THE FORMATION OF THYROXINE IN IODINATED PROTEINS

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THE iodination of proteins has been the subject of investigations covering a period of half a century. In many respects, progress in this field has gone hand in hand with the development of knowledge concerning the natural secretion of the thyroid hormone. Immediately following the discovery of iodine in organic combination in the thyroid by Baumann in 1895,[1] investigations were conducted to determine whether a thyroidal substance could be formed by the simple iodination of proteins. This early work has recently been reviewed in detail by Salter[2] and Reineke.[3] Although the early attempts were not successful in the formation of an active product, considerable information was gained on the types of combination of iodine with proteins, and the methods by which the combination could be effected. Of the various iodination methods employed, the most noteworthy is that of Blum and Vaubel,[4] who buffered their protein solutions with sodium bicarbonate in order to neutralize the hydriodic acid that forms as a side product from the substitution of iodine. With proper control of conditions, this method can now be used for the formation of iodinated proteins possessing marked thyroidal activity.

In the light of present knowledge, it is difficult to evaluate early claims of thyroidal activity in iodinated proteins, since the biological assay methods employed were not well standardized, nor were the conditions employed in forming these substances fully controlled. Blum[5] reported that his iodinated albumin produced curative effects in myxedema, but this claim was later withdrawn.[6] Following the discovery that thyroid substance will accelerate the metamorphosis of frog tadpoles,[7, 8] it was reported that comparable effects were produced with iodinated proteins.[9–11] Alkaline hydrolysis was reported to destroy the activity of such preparations.[12] Since we now know that thyroxine is relatively resistant to destruction during alkaline hydrolysis, it appears unlikely that it had actually been formed in these preparations. In fact, the effects observed were considered at the time as merely indicating a special reaction of such preparations on tadpoles, and not a true thyroidal effect.

Thyroidal Activity of Iodinated Proteins and Their Hydrolysates. With the isolation of thyroxine by Kendall[13] and its synthesis by Harington and Barger,[14] it appeared extremely unlikely that a compound of this nature could be formed simply by the iodination of proteins. Conse-

quently, it is not surprising that subsequent results suggesting such a possibility were viewed with some skepticism.

However, Brandt, Mattis, and Nolte[15] reported that an acid-insoluble precipitate obtained from an iodinated protein after hydrolysis with barium hydroxide exerted a thyroid-like action on frog tadpoles. Likewise, Abelin and co-workers presented a series of reports[16–21] in which they gave convincing evidence that the acid-insoluble concentrates obtained from iodinated proteins after hydrolysis with alkali produced many effects that were qualitatively indistinguishable from those elicited by thyroxine.

The isolation from iodinated proteins of thyroxine in crystalline form was finally reported by Ludwig and von Mutzenbecher[22] and confirmed by Harington and Pitt Rivers.[23] The importance of conducting the iodination process under exact, but rather empirically selected conditions was emphasized. Little attention was given, however, to the possible activity of iodinated proteins prior to hydrolysis or the influence of varying the reaction conditions on the activity of the resulting product. Although it had been reported[15, 17] that iodinated proteins produced thyroidal effects only after hydrolysis, the reports of Kaer,[24] Lerman and Salter,[25] Harington and Pitt Rivers,[23] and Reineke and Turner[26] indicated that some whole iodinated proteins produce significant thyroidal effects. Consequently, our attention was turned to the possibility of increasing the activity of iodinated proteins by suitable control of the reaction conditions.

Factors Affecting the Formation of Active Substance. In the subsequent investigations, the procedure was adopted of varying single factors in the iodination and incubation processes while maintaining other conditions constant in so far as possible. Until the applicability of chemical methods for the determination of thyroxine in such preparations had been established[27] biological assay methods were employed to determine their thyroidal potency.

In making the preparations, 20 gm. of casein was placed in 700 ml. of distilled water containing sodium bicarbonate, and dissolved by stirring. The solutions were then placed in a constant-temperature water bath, and finely powdered iodine was added slowly over a period of 3 to 4 hours with vigorous stirring. The mixture was then incubated for a period of 18 to 20 hours at constant temperature, the stirring being continued throughout the process. The solutions were finally dialyzed and the iodinated protein was recovered by isoelectric precipitation.

When the sodium bicarbonate added in the above procedure was varied over a broad range in succeeding preparations,[28] good potency was observed as long as the bicarbonate used was sufficient to maintain a pH of 7.0 or above. Even though normal amounts of iodine were combined at lower pH values, there was a pronounced decline in thyroidal activity.

By a similar procedure, it was found that the amount of iodine added has a controlling influence on the amount of thyroidal substance formed. The potency of the iodinated protein increased progressively with increasing iodine input until 4.5 to 5.0 atoms of iodine had been added per mole of tyrosine in the protein.[29, 30] Iodination beyond this point resulted in pronounced decreases of activity. In the medium employed, only one-half of the reacting iodine is substituted on the tyrosine radical, the remainder being used to form hydriodic acid. Consequently, the optimal iodine input under these conditions would be slightly in excess of the amount required to substitute two atoms per mole of tyrosine in the protein. The excess would be available for oxidation in the coupling of two molecules of diiodotyrosine to form thyroxine. With a greater excess of bicarbonate, more iodine was required to reach the point of maximum potency.[31]

Proteins iodinated in a more alkaline ammoniacal medium by Muus *et al.*[31] did not reach their peak activity until considerably more iodine had been combined, and failed to show a decline in potency with excessive iodination. In the opinion of the author, this difference in results can be explained by the fact that the reactivity of diiodotyrosine as well as the oxidative action of iodine declines with increasing alkalinity of the medium.[32]

In all the earlier studies, the iodination and incubation procedures were conducted at physiological temperature on the assumption that this temperature would be optimal for thyroxine formation. Further investigation revealed that quite the reverse was true. When the temperature was increased to 60° to 70°C. during either the iodination or incubation steps and maintained at the elevated level for 18 to 20 hours,[28] a pronounced rise in the thyroidal potency of the resulting product occurred. At temperatures in excess of 90°C., little active substance was formed.

Two additional factors were found[30] to influence significantly the formation of active iodinated proteins, namely, the amount of stirring or aeration and the inclusion of any one of a series of manganese compounds as a catalyst (TABLE 1). With other conditions held constant, there is a considerable increase in the apparent thyroxine content when the amount of agitation is increased sufficiently to whip air into the solutions. Still another increase in potency occurs if the incubation is conducted in the presence of a manganese compound. Under the conditions employed, manganese tetroxide (Mn_3O_4) and the oxides obtained by the reduction of potassium permanganate with glucose exerted the greatest effect.

The combined influence of several interacting factors on thyroxine formation is shown in FIGURE 1. Manganese tetroxide appears to be effective over a considerable range of iodine concentrations. Further, the amount of iodine added remains a critical factor in the presence of manganese.

TABLE 1

EFFECT OF INCUBATION TEMPERATURE, MANGANESE COMPOUNDS, AND AMOUNT OF AGITATION ON FORMATION OF THYROXINE IN IODINATED PROTEIN

(From *J. Biol. Chem.* **161**: 613. 1945.)

Catalyst	*Stirring r.p.m.*	*Thyroxine content per cent*	*Average per cent*
Series I. Skim milk proteins iodinated and incubated at 37°			
None	Very gentle	0.33	
None	Very gentle	0.26	
None	Very gentle	0.27	0.29
Series II. Casein iodinated at 38–40°, incubated at 70°			
None	300	1.67	
None	600	1.73	
None	600	1.80	
None	600	1.75	
None	600	1.84	1.76
Mn_3O_4	300	1.94	
Mn_3O_4	300	1.99	1.96
Mn_3O_4	600	2.72	
Mn_3O_4	600	2.93	
Mn_3O_4	600	3.03	
Mn_3O_4	600	2.78	
Mn_3O_4	600	2.80	
Mn_3O_4	600	3.04	2.88
Oxides from reduction of $KMnO_4$	600	2.97	
	600	2.96	
	600	2.60	2.84
MnO_2	600	2.16	
MnO_2	600	2.19	2.17
Mn_2O_3	600	2.26	
Mn_2O_3	600	2.33	2.30
$MnSO_4$	600	2.00	
$MnSO_4$	600	2.13	2.07

Although it is possible to demonstrate some thyroidal activity in iodinated proteins prepared under a variety of conditions, all of the factors discussed appear to be critical and must be maintained at the optimum in order to obtain preparations of high potency.

Further information on the control of thyroxine formation is provided by results obtained in the direct synthesis of thyroxine from diiodotyrosine. It was first reported by von Mutzenbecher[33] that, when diiodotyrosine is incubated in mildly alkaline solution at 37°C. for a period of two weeks, crystalline thyroxine equivalent to about 0.1 per cent of the diiodotyrosine used initially is formed. This was fully confirmed by others[34–36] using an identical procedure. Harington[37] stated that, when diiodotyrosine was oxidized with hydrogen peroxide on the steam bath, the solution meanwhile being shaken constantly with *n*-butanol to extract the thyroxine as it was formed, a greatly improved yield was obtained.

When diiodotyrosine was dissolved in N/10 sodium hydroxide and

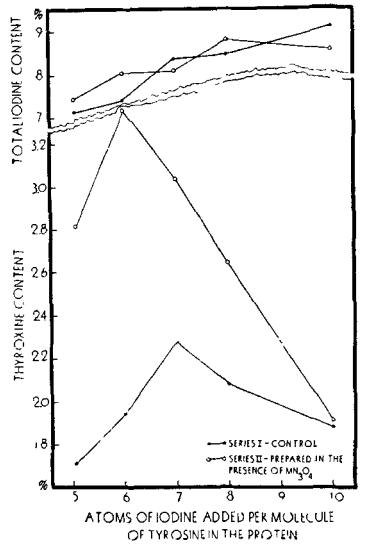

FIGURE 1. The effect of progressive iodination in the presence of excess bicarbonate and manganese oxide on the thyroxine content of iodinated casein. (From J. Biol. Chem. 161: 613. 1945.)

incubated for 18 to 20 hours with vigorous stirring or aeration,[32] the results were very similar to those obtained with iodinated casein (FIGURE 2). Quite appreciable yields of thyroxine were obtained under optimum conditions, amounting to 0.85 per cent of the diiodotyrosine incubated. If allowance was made for the unaltered diiodotyrosine that could be recovered, the net yield amounted to 2.8 per cent. The incubation temperature is highly critical, with an optimum at about 60°C. Thyroxine

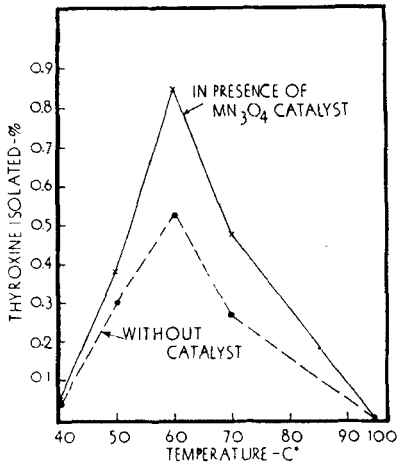

FIGURE 2. Gross yield of thyroxine isolated after incubation of diiodotyrosine at various temperatures. (From J. Biol. Chem. 162: 369. 1946.)

formation was increased somewhat in the presence of manganese tetroxide at all points in the effective temperature range. In addition, it was found that thyroxine formation was negligible unless the solutions were either stirred or aerated directly, and also that manganese tetroxide catalyzed the reaction only when air was introduced. It appears probable, therefore, that the effect of manganese is due to its acceleration of an atmospheric oxidation taking place at some point in the chemical system involved. Further investigation will be required to determine the site of action.

Nature of the Active Substance in Iodinated Proteins. From the fact that thyroxine can be isolated readily from iodinated proteins prepared under suitably controlled conditions, there can be no doubt that their thyroidal activity is due at least in part to their thyroxine content. Ludwig and von Mutzenbecher,[22] as well as Harington and Pitt Rivers,[23] reported that subsequent to hydrolysis of iodinated proteins with barium hydroxide approximately 0.1 per cent of crystalline thyroxine was recovered.

By the use of highly active iodinated casein and a similar method of hydrolysis and isolation, Reineke and Turner[38] obtained a yield of 0.424 per cent of crystalline *dl*-thyroxine. In a more recent attempt by the author (unpublished), 0.5 per cent of thyroxine was isolated. The identity of the compound is fully established by its characteristic crystalline structure (FIGURE 3), by the fact that it shows an ultraviolet absorption curve identical with that of synthetic thyroxine (FIGURE 4), by its iodine content of 64 to 65 per cent, and finally by its high metabolic potency when administered to test animals.

The maximum yield isolated actually represents about 5 times the thyroxine content of USP thyroid. However, it is only about 1/6 of the amount of thyroxine that is apparently present, as judged from the results of chemical and biological assays. The discrepancy can be accounted for, in part, in the sizable losses of thyroxine involved in its isolation and purification. In addition, there are apparently variable losses during the hydrolysis in strong boiling barium hydroxide solution that is required to liberate the thyroxine. There is also the possibility that a part of the activity is due to the presence of an as yet unidentified thyroxine-like compound.

As would be expected from its method of formation, thyroxine exists in iodinated proteins in the natural *l*-form. In the usual alkaline hydrolysis, racemization occurs, so that a *dl*-mixture is obtained. When active iodinated protein was hydrolyzed in a mixture of sulfuric acid and *n*-butanol, racemization was avoided and pure levorotatory thyroxine was isolated quite readily.[39]

The relative potency of *l*-thyroxine compared with that of the racemic mixture that is more easily available for use as a standard, is of considerable importance in evaluating the results of biological assays. Biological

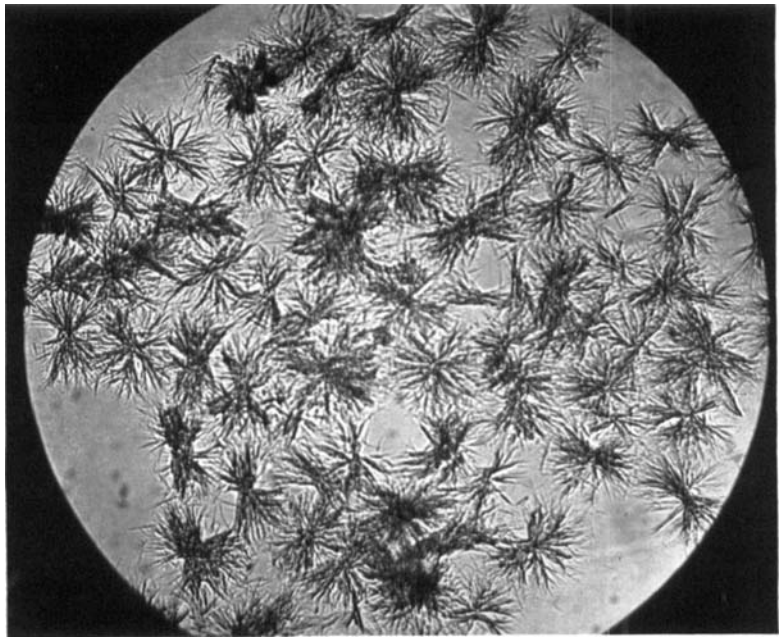

FIGURE 3. Spectrographic absorption curves of synthetic thyroxine and thyroxine isolated from a barium hydroxide hydrolysate of iodinated casein. (From J. Biol. Chem. 149: 555. 1943.)

assays of the compounds separated by Harington from a racemic mixture indicated[40–42] that *d*-thyroxine has 1/3 or more of the activity of the *l*-form. However, it was pointed out in the original report on the resolution of these compounds[43] that the separation of isomers was probably not complete. The higher specific rotation of *l*-thyroxine tested more recently[39, 44] indicates better optical purity. Thus, it seems that the activity attributed to *d*-thyroxine could be accounted for by contamination with *l*-isomer. It should be noted, however, that Deanesly and Parkes[45] tested on *Xenopus* tadpoles a specimen of synthetic *l*-thyroxine that showed a high specific rotation, and failed to find it more active than the *dl*-mixture.

The chart shown in FIGURE 5 is typical of results we obtained[46] when the potency of *l*-thyroxine isolated from iodinated casein was compared with that of a *dl*-mixture. In this instance, the ability of the thyroxine to prevent the increase in the thyroid weight of thiouracil-treated chicks was used as the measure of response. When the data are plotted so that the *dl*-thyroxine dosage scale is twice that used for *l*-thyroxine, the response curves are identical, demonstrating that the latter preparation has twice the activity of the former. Similar results were obtained in tests on thiouracil-treated rats, by the metabolic stimulation of guinea pigs, and by the metamorphosis-stimulating effect in *Rana pipiens* tad-

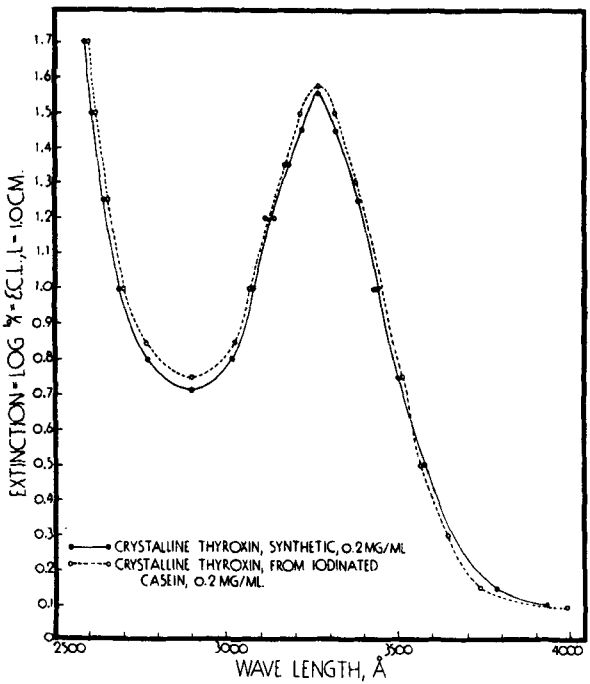

FIGURE 4. Crystalline *dl*-thyroxine isolated from thyroactive iodinated casein subsequent to hydrolysis with barium hydroxide (x 400).

poles. Thus, it was concluded, in agreement with Foster *et al.*,[44] that all of the activity of *dl*-thyroxine can be accounted for by its *l*-component. In attempting to determine the thyroxine content of iodinated proteins by biological assays, comparisons have either been made with an *l*-thyroxine standard, or, where a *dl*-standard was used, the apparent thyroxine content of the preparation under test has been divided by two to convert the value to the *l*-thyroxine basis. The biological assay of thyroidal preparations is further complicated by differences in the absorption of different substances, particularly when administered orally. Quite surprisingly, it was found[28] that, when dissolved in mildly alkaline solution, iodinated proteins are highly effective when given by injection. Therefore, in attempting to estimate the active substance actually present in the iodinated protein, it was injected and the response compared directly with that obtained with injected thyroxine.

The specificity of chemical methods for the determination of thyroxine, when applied to iodinated proteins, must be well established before the results obtained can be interpreted properly. Measurement of the acid-insoluble iodine of iodinated protein hydrolysates provides only a rough index of potency because considerable amounts of non-thyroxine iodine are included in this fraction.[45] Preliminary results with Blau's

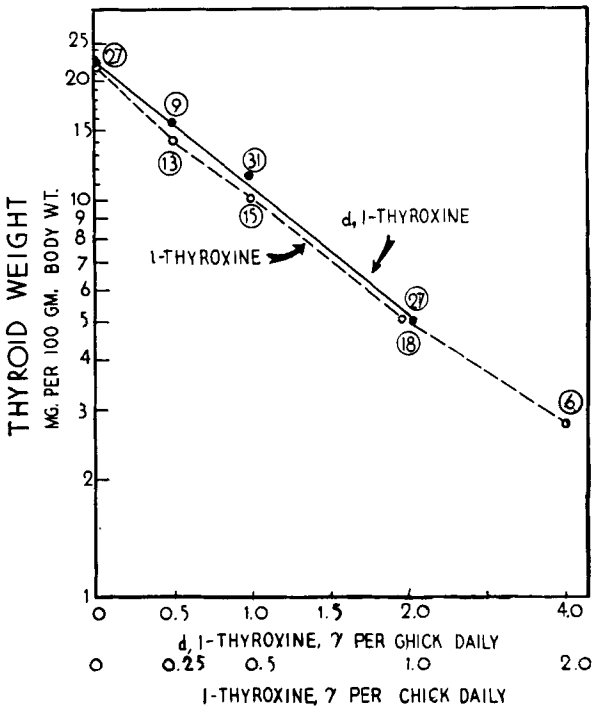

FIGURE 5. The relative potency of *l*- and *dl*-thyroxine in reducing the thyroid weight of thiouracil-treated male chicks. The encircled numerals indicate the number of animals per dosage group. (From Endocrinology 36: 200. 1945.)

n-butanol extraction procedure for the determination of thyroxine in thyroid substance failed to give good agreement with biological assays when applied to iodinated proteins. When a more vigorous hydrolysis was used, however, excellent agreement was found (TABLE 2) between the chemical thyroxine analysis and the bioassay value obtained by determining the metabolic response to intraperitoneally injected iodinated protein. The biological assay method yielded values that averaged about 8 per cent below the chemical method. This difference might be accounted for either by the inclusion of a small amount of non-thyroxine iodine in the chemical determination, or by a lower absorption, from the injection site, of the iodinated protein than of the thyroxine used as a standard. By suitable control of conditions, iodinated proteins that apparently contain about 3 per cent of thyroxine as determined by either method can now be prepared quite consistently. Biological assays made at various stages of the *n*-butanol extraction procedure indicated that all the active substance in the iodinated protein is recovered in the extraction process.

Even though close agreement is obtainable between the chemical and biological methods, the possibility of the occurrence in iodinated pro-

TABLE 2

DATA DEMONSTRATING THE CORRELATION BETWEEN THE CHEMICAL AND BIOLOGICAL ASSAY METHODS FOR THYROXINE

(From *J. Biol. Chem.* **161**: 599. 1945.)

Preparation No.	*Iodine added per mole tyrosine in protein, atoms*	*Iodinated protein injected, γ/100 g. body wt.*	*Increase in CO_2 output, per cent*	*Thyroxine found*		*Difference, per cent*
				Bioassay, per cent*	*Chemical analysis, per cent*	
1		223	25.4	2.46	2.69	−8.6
2		138	24.7	3.80	3.91	−2.8
3		176	20.8	2.46	3.06	−19.6
4		300	27.6	2.01	2.06	−2.4
5		150	20.9	2.90	3.88	−25.3
6		145	25.1	3.71	3.73	−0.5
6		161	25.0	3.31	3.73	−11.3
6		161	25.1	3.34	3.73	−10.5
7	4.51	243	21.7	1.86	2.21	−15.8
8	5.01	198	23.4	2.50	2.71	−7.7
9	5.51	201	26.8	2.90	2.69	+7.8
10	6.01	190	23.0	2.55	2.83	−9.9
11	6.51	175	23.1	2.78	3.09	−10.0
12	7.01	174	22.2	2.67	3.11	−14.1
13	8.01	191	26.4	2.98	2.83	+5.3
14	9.01	194	23.2	2.53	2.78	−9.0
15	10.01	209	25.8	2.66	2.58	+3.0
	Weighted average			2.79	3.04	−8.1

* Estimated from standard response curve for intraperitoneally injected *l*-thyroxine.

teins of an active compound other than thyroxine is not wholly excluded. Such a compound, if present, however, would need to have a thyroidal activity per unit of iodine that is very similar to that of thyroxine iodine. On the other hand, if all of the substance measured by these methods is actually thyroxine, it should be possible to isolate more than the 0.5 per cent yield thus far recovered subsequent to hydrolysis.

Thyroactive iodinated proteins are effective when given orally in all species in which they have been tested to date. The oral effectiveness is considerably less than by injection and will probably vary in different species. Reineke and Turner[47] reported that iodinated protein was only about 5 per cent as effective when given to sheep orally as by subcutaneous injection. Even thyroxine given in alkaline solution was only about 12 per cent as effective by oral as by parenteral administration in this species. Deanesly and Parkes[48] point out that iodinated proteins apparently are not utilized as effectively as thyroid substance when administered orally to cattle. Species with a simple digestive system appear to utilize the active substance in iodinated proteins far more effectively than do ruminant animals. However, exact comparisons of the relative oral potency of iodinated protein, thyroid substance, and thyroxine in other species, including man, have not been reported.

Mechanism of Thyroxine Formation. When publishing their classical experiments on the constitution and synthesis of thyroxine, Harington

and Barger[14] proposed the theory that thyroxine is synthesized *in vivo* by the iodination of tyrosine, followed by the oxidative coupling of two molecules of diiodotyrosine and the elimination of one side chain.

With the now well-established finding that thyroxine can be formed simply by the incubation of diiodotyrosine, or by the iodination of proteins under suitable conditions, it is proved beyond a doubt that this overall reaction will take place quite readily even without the intervention of a biological enzyme system.

However, there is little experimental evidence on the actual mechanism involved. By analogy with the findings of Pummerer *et al.*[49] on the oxidation of *p*-cresol, Johnson and Tewkesbury[35] proposed a series of reactions that would account for the oxidative conversion of thyroxine to diiodotyrosine (FIGURE 6). This would involve the oxidative coupling of

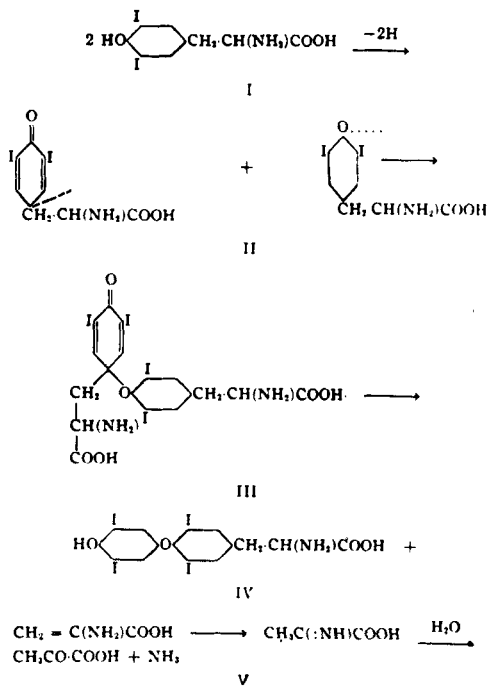

FIGURE 6. A mechanism for the conversion of diiodotyrosine to thyroxine. (From Proc. Nat. Acad. Sci. **28**: 73. 1942.)

two molecules of diiodotyrosine (I and II) to form the intermediate compound III. Compound III could follow one of two pathways, namely, (*a*) molecular dissociation with loss of one alanine side chain and formation of thyroxine, IV, and aminopyruvic acid, or (*b*) hydrolysis, with production of serine. Qualitative tests indicated the presence of pyruvic acid and ammonia, but not serine, as secondary products in the reaction mixture. Harington[38] developed still further the theoretical background sup-

porting this theory. However, no actual evidence has been obtained on the intermediate compounds that would be necessary to fully confirm the proposed mechanism.

All the facts established thus far are consistent with the view that in the iodination of proteins, thyroxine is formed *within the protein molecule* by the oxidative coupling of two diiodotyrosine radicals, and the subsequent splitting-off of one side chain.

Although iodine can be substituted on the tyrosine combined in proteins under a variety of conditions, the secondary reactions involved in the formation of thyroxine are dependent upon the maintenance of mildly oxidative conditions in the reaction medium. It has been suggested by a number of workers that the coupling reaction involved is actually effected by the oxidative action of iodine. It is now established that thyroxine formation can be accelerated by incubation at 60° to 70°C. in the presence of vigorous stirring or aeration. The catalytic effect of manganese is apparent only in the presence of oxygen. It can easily be demonstrated that all of the iodine added combines either with the protein or as iodide during the iodination step. Consequently, no free iodine would be present for oxidative purposes during the incubation period when most of the thyroxine is formed. If we accept the idea that iodine is the effective agent in the conversion of diiodotyrosine to thyroxine, the influence of oxygen and manganese at high incubation temperatures might be explained by their oxidation of iodide to a more highly oxidized form such as hypoiodite. This compound would then be available for the oxidative coupling reaction. From the evidence now available, it seems clearly established that the thyroxine formed during iodination remains in firm combination in the protein molecule. Quite a vigorous hydrolysis with either alkali or acid is required to liberate the thyroxine so that it may be isolated in crystalline form. Furthermore, no loss of potency is observed after long-continued dialysis of the iodinated protein, indicating again that the thyroxine is combined in a large non-dialyzable molecule.

Our knowledge of protein structure is, of course, too meager to justify much speculation on the mechanism whereby two diiodotyrosine radicals, both of which are already combined in a protein molecule, could undergo the coupling reaction involved in the formation of thyroxine. It seems reasonable to believe, however, that only a certain proportion of the diiodotyrosyl radicals would be arranged spatially in such a position that they could undergo the reaction.

It can be calculated, for example, that in casein containing 5.65 per cent of tyrosine the theoretical thyroxine yield, if all of the tyrosine were iodinated and subsequently converted to thyroxine, would be about 10.6 per cent. Iodinated casein containing slightly more than 3 per cent of thyroxine-like substance, as determined by chemical analysis, can be prepared quite consistently, but no method of treatment has been found that will increase the conversion much beyond this point. The formation

of additional thyroxine may, then, be impossible because of spatial incompatibilities.

Many parallels could be drawn between the formation of thyroxine in iodinated proteins and in the thyroid gland. In both instances, it appears well established that the synthesis consists, first, of the substitution of iodine on the tyrosyl radicals of the protein, and, secondly, the oxidative coupling of two diiodotyrosyl radicals within the protein molecule to form a thyroxyl radical.

Both reactions will take place in proteins iodinated artificially, without the intervention of enzymes. In the process occurring *in vivo*, it would presumably be necessary to have an enzyme system capable of oxidizing iodide to iodine to permit its substitution on tyrosine. Whether the oxidative coupling reaction involved in the formation of thyroxine from diiodotyrosine is actually effected by hypoiodite or a more highly oxidized form of iodine in either iodinated proteins or the thyroid, remains to be established by further investigation.

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Discussion of the Paper

Dr. Samuel Dvoskin (*Columbia University, New York, N. Y.*):

The experiments of Dr. Reineke have clearly elucidated the optimal conditions under which thyroxine is formed *in vitro* by the iodination of proteins. It may be of interest to present the results of recent experiments with the rat and chick, in which elemental iodine was injected subcutaneously *in vivo*.

Injections of a solution containing elemental iodine served to reinitiate bone growth and increase body length in young thyroidectomized female rats. Equivalent dosage of iodide only exerted minimal effects. In normal young female rats, the elemental iodine injections caused a marked decrease in cell height of the thyroid epithelium and a decrease in the weight of the thyroid gland. Iodide injections, in equivalent dosage, failed to alter gland weight and only slightly lowered cell height. In thiouracil-fed chicks, or thiouracil- or sulfadiazine-fed young female rats, the subcutaneous injection of a solution containing elemental iodine caused a complete inhibition of the goitrogenic effects. Iodide injections, in equivalent dosages, failed to decrease the thyroid cell height, but partially reduced the thyroid gland weight.

Oral administration of solution containing elemental iodine was no more effective than oral administration of iodide solutions in influencing thyroid weight and structure in normal or thiouracil-fed rats.

From the evidence, it appears that the subcutaneous administration of elemental iodine to the rat and chick has an action similar to thyroxine. It is possible that the action of elemental iodine results from the formation of an iodoprotein, *in vivo*, having thyroxine-like action. However, no evidence is at hand at present to prove this hypothesis.*

DR. VICTOR M. TRIKOJUS (*University of Melbourne, Melbourne, Australia*):

Since the lactic acid analogue of 3:5-diiodotyrosine has been shown by Foster and Gutman to be a metabolite of the amino acid, and Saul and Trikojus[1] have demonstrated its conversion by incubation to the corresponding analogue of thyroxine, it is pertinent to ask whether such a transformation would be possible in the body. Salter[2] has referred to the presence of a substance in hydrolysates of iodinated serum protein similar to thyroxine as regards its iodine content, but being nitrogen-free.

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DR. FREDRICK GUDERNATSCH (*New York, N. Y.*):

The new results which Dr. Reineke obtained in several species, amphibian, avian, and mammalian, when assaying his various iodinated proteins for thyroid-hormone activity, are very impressive. The formation in such proteins of the actual hormone, thyroxine, is now well understood. Very early, experimenters had realized the necessity of iodine for the physiological activity of the thyroid hormone, and especially the importance of the combination of iodine in organic form. Some investigators, however, maintained that the tadpole effect could be elicited by uncombined inorganic iodine. These views were found to be erroneous, for it was soon recognized that the animals treated with I_2 were maintained on protein foods, the latter being fed in association with iodine. Iodized peptides also elicited a semblance of the tadpole effect. Further, the possibility (almost certainty) that some inorganic iodine, after entering the tadpole, would become organically combined, could not be ruled out.

In 1916/17, we carried out numerous series of tadpole experiments, later interrupted by the War, with fractionated thyroid derivatives (nucleoproteins, globulins, coagulable proteins, etc.). These elicited the typical thyroid effect, albeit in varying degrees, nucleoproteins being the most potent ones. Unfortunately, we equalized the concentrations used according to the nitrogen, not the iodine, content of the fractions. However, the connection between iodine content and activity was readily seen. When graded according to their iodine content (mg./cc.), the fractions lined up exactly as when arranged according to hormonal potency. Yet, "it is not the iodine itself which provides this activity, but the

* Dr. S. Barker has recently presented evidence that iodoproteins are formed at the injection site.

special coupling of iodine with some particular protein fraction, the most potent combinations being those present in the thyroid. Thyroxine contains only twice as much iodine per molecule as diiodotyrosine, yet it is not just twice but several hundred times as active as the latter. As an example, tadpoles will respond to a determinable minimum quantity of thyroxine, but when we treat them with twice the quantity of diiodotyrosine they will show no thyroid response whatsoever, though getting the same amount of iodine. We would have to apply a much higher (more than a thousand times higher) concentration of diiodotyrosine. Likewise, tetra-iodothyronine (thyroxine) with its four I atoms is far more than twice as potent as diiodothyronine with two I atoms, though otherwise the molecules are similar."*

The effectiveness of iodine when coupled with protein constituents was again shown in later experiments extending over a number of years (Gudernatsch and Olive Hoffman, 1929–36), when we studied the effects of amino acids, singly and in various mixtures, in tadpole development. Some of the simpler acids proved to be an adequate food for mere maintenance, some of higher molecular weight (arginine, lysine, cystine) supported growth, while the aromatic acids (phenylalanine, hydroxyphenylalanine, tryptophane) showed signs of a differentiation effect. All are alanine derivatives, the same as diiodotyrosine and thyroxine. In some experiments, iodine was added to the solution of these acids and the animals were reared in very dilute mixtures. The rate of development became more rapid in every case. In increasing degree of effectiveness, the best groups ranged as follows: tyrosine alone; phenylalanine+iodine; tryptophane+iodine; tyrosine+iodine. The next step toward a much greater and true hormonal effectiveness would be diiodotyrosine and thyroxine. Dr. Reineke's iodinated proteins would range at the true hormone end of such a graded series.

* From: F. GUDERNATSCH. Endocrine and Amino Acid Studies in the Physiology of Development—A Review of the author's experiments. 1936.