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Edited by

GREGORY PINCUS

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PREFACE

The twelfth annual Laurentian Hormone Conference was held at Estes Park, Colorado, during the period September 6 to 11, 1955. The holding of the meeting in the Rocky Mountains was to enable attendance by investigators in the West and far West who ordinarily find it difficult to make the trip to the usual eastern site. The response to this move was the largest attendance of any meeting thus far held, in part due to the fact that attendance by easterners was scarcely diminished.

Contributions to the support of the Conference were made by the following companies: The Armour Laboratories; Ayerst Laboratories; Baxter Laboratories, Inc.; Carroll Dunham Smith Pharmacal Company; Ciba Pharmaceutical Products, Inc.; Ciba Company Limited; Endo Products, Inc.; Charles E. Frosst & Co.; Hoffman-La Roche, Inc.; Frank W. Horner Limited; Lederle Laboratories Division; The Eli Lilly and Company Research Laboratories; Merck & Company, Inc.; The Wm. S. Merrell Company; Organon, Inc.; Ortho Research Foundation; Parke, Davis & Company; Chas. Pfizer & Co., Inc.; Schering Corporation; Schieffelin & Co.; G. D. Searle & Company; Sharp & Dohme; Smith, Kline & French Laboratories; Squibb Institute of Medical Research; Sterling-Winthrop Research Institute; The Upjohn Company; Warner-Chilcott Research Laboratories; Wyeth Laboratories, Inc.; Syntex, S.A.; Chemical Specialties, Inc.; and Root Chemicals, Inc. Their assistance enabled the Committee on Arrangements to invite as special guests from abroad Dr. Jean Roche of the College de France and Dr. Thaddeus Mann of Cambridge University.

The Committee is indebted to Drs. T. Dougherty, S. Lieberman, L. Engel, G. Wolstenholme, R. Levine, W. O. Nelson and A. Segaloff who served with vigor and tact as chairmen at the various sessions. Miss Joanne Sanford, Mrs. Jacqueline Foss, Miss Marjorie Riches and Miss Florence Frey ably assisted as secretaries to the Conference, and Mrs. L. P. Romanoff has been kind enough to prepare the index of this volume.

The results of this meeting in a literally rarefied atmosphere are herein incorporated. We believe they indicate no impairment of thought or activity, and that they fulfill the objective of the presentation and discussion of scientific endocrinology in its pioneering and solid accomplishment.

GREGORY PINCUS

Shrewsbury, Massachusetts
July, 1956

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I. HORMONE BIOSYNTHESIS AND METABOLISM

Nature and Metabolism of Thyroid Hormones

JEAN ROCHE AND RAYMOND MICHEL

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Important advances have been achieved in the last few years in the biochemistry of thyroid hormones, chiefly owing to the extensive use of radioactive iodine in chromatographic studies. It is not possible to review here all the recent papers devoted to the subject, partly covered by a series of surveys (9, 27, 35, 48, 70-73, 93). Therefore, it seems preferable to present the actual state of the problems concerned in two fields of thyroid biochemistry which are rapidly progressing: the nature of thyroid hormonal secretion, and the metabolism of the active products. Both can be connected with the eventual formation in receptors cells of active substances originating from precursors elaborated in the gland.

I. NATURE OF THYROID HORMONAL SECRETION

If one defined as the physiological hormone the mixture of active substances secreted in blood, the nature of these substances would seem to be clarified at least for the major part of organically bound iodine of glandular origin present in the plasma. Iodide ions circulating in the plasma are likely trapped by epithelial cells in the gland and enzymically oxidized to iodine, probably on the border of cells oriented toward the colloid vesicles, containing a specific glycoprotein, thyroglobulin (47, 101). This protein reacts immediately with free iodine, by means of a process apparently identical to that realized *in vitro* by direct iodination of proteins by I_2 solutions and leading to the formation of iodinated amino acids (1), some of which have been identified only recently. Thyroglobulin is stored in the colloid vesicles where its iodination proceeds, but it is never secreted as a precursor of active material except in abnormal conditions—for example, after an overdosage of radioactive iodides collected by the gland (61, 63). In physiological conditions the protein is hydrolyzed by a mixture of catheptases (7, 52, 53, 64, 75), and this process liberates the iodinated amino acids, some of which are secreted as the hormonal products of thyroid activity. All the iodinated amino acids included in the specific protein have been found free in the *n*-butanol extract of the gland (31), but only some are secreted in blood (98), owing to a sort of choice due to the dehalogenation of the others by an enzymic mechanism (77).

Iodinated derivatives of three amino acids have been isolated and characterized as thyroglobulin constituents. Two series are substitution products of common amino acids: L-tyrosine and L-histidine. The third derives from a specific amino acid, found until recently only in the thyroid gland: L-thyronine, or β-4-(4′-hydroxyphenoxy)phenyl-α-aminopropionic acid (abbreviated symbol, Th). Only the iodothyronines have the definite physiological effect of thyroid preparations, especially on heart beat and on oxygen consumption, and they are the sole iodinated products secreted by the gland in chromatographically detectable amounts. The L-2(or 4)-monoiodohistidine (68) contains no more than 2 to 3% of the total iodine of the protein and can be neglected for the present discussion; its presence is not directly connected to hormogenesis, of which it is a by-product. A radioautogram obtained with suitable solvents illustrates the complexity of the mixture of

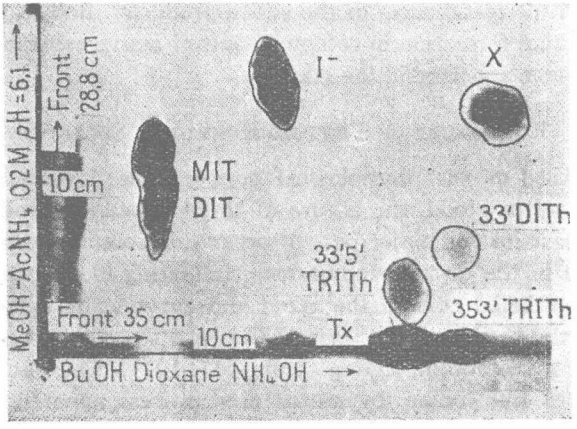
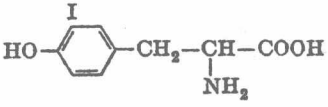


FIG. 1. Radioautogram of bidimensional chromatogram of thyroglobulin hydrolyzate (successive action of total pancreatic proteinases and of papain) of the thyroglobulin of rats treated with I^{131} 24 hours before they were killed. Solvents: (1) *n*-butanol-dioxane (4: 1) saturated with NH_4OH, 2 *N*, descending, $t° = +17°C$. (2) Methanol-ammonium acetate, 0.2 *M* (1: 2.5), pH = 6.1, ascending, $t° = +3°C$. I^- = iodides, MIT = L-3-monoiodotyrosine, DIT = 3,5-diiodotyrosine, 3,3′-DITh = 3,3′-diiodothyronine, 3,5,3′-TRITh = 3,5,3′-triiodothyronine, 3,3′,5′-TRITh = 3,3′,5′-triiodothyronine, and Tx = thyroxine. The weakness of the 3,3′-DITh spot is due to length of time elapsed before killing.

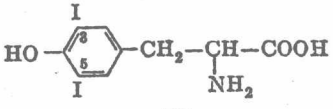
iodinated compounds of enzymic hydrolyzates of a thyroglobulin of rat injected with I^{131} (Fig. 1).

L-3-Monoiodotyrosine (I) or MIT (L-3-iodo-4-hydroxylphenyl-α-aminopropionic acid) was found for the first time in 1948 in the fibrous scleroproteins of the corneous skeleton of sea fans, the *Gorgonia* (26), in the

spongins of sponges (91), in thyroglobulin (22, 65), and in thyroid extracts (81). It contains 10 to 15% of the total iodine of the gland protein and is formed as the initial step in its halogenation. The structure of L-3,5-diiodotyrosine (II) or DIT (L-3,5-diiodo-4-hydroxyphenyl-α-aminopropionic acid, previously called iodogorgonic acid), isolated in 1896 from a Gorgonin (21), was identified in 1910. It was extracted in 1929 in pure form from thyroid gland and found to contain 30 to 40% of the total iodine of this organ (38). These two derivatives are devoid of hormone activity. It has been claimed that the DIT is a weak antagonist of L-thyroxine, but this effect is probably due to iodides produced by its dehalogenation (inhibition effect on iodine fixation, exerted through action on thyrotropic hormone secretion by the antehypophysis).



(I)
L-3-Monoiodotyrosine, or MIT



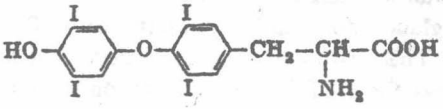
(II)
L-3,5-Diiodotyrosine, or DIT

Amino acids of the L-iodothyronine series have been identified as constituents of thyroglobulin and found in the free state in thyroid extracts. Their mixture can be considered as the physiological hormone, as they are biologically active and are not deiodinated by the dehalogenase acting on the free iodothyronines in the gland (76). It may be that very small amounts of other iodothyronines as yet unknown are also present, but one can be reasonably sure that at least 95% of hormone iodine is found in four substances: L-thyroxine (III), or Tx; L-3,5,3′-triiodothyronine (IV), or 3,5,3′-TRITh; L-3,3′,5′-triiodothyronine (V), or 3,3′,5′-TRITh; and L-3,3′-diiodothyronine (VI), or 3,3′-DITh. A fourth of the total iodine of the gland is included in the iodothyronines.

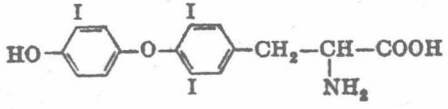
L-Thyroxine, or 3,5,3′,5′-tetraiodothyronine, was isolated in 1915 (43), and its structure was established in 1927 (37). It contains about 20% of the total iodine of thyroglobulin and has for a long time been considered to be the sole hormone. L-3,5,3′-triiodothyronine was identified and isolated from the gland in 1952 simultaneously by two groups of biochemists, working separately in London (33, 34) and in Paris (66, 69). Its activities are five to ten times as great as those of Tx, according to the test used for the bioassay. It is never present in an amount exceeding 5 to 7% of the total iodine in thyroglobulin or in *n*-butanol extracts of the organ.

One could expect that other iodothyronines would be found as unknown constituents detected by chromatographic analysis of thyroid gland hydrolyzates or extracts from animals treated with radioactive iodides (30). Re-

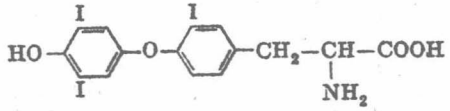
search in this field has been rendered possible by preliminary work including preparation by synthesis of a series of new iodothyronines (82) and a study of their chromatographic behavior in numerous solvents (87, 88). As some of these have been until now used only by our co-workers and us, informa-



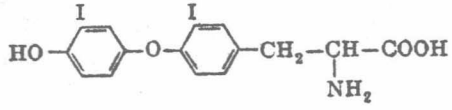
(III)
Thyroxine, or Tx



(IV)
3,5,3′-Triiodothyronine, or 3,5,3′-TRITh



(V)
3,3′,5′-Triiodothyronine, or 3,3′,5′-TRITh



(VI)
3,3′-Diiodothyronine, or 3,3′-DITh

tion concerning the R_f of iodothyronines in the presence of these solvents has been summarized in Table I.

Isopentanol saturated with NH_4OH, 2 N, is very suitable for the separation of 3,5,3′-TRITh and 3,3′,5′-TRITh; n-butanol-dioxane saturated with NH_4OH, 2 N, for the identification of 3,3′,5′-TRITh; and methanol-ammonium acetate, 0.2 N (pH = 6.1, $t°$ = +3°C.), for 3,3′-DITh. Autograms reproduced in Figs. 1, 2, and 3 show the results obtained in bi-dimensional chromatography with some of these mixtures.

Two new iodothyronines have been recently identified in thyroglobulin and as free amino acids in the gland extracts (87, 88), as a result of the use of appropriate solvents. 3,3′,5′-Triiodothyronine is present in very small

TABLE I

R_f of Iodothyronines

Products	*n*-butanol-AcOH-H_2O (78: 5: 17), descending, 17°C.	*n*-butanol sat. NH_4OH, 2 *N*, descending 17°C.	*n*-butanol-dioxane (4: 1) sat. NH_4OH, 2 *N*, descending 17°C.	Methanol-$AcONH_4$, 0.2 *N* (1: 2.5) ascending, 3°C., carrier 10 μg.	Isopentanol sat. NH_4OH, 2 *N*, descending, 13°C.	Collidine-H_2O (35. 5: 100) sat. NH_3 vap., ascending, 17°C.	*tert*-Pentanol sat. NH_4OH, 2*N*, ascending, 17°C.
3′-MITh	0.68	0.41	0.40	—	—	0.58	—
3-MITh	0.70	0.60	0.65	0.00	0.70	0.65	—
3,5-DITh	0.65	0.72	0.70	0.00	0.75	0.68	0.50
3,3′-DITh	0.70	0.51	0.48	0.20	0.50	0.58	0.32
3′5′-DITh	0.75	0.43	0.40	—	—	0.38	—
3,3′,5′-TRITh	0.68	0.43	0.41	0.00	0.40	0.63	0.20
3,5,3′-TRITh	0.70	0.65	0.62	0.00	0.60	0.70	0.40
Tx	0.75	0.48	0.46	0.00	0.50	0.59	0.25
Thyronine	0.60	0.50	0.55	—	—	0.57	—
Iodides	0.20	0.31	0.37	0.60	0.20	0.85	0.18

amounts, not exceeding 2% of the total iodine. 3,3′-Diiodothyronine is much more abundant and frequently contains one-fourth of the total hormone iodine. As Tx is twice as rich in halogen, being tetraiodinated, one can estimate that sometimes half the thyronine secreted in the hormone is 3,3′-DITh. This derivative has been constantly found in blood plasma (Fig. 2) (81). Whereas 3,5,3′-TRITh exists irregularly and in very small amounts in normal subjects (5, 13, 15, 19, 33), 3,3′,5′-TRITh has not been detected* in plasma but traces are certainly present because none of the iodothyronines studied is enzymatically deiodinated in the gland. 3,3′-DITh is slightly less active than

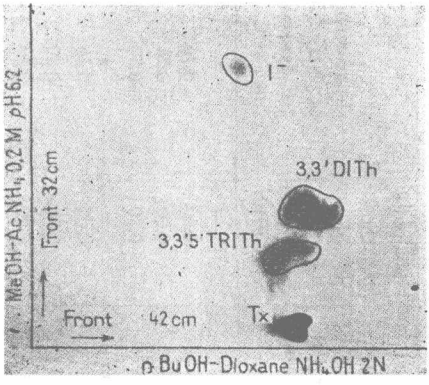


FIG. 2. Radioautogram of bidimensional chromatogram of an enzymatic hydrolyzate fraction of total thyroid extract of rats treated with I^{131} 12 hours previous to being killed. Solvents: (1) *n*-butanol-dioxane (4: 1) saturated with NH_4OH, 2 *N*, descending, $t° = +17°C$. (2) Methanol-ammonium acetate, 0.2 *M* (1: 2.5), pH = 6.1, ascending, $t° = +3°C$. I^- = iodides, Tx = thyroxine, 3,3′-DITh = 3,3′-diiodothyronine, 3,3′,5′-TRITh = 3,3′,5′-triiodothyronine.

Tx as an antigoitrogenic substance (75% in assays on rats), and 3,3′,5′-TRITh has only very small efficiency (5% in the same assay) (89).

In the present state of our knowledge, the hormone mixture produced by the thyroid gland appears to include the four iodothyronines mentioned above. The proportion of these is probably more or less variable, according to influences exerting their effects on the speed of the iodination of thyroglobulin. Tx is always the most abundant, as well as its precursor DIT. Both TRITh isomers are always present in very small amounts, distinctly less than those of 3,3′-DITh. The physiological importance of these four iodothyronines holds not only for the amounts secreted but also for their

* 3,3′,5′-TRITh has been recently identified in plasma of rats treated by I^{131} (91a).

utilization by cells, the speed of which is much higher for 3,3'-DITh and 3,5,3'-TRITh than for Tx. Formation of these two from more halogenated precursors in peripheral cells is also possible, as will be discussed below, but the scope of this section has been to review the nature of the active product elaborated and secreted by the gland. The nature of the products acting in receptors cells is certainly a more complicated subject and will also be discussed later.

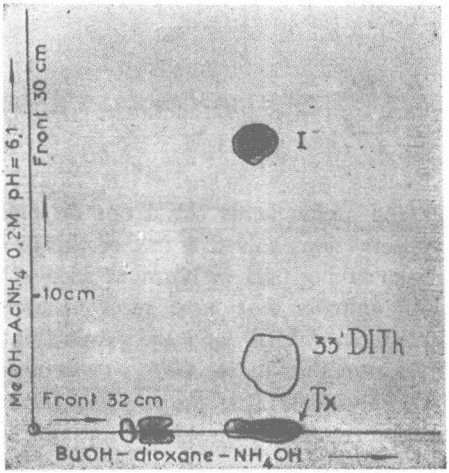


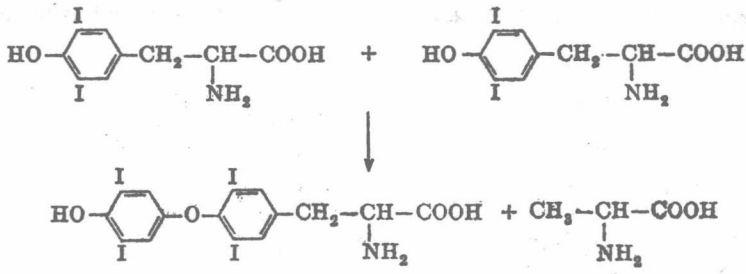
FIG. 3. Radioautogram of bidimensional chromatogram of a *n*-butanol extract of rat plasma prepared 24 hours after administration of I^{131} as iodides. Solvents: (1) *n*-butanol-dioxane (4: 1) saturated with NH_4OH, 2 *N*, descending, $t° = +17°C.$; (2) methanol-ammonium acetate, 0.2 *M*, (1: 2.5), pH = 6.1, ascending, $t° = +3°C.$ I^- = iodides, Tx = thyroxine, 3,3'-DITh = 3,3'-diiodothyronine.

II. BIOSYNTHESIS OF HORMONES IN THE GLAND

Two important aspects of the biosynthesis of thyroid hormone can be mentioned: the formation of thyronine, and its iodination. Both were previously included in the classical scheme proposed for L-thyroxine formation from L-3,5-diiodothyronine when these were the only iodinated amino acids known as thyroglobulin constituents (36).

Kinetic studies on the formation of iodotyrosines and iodothyronines in the gland (16) confirmed this hypothesis and led to the possibility that Tx originates from the condensation of two molecules of DIT with elimination of one alanine residue. The intimate mechanism of this reaction has been discussed on many occasions (56); it nevertheless remains practically unknown, and further study is urgently needed. It could be expected that the

same reaction would apply to MIT and produce 3,3′-DITh and that the biosynthesis of 3,5,3′-TRITh and of 3,3′,5′-TRITh would take place by the same process, that is, between one molecule of MIT and one of DIT. The technical difficulty of the simultaneous determination of the relative amounts



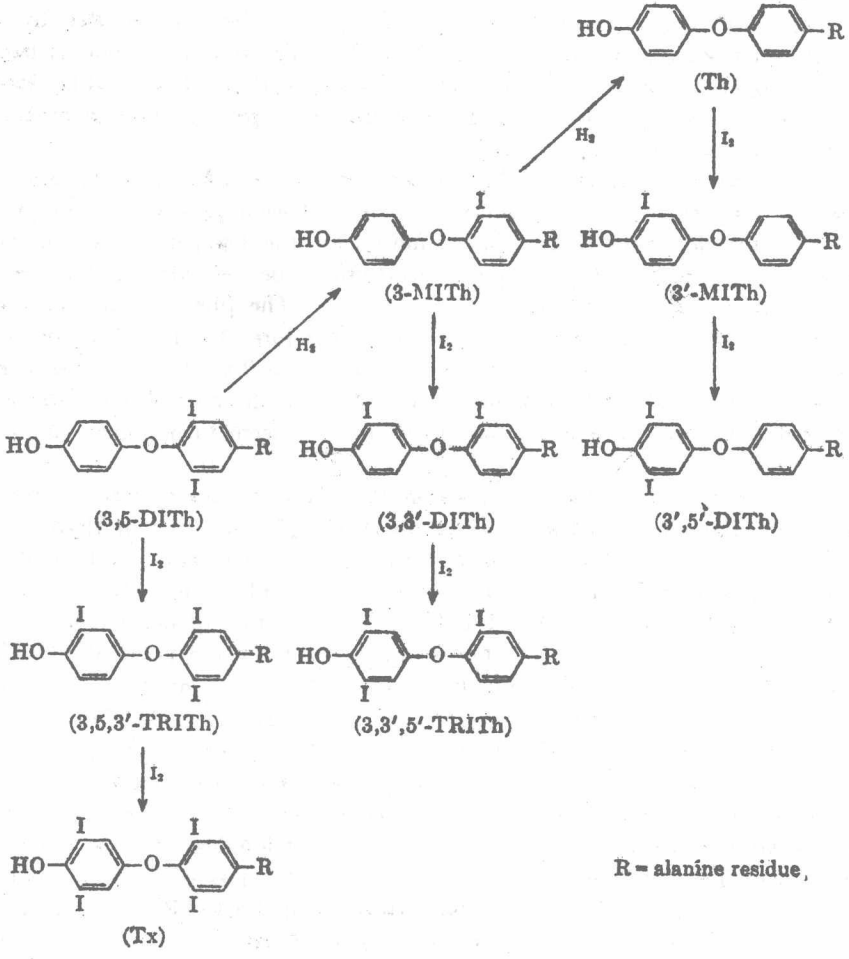
of these various iodinated amino acids could not be completely overcome, but some significant observations solved a few of the problems involved in the sequence of processes taking part in hormone biosynthesis. Radioactive 3,3′-DITh is formed in animals that have received labeled iodide during the time of maximal content of MIT, and the proportion of radioactive Tx increases when MIT is transformed to DIT. Therefore MIT behaves as the precursor of 3,3′-DITh, and DIT as that of Tx. It can be assumed that the condensation reaction is also responsible for the formation of both TRITh in the gland.

Since 1952 the presence of products less iodinated than Tx has raised the question of their origin by a dehalogenation process and, at the same time, that of their role as biological intermediary products in the formation of more iodinated hormones. Enzymic dehalogenation of MIT and DIT by thyroid slices (76) or by purified extracts (77) is very efficient, but the iodothyronines are not deiodinated under the same conditions (76). Therefore it is not likely that Tx is a physiological source of less iodinated homologs in the gland. On the contrary, the iodination process appears to affect poorly the halogenated thyronines in thyroglobulin, as indicated by chemical models.

The scheme on following page (72) summarizes reactions recently studied, starting from 3,5-DITh, in addition to its well-known iodination to Tx, through 3,5,3′-TRITh.

The series of formulas to the left illustrates the iodination of 3,5-DITh as it proceeds for the preparation of 3,5,3′-TRITh and for the last step of the classical thyroxine synthesis (37). As 3,5-DITh is not a natural precursor of 3,5,3′-TRITh, the transformation of 3,5,3′-TRITh to Tx is the only

biological process involved in this sequence. Reduction (Raney's nickel) of 3,5-DITh leads to 3-MITh and to thyronine (Th). The direct iodination of these two amino acids is governed by the orienting power of phenolic groups, and its results are of interest in the interpretation of the origin of biological iodothyronines (86).



Thyronine is not found in thyroglobulin, and therefore the immediate products of its direct iodination, 3′-MITh and 3′,5′-DITh, are also absent from the protein. 3-MITh is halogenated to 3,3′-DITh, and this to 3,3′,5′-TRITh. Further iodination of 3′,5′-DITh and 3,3′,5′-TRITh has not been obtained under conditions whereby transformation of 3,5-DITh to Tx pro-

ceeds, and oxidation reactions partly destroy the organic structure if an excess of halogen reacts. Thus 3′,5′-DITh, 3,3′,5′-TRITh, and Tx are in some respects final products of iodination, the benzene ring of Th bearing the alanine residue being much less reactive than that bearing the phenol group. These results have been applied for analytical purposes in biological interpretations.

Analytically 3,5,3′-TRITh and 3,3′,5′-TRITh can be differentiated by a halogenation assay. The first is transformed to Tx, and the second is not, under properly standardized conditions. The iodination of 3,3′-DITh into 3,3′,5′-TRITh is also specific, and chromatography permits safe identification of all these substances (90).

In the thyroid gland, identical processes seem to take place in the phenolic amino acids present in thyroglobulin, the rings of each participating in substitution reaction with their proper affinity for the halogen. There is no chance for L-tyrosine only (or for L-histidine) to be iodinated in the presence of I_2 freed by enzymic oxydation of I^-. The phenolic ring of the iodothyronines shows identical behavior. Therefore 3,3′-DITh is partly transformed to 3,3′,5′-TRITh, and 3,5,3′-TRITh to Tx. The variations in the respective levels of labeled molecules, observed in the thyroid gland at different times after administration of I^{131} to rats, seem to be partly due to this process.

The biosynthesis of the hormones in the gland probably involves condensation of iodotyrosines and iodination in the phenolic ring of thyronine derivatives having position 3′ or 5′ free. The following scheme can be tentatively proposed: L-3,3′-DITh is formed only by condensation of two molecules of L-3-MIT, as is L-3,5,3′-TRITh by condensation of one molecule of L-3-MIT and one of L-3,5-DIT. On the contrary, L-Tx and L-3,3′,5′-TRITh seem to be of double origin, including condensation reactions (2, L-3-5-DIT $\rightarrow$ L-Tx and L-3,5,3′-TRITh $+ I_2 \rightarrow$ Tx; 2 L-3-MIT $\rightarrow$ L-3,3′-DITh and L-3,3′-DITh $+ I_2 \rightarrow$ L-3,3′,5′-TRITh). This conception can be proposed only for hormonal synthesis in the gland, where the halogenation reaction is very active. Deiodination of the secreted products by the receptor cells was shown a long time ago by urinary excretion of iodides after the administration of Tx. Therefore one might think that the loss of halogen would proceed step by step, leading to the formation of 3,5,3′-TRITh from Tx, inversely to the substitution reaction, discussed above. Thus, two ways for the formation of incompletely halogenated iodothyronines may be considered: the condensation and iodination process takes place in the gland, and the deiodination reaction is chiefly, if not strictly, peripheral. It must also be pointed out, in defining the physiological significance of molecular changes of idothyronines in receptor cells, that the nature of the thyroid

hormones secreted and that of the active products are not necessarily identical.

III. Metabolism of Thyroid Hormones

Many questions concerning the metabolism of the iodothyronines produced by the thyroid gland have not been completely solved. Their circulation in plasma, recently reviewed (10), will not be discussed here, but only their metabolism and its incidence on the hormonal activity. Owing to enterohepatic circulation of the hormones and to their destruction and use by the liver, liver metabolism will be presented first.

1. Hepatic Metabolism of Thyroid Hormone

It was established in 1919 (44) and has been frequently confirmed since (1, 8, 40, 46, 103) that circulating L-thyroxine is partly collected by the liver and can be recovered in the bile in free iodides (11, 12, 35). Physiological proof of liver degradation of L-thyroxine has been provided by the fact that it is much less active in hepatectomized animals than in controls (42). By means of physiological doses of the hormones, rather than pharmacological amounts as in earlier experiments, the role of the liver has been shown to be twofold: destruction of the excess plasma hormone, and regulation of its blood level by enterohepatic circulation. The nature of the iodinated constituents of the bile in animals injected with L-thyroxine; L-3,5,3′-triiodothyronine, and DL-3,3′-diiodothyronine and the physiological significance of their excretion have been studied. The results are qualitatively very similar, if not identical, but important differences exist in the rate of excretion and in the proportion of various hormone derivatives, according to the nature and the amount of product injected.

Hepatic metabolism has been chiefly studied with substances labeled by I^{131} (3, 45, 83, 96). Researches with C^{14}-labeled molecules have a most promising future. Thyroidectomized rats bearing a biliary fistule have been injected with physiological amounts (0.5 μg. to a few μg) of DL-3,3′-DITh (74), L-3,5,3′-TRITh (80), D-Tx (84), or L-Tx (80) labeled by I^{131} in various positions, and the excretion of radioactive products in the bile has followed for 48 hours. In addition to iodides and free hormone, several substances, specific for each injected hormone, have been found. In no case are those produced from L- or D-thyroxine identical to those derived from less iodinated homologs, which is an indirect but important argument against an intense formation of 3,5,3′-TRITh from Tx in the liver. Even the existence of this reaction can be questioned, the alleged characterization of 3,5,3′-TRITh in the bile after injection of Tx (17, 23, 24) requiring analytical confirmation because of the analogy of the chromatographic behavior of 3,5,3′-TRITh to the other iodo derivative present in the bile (see below).