur metabolite. Unlike the metabolism of thiourea (207) by a thyroid particulate fraction *in vitro*, there is no significant protein-bound sulfur formed. The optimal requirements for the degradation of thiocyanate *in vitro* are: tissue fraction, *ca.* 0.5 mg protein; tris buffer pH 7.4, 1.5×10^{-1} M; ascorbic acid, 4×10^{-4} M; thiocyanate, 1.7×10^{-4} M, and water to a final volume of 1.0 ml. The tissue fraction is a cytoplasmic particulate derivative of thyroid tissue. Similar preparations of liver and kidney tissue are inactive. Inhibition studies have suggested that thiourea is a competitive inhibitor of the desulfuration of thiocyanate.

The metabolism of thiocyanate by thyroid tissue is not related to its action in discharging iodide from this organ, since this effect of thiocyanate can be demonstrated in mammals pretreated with thiocarbamides which have been shown to inhibit completely the oxidation of thiocyanate. Nevertheless, further studies concerning the metabolism of thiocyanate in thyroid tissue are indicated as another means of evaluating the iodination reaction.

2. Other monovalent anions. Wyngaarden *et al.* (362) investigated a series of anions to determine whether their action on the thyroid was similar to that of thiocyanate. Anions which behaved as iodide in the Hofmeister series or in their interaction with serum albumin were selected initially. Then the series was extended to include higher or lower oxidation forms of active ions. Scatchard and Black (261), using a pure solution of albumin, found that the combining capacity of this protein for several anions was $ClO_4^- > SCN^- = I^- > NO_3^-$. Anions which discharged iodide from the thyroid of rats which had been pretreated with propylthiouracil were found to be, in decreasing order of potency: $ClO_4^- > IO_4^- > IO_3^- > ClO_3^- > H(IO_3)_2^- > ClO^- > NO_3^- > NO_2^-$. Fluoride, Cl^-, Br^-, MnO_4^-, $S_2O_8^=$, $SO_4^=$, $NaPO_4^-$, acetate, ascorbate, and dehydrocholate were ineffective in 100 μmol doses. Perchlorate was found to be ten times as

active in depressing the T/S ratio of iodide as equimolar doses of thiocyanate (361).

Perchlorate. Anbar *et al.* (7) have extended the observations with perchlorate, especially as to its concentration in the thyroid and its metabolism. Using doubly-labeled ClO_4^- (Cl^{36} and O^{18}), ClO_4^- was found to concentrate in the thyroid at a T/S ratio of about 3 to 4/1. Urinary studies of radioactivity showed that ClO_4^- was probably not metabolized in four people following the administration of 200 mg.

Anbar *et al.* (8, 9) pursued the studies of anions which inhibit I^- transport to those with monovalency and volume and tetrahedral configuration similar to that of ClO_4^-. The calculated ionic volumes of ClO_4^-, I^-, TcO_4^-, ReO_4^-, MnO_4^-, SO_3F^-, $PO_2F_2^-$, and BF_4^- were all about 4.0×10^{-23} cm^3 (8, 9). It was found that SO_3F^-, $PO_2F_2^-$ and BF_4^- inhibited the concentration of iodide by the thyroid (8). Radioactively-labeled BF_4^- was apparently concentrated by the thyroid (T/S = 36/1 at 120 min) (9). This measurement is open to some criticism since only radioactivity was assessed, and it is not clear from the data presented that this radioactivity represented BF_4^- *per se*. On the basis of their data, Anbar *et al.* (8, 9) suggested that the trapping mechanism in the thyroid involves a factor which does not distinguish between different anions having the same charge and a comparable volume, and does not involve any special energy expenditure or active carrier system. They considered that the important component is probably a protein with a spatial arrangement that can chelate those anions which fulfill size requirements. Halmi (137) has reviewed this interesting problem and has listed five arguments against this theory. The most important of these concerns the thiocyanate ion, which is a planar, not a tetrahedral ion; its ionic volume was not listed by Anbar (8, 9). This is no doubt due to the fact that it is impossible to determine a precise radius for such an anion.

D. Peripheral effects of various compounds

It is pertinent to evaluate briefly several compounds which may affect thyroid function primarily by producing alterations in the periphery. Robbins and Rall (249) have introduced the concept that it is the level of free, not bound, thyroxine in the serum which is metabolically active. Compounds which affect the binding of thyroxine and its metabolism peripherally will be discussed.

1. Thiocarbamides. There is evidence that some thiocarbamides have a peripheral action. Propylthiouracil (155, 322), but not 1-methyl-2-mercaptoimidazole (327), inhibits the peripheral deiodination of exogenously administered thyroxine in rats. This has also been demonstrated *in vitro* (45, 176) since thiouracil derivatives (3×10^{-7} M to 3×10^{-6} M) were found to inhibit the conversion of L-thyroxine to a compound chromatographically similar to triiodothyronine by rat kidney slices. The role that these peripheral effects of the antithyroid compounds play in altering the metabolic rate of patients is not clear since it has not been shown that thyroxine needs to be deiodinated to be metabolically active.

2. Estrogens and androgens. Suitable doses of estrogens in man produce an increase in the levels of serum protein-bound iodine (86), an increase in the

binding capacity of the thyroxine-binding protein for thyroxine (76), and a decreased binding by erythrocytes of I^{131}-thyroxine and -triiodothyronine in an *in vitro* assay (138). These changes simulate findings in pregnancy (75). In spite of the elevated protein-bound iodine, the thyroid uptake of I^{131} is not depressed (76) and the pregnant patient is not hyperthyroid, presumably due to the fact that the level of free thyroxine in the serum is normal (249).

Androgens seem to produce effects opposite to those of estrogens. Federman *et al.* (90) administered 100 mg of methyltestosterone daily to four subjects for seven weeks and noted a decrease in the uptake of I^{131} by the thyroid, a slight decrease in the protein-bound iodine of the serum, a decrease in the thyroxine-binding capacity of the thyroxine-binding globulin, an increase in the calculated "free thyroxine," and an increase in daily thyroxine degradation. Keitel and Sherer (161) also noted a fall in the serum protein-bound iodine and a decreased uptake of I^{131} by the thyroid following the use of methyltestosterone.

3. Compounds which bind to the thyroxine-binding protein. A number of compounds such as 2,4-dinitrophenol (349), salicylates (14), DL-tetrachlorothyronine (183, 245), diphenylhydantoin (223), and trypan blue (363, 364) have been shown to lower the serum protein-bound iodine level. From the observations with DNP and salicylates, it was postulated that these compounds act, in part, by inhibiting the TSH mechanism at the pituitary or at a higher level and, in part, by accelerating the loss of thyroxine from the circulation (9, 344), possibly through an increase in the level of "free thyroxine" (44). Wolff *et al.* (350) finally concluded, from a series of binding studies, that the loss of binding sites on the major thyroxine-binding proteins in serum could explain the lowering of the serum protein-bound iodine produced by salicylates, 2,4-dinitrophenol, and DL-tetrachlorothyronine. A somewhat similar mechanism has been postulated for trypan blue (363, 364).

4. Reserpine. It has been suggested that reserpine may alter thyroid function directly, or indirectly, by acting on subcortical areas, especially the reticular system and the hypothalamus, or peripherally by its action on the metabolism of thyroxine. Reserpine (1.4×10^{-4} M) has been reported to inhibit the formation *in vitro* of protein-bound iodine in calf thyroid slices (210). However, the administration of large doses of reserpine (0.75 to 4.0 mg/day) to hyperthyroid patients for as long as 104 days led to the amelioration of symptoms without altering the serum protein-bound iodine, the basal metabolic rate, or the uptake of I^{131} by the thyroid (38). Yamazaki *et al.* (366) have administered reserpine to mice and concluded that it may inhibit the release of TSH and thus depress thyroid function. It has also been shown that reserpine, as well as certain metabolites of tryptophan, inhibit the deiodination of thyroxine and several of its analogues by homogenates of mammalian and amphibian tissues (49).

XII. CONCLUSION

It is clear from this review that some progress has been made in defining thyroid function at a basic level. A stumbling block to the precise localization of various mechanisms is the anatomical structure of the thyroid, *i.e.*, a division into two

major components—follicular cells and follicular colloid. Detailed observations of isolated functional follicular cells should help solve this dilemma. Nevertheless, it appears likely that the follicular cell with its complement of cytostructural components is the initiating locus of the important reactions in the thyroid.

Progress has been made regarding carbohydrate and lipid metabolism in thyroid tissue. Further studies along these lines should be forthcoming and will be invaluable in defining the energetics of thyroid tissue. Similar progress in protein synthesis, in particular, thyroglobulin synthesis, is lacking.

New and systematic methods of study are contributing greatly to the evaluation of the iodide-concentrating mechanism. A maze of data concerning the iodination reaction has been published and it is difficult to evaluate them critically because of the wide variety of techniques and systems employed by various investigators. Although the weight of evidence suggests that a peroxidase is involved in the iodinating system, the precise role of the peroxidase remains to be elucidated. It will be important to acquire knowledge concerning the endogenous hydrogen peroxide-generating system within the thyroid. Furthermore, the actual concentration of peroxide as well as the factors which control its level must be determined. The nature of the iodinating intermediate remains an enigma, but on the basis of preliminary studies, a sulfenyl iodide (RSI) suggests itself as a likely possibility.

This review of antithyroid drugs has been largely limited to specific compounds, the metabolic pathways of which have been studied. This obviously has limited the report. Observations involving the thiocarbamides continue to play a crucial part in evaluating thyroid function. Recent studies with the thiocarbamides, thiourea in particular, have been informative and have led to a new concept in thyroid physiology, *i.e.*, the possible role of a disulfide bond in thyroid tissue, not only as a locus for the inhibitory action of these compounds but as an initiating site for the iodination reaction.

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