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Thyroactive Iodinated Proteins

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I. Introduction

Investigations on the iodination of proteins date back to the latter part of the nineteenth century following Baumann's (14) discovery of iodine in

organic combination in the thyroid gland. Further research, particularly by Hutcheson (67) and Oswald (108), indicated that the greater part of the iodine of the thyroid occurs in combination with thyroglobulin, thus establishing the fact that the active substance is actually an iodoprotein.

In addition to giving much impetus to investigations on the natural thyroid hormone, these early discoveries led to a number of attempts to duplicate the active substance artificially by the iodination of ordinary proteins. In fact, some early claims for the formation of active iodinated proteins were made (21, 22) but these were subsequently withdrawn when the results could not be confirmed. Attempts to increase the activity of thyroid protein by further iodination (68) were also unsuccessful. However, a long series of investigations on the chemistry of the iodination process, inaugurated by the pioneer researches of Hofmeister, Oswald, and Blum, and continuing over a period of more than thirty years, provided the basis for much of our present knowledge of the groups affected by iodination within the protein molecule.

In the meantime, the classical experiments of Kendall (76) resulted in the isolation of crystalline thyroxine in 1915. As a result of a series of brilliant investigations, the chemical configuration of thyroxine was deduced by Harington (50) in 1926. Shortly thereafter *dl*-thyroxine was synthesized by Harington and Barger (54).

Parallel experiments on artificially iodinated proteins finally resulted in isolation of crystalline thyroxine by Ludwig and von Mutzenbecher (92, 93). Subsequent research has been directed principally toward elucidation of the mechanism involved in the formation of thyroxine by this means, the improvement of the iodination method to form products with greater thyroidal activity and higher thyroxine content, and determination of the effects of active iodinated proteins on various physiological processes.

II. The Iodination of Proteins

1. Iodination Methods

Proteins may be iodinated in neutral, basic or acid media, though the iodine content and the reactive protein groups affected, will vary according to the conditions employed. Although it has long been known that iodine would combine with proteins (27), the awakened interest occasioned by the discovery of iodine in the thyroid led to intensive investigations of this reaction.

Liebricht (90) combined iodine with casein by mixing the two substances together in the proportion of 20 g. of iodine to 80 g. of casein, and stirring at 100°C. The brown powder obtained was extracted repeatedly with ether to remove excess iodine. This product, designated as *periodocasein* contained

17.8% iodine, of which a large proportion was in loose combination. The loosely combined iodine could be removed by treatment with alkaline bisulfite, resulting in a preparation containing 5.7% iodine, and termed *iodocasein*. Brief digestion of *periodocasein* with 10% sulfuric acid produced *caseoiodine*, a split-product containing 8.5 to 9.3% iodine and having many chemical properties in common with the *iodothyrin* of Baumann.

It was discovered shortly afterwards, by Blum and Vaubel (26), that hydriodic acid liberated as a side product prevented the reaction of iodine with protein from going to completion. To overcome this they conducted the iodination in a solution buffered with sodium bicarbonate. By this means the hydriodic acid is neutralized continuously, thus permitting the reaction to proceed. It is of considerable interest that more than forty years later this method, with but slight modifications, has been found to provide optimal conditions for the formation of iodinated proteins with high thyroxine content and marked thyroidal properties.

Proteins were also iodinated (62, 84) by the addition of potassium iodide and iodate together with sufficient sulfuric acid to liberate iodine by the well known equation:

$$5KI + KIO_3 + 3H_2SO_4 \rightarrow 3I_2 + 3K_2SO_4 + 3H_2O$$

Iodinated egg albumin formed by this method contained from 7 to 12% iodine, the amount combined depending on the excess of iodine added, the temperature, and the time permitted for the reaction to go to completion.

Iodination in an ammoniacal medium (25) was believed to produce less alteration in the protein than did the bicarbonate method. In the formation of thyroidally active iodinated proteins, to be reviewed in later sections, both of these methods have been employed.

2. Iodine-binding Groups in the Protein Molecule

With the discovery of iodination methods, attention naturally turned to investigations on the mode of combination of iodine with proteins. It was noted (62, 84) that iodination caused both the Millon and Adamkiewicz reaction to become negative, indicating alteration of both the tyrosine and tryptophane. Evidence for the oxidation of sulfur was presented (62). Iodination of oxyhemoglobin (85) caused an increase in the ratio of carbon to nitrogen, leading to the assumption that an albumose-like fraction was split off in the process.

The isolation of iodogorgonic acid by Drechsel (37) in 1896 from a hydrolyzate of gorgonin, a protein derived from the axial skeleton of the coral, and its identification as diiodotyrosine by Wheeler and Jamieson (162), and Henze (59) drew attention to tyrosine as an iodine-binding group.

Final proof of the iodination of tyrosine in proteins was provided by

Oswald when he isolated this compound from the hydrolytic products of iodoalbumin (109), iodogliadin (110) and iodocasein (111). It was not until twenty years later that Harington and Randall (57) succeeded in isolating the same compound from a hydrolyzate of the thyroid gland.

From the known tyrosine composition of a number of proteins it could be calculated that more iodine was combined than could be accounted for by substitution of tyrosine. The key to the solution of this problem was provided by Pauly (114) with the discovery that iodine can be substituted on the imidazole ring of histidine. Imidazole itself was shown to substitute one atom of iodine on the imino group and three atoms on carbon. Since one carbon position is blocked in histidine by the side chain, two carbons and the imino nitrogen would be available for the substitution of iodine in this amino acid. The amount of iodine that will actually combine with histidine appears to depend in large measure on the conditions used. Pauly found that imidazole will take up iodine more readily the more alkaline the solution.

TABLE I

Iodine Content of Some Artificial Iodoproteins

Preparation	Iodine Content "A" substance	"B" substance	"C" substance	Ratio of Nitrogen-I to Carbon-I
Iodoovalbumin	7.55	5.12	4.91	1:2
Iodoserumalbumin	8.96	6.73	6.70	1:3
Iodoserumglobulin	8.30	6.64	—	1:4
Iodothyroglobulin	6.14	4.88	4.96	1:4
Iodocasein	7.51	7.51	7.51	0:2

(From *Z. physiol. Chem.* (24).)

Though he was not able to iodinate free histidine directly, benzoyldiiodohistidine and *p*-nitrobenzoyldiiodohistidine, which served as structural analogues, were formed. The iodine taken up by carbon was in firm combination, but that attached to nitrogen could be removed easily with bisulfite.

Blum and Strauss (24) suggested that the extent to which iodine can be substituted on the imino group or on the unsaturated carbon atoms in histidine varies with different proteins according to the accessibility of these groups within the protein molecule. Various proteins iodinated by the bicarbonate method yielded characteristic iodine numbers which varied with the protein used. The fully iodinated protein was designated as substance A. Removal of the nitrogen-bound iodine by treatment with bisulfite resulted in a product with iodine bound only to carbon, and designated as substance B. Rapid iodination over a short period produced a C-substance with an iodine content similar to that of B, as shown in Table I.

In proteins treated with the maximum amount of iodine a number of side

reactions were shown to take place. Negative tests for tryptophane indicated oxidation of this compound. Negative tests for reduced sulfur, and the formation of iodoform indicated the oxidation of cystine. The hydriodic acid formed was more than four times the amount that would be expected from substitution alone. It was pointed out that oxidation of the sulfur from one molecule of cysteine to cysteic acid ($R\text{-}SO_3H$) would result in the formation of six molecules of hydriodic acid. In summary, the reaction of iodine with proteins was pictured by Blum and Strauss as follows:

I. Main reactions
   a. Full carbon iodination (negative Millon reaction)
   b. Destruction of ½ the groups giving the biuret reaction
   c. HI formation from substitution

II. Side reactions
   a. N-iodination
   b. Oxidation of cystine and tryptophane. Splitting off of sulfur
   c. Iodoform formation
   d. Further HI formation from oxidation

By virtue of its high histidine and low tyrosine content the iodination of globin is of particular interest in demonstrating the mode of substitution of histidine. Maximally iodinated globin (153) contained 11.4% of iodine; upon removing the N-iodine by treatment with bisulfite 7.6% of iodine remained bound to carbon. Re-iodination of this preparation restored the iodine content to 11.4%. Globin was shown (10) to combine with twice as much iodine as could be accounted for by substitution on the tyrosine alone, the remainder being accounted for by substitution on histidine. Nitroglobin (12) combined with only three-fourths of the theoretical amount of iodine because one of the reactive carbon atoms of tyrosine was already occupied by an NO_2 group. On the basis of quantitative analyses it was calculated (11) that globin would have six possible iodine numbers depending upon the extent of iodination of the histidine. By variation of the iodination method it was shown that all six possible steps can be brought about in stoichiometrically exact and reproducible proportions.

III. Thyroidal Activity of Iodinated Proteins

With the awakened interest in iodinated proteins resulting from the discoveries on the nature of the thyroid hormone in the 1890's, claims for the formation of thyroidally active preparations were made from time to time. These were subsequently abandoned, apparently due to lack of confirmation. In the light of present knowledge of the many conditions affecting the formation of such substances this is not surprising. Biological tests which would indicate thyroidal activity were made on only a few iodoproteins, with little regard for the method of preparation.

1. Early Evidence of Thyroidal Activity

Blum (21) asserted that his iodinated albumin produced the same effects in myxedema as thyroid substance. In a later report (23) this claim of specific thyroidal action was apparently withdrawn. The *caseoiodine* of Liebricht (90) was stated (167) to be wholly ineffective when tested on thyroidectomized dogs. Since the function of the parathyroid was unknown at that time, and the criterion of thyroidal activity used was the prevention of tetany and final death, this result is inconclusive.

The specific effect of thyroid substance in stimulating the metamorphosis of frog tadpoles was first reported by Gudernatsch (47, 48) in 1913. In the next year Morse (103) reported that comparable effects were produced by iodinated proteins. Lenhart (89) tested a commercial preparation of iodoalbumin containing 21% iodine, much of it loosely combined, on tadpoles. Even though stimulation of metamorphosis was observed, toxic side effects were also noted and, therefore, the results were not accepted as establishing a thyroid-like action. From tests on a similar preparation, Rogoff and Marine (139) concluded that iodinated albuimin has a thyroid-like action on tadpoles, but that the effect develops more slowly than with thyroid substance. Further tests on a series of iodinated proteins (140) showed evidence of some activity for all of them. Alkaline hydrolysis was reported to destroy the activity of all these preparations, however. Unfortunately no confirmatory assays on other types of test animals were conducted, with the result that these early findings came to be regarded merely as indicating a special action of such preparations on tadpoles.

2. Hydrolysis and Concentration of the Active Substance

The successful hydrolysis and concentration of a physiologically active substance from iodinated protein was first reported by Brandt, Mattis and Nolte (29). Ordinary iodinated proteins were stated to have no effect on the metamorphosis of tadpoles when fed for as long as four weeks. The acid-insoluble precipitate obtained after hydrolysis of the iodoprotein with barium hydroxide, however, exerted a metamorphosis-stimulating effect similar to that of thyroid substance.

The concentration of a thyroidally active substance from hydrolyzates of iodinated proteins was investigated extensively by Abelin and coworkers, their initial report appearing in 1933. The acid-insoluble substance obtained after hydrolysis with alkali was designated as *homothyroxine* (5). Physiological and chemical tests of this substance showed that, qualitatively, it possessed many of the properties of thyroxine. When given in high dosage, homothyroxine caused moulting and the appearance of white feathers in black chickens (1). It also counteracted the decrease in body temperature

ordinarily caused in guinea pigs by the injection of novocaine. Both of these effects can be duplicated by administering thyroxine. An active concentrate containing more than 26% of iodine and capable of producing pronounced stimulation of metabolism in the rat was prepared (2). Further purification (3) resulted in the isolation of a crystalline compound similar to thyroxine in microscopic appearance, and possessing high thyroidal activity. Complete identification of the compound was not made, however.

Finally, the isolation from iodinated proteins of thyroxine in crystalline form was reported by Ludwig and von Mutzenbecher (92, 93), proving that the active principle formed in the iodination of ordinary proteins under certain conditions is identical with that of the thyroid gland.

3. Formation of Iodinated Proteins Which are Effective Without Hydrolysis

In the experiments leading to the isolation of thyroxine the idea was advanced by Brandt, Mattis and Nolte (29) that hydrolysis of the protein was required to obtain an active product. Huge doses of iodinated protein given orally to a rat had no effect on its metabolism; small amounts of a hydrolyzate produced a pronounced increase (Abelin, 2). On the other hand, Kaer (74) reported that a commercial iodinated protein containing 5% of iodine produced thyroidal effects when fed to both tadpoles and guinea pigs. Both iodinated serum proteins and their degradation products were reported by Lerman and Salter (91) to be effective in the relief of myxedema in man. The iodinated casein prepared by Harington and Pitt Rivers (55) for the isolation of thyroxine was stated to increase the metabolism of rats when administered orally.

Iodinated proteins prepared in the author's laboratory (124) consistently produced thyroidal effects when given orally to either normal or thyroidectomized animals. Iodinated protein differed from thyroid or thyroxine (137) in that no response was produced in tadpoles placed in a solution containing the material in the form of a suspension. Pronounced metamorphosis was induced, however, when iodinated casein was given either orally or by intraperitoneal injection.

In view of the crude nature of this material, the marked thyroidal response, and the lack of toxic side effects when it was injected without a preliminary hydrolysis were quite surprising. Subsequent investigations revealed that guinea pigs (126) and mice and rats (82) responded similarly when injected with thyroactive iodinated casein. Discovery of the effectiveness of these products when given parenterally greatly facilitated the quantitative assay of experimental preparations, since injected iodoprotein could be compared directly with injected thyroxine, thus avoiding the differences in digestion and absorption which would be encountered in comparisons made by oral administration.

4. Methods of Forming Highly Active Iodinated Protein

In view of the contradictory information as to the possible thyroidal nature of various iodinated proteins, and also the lack of knowledge of the influence of variables in the iodination process on the amount of thyroidal substance formed, a series of investigations were undertaken by the author in collaboration with Dr. C. W. Turner and others in order to determine the factors influencing this reaction. The general iodination method was similar to that devised by Blum and Vaubel (26) and used subsequently (92, 93, 55) in preparing iodinated proteins for the isolation of thyroxine.

In this recent work the principal departures from earlier procedures have been (a) limitation of the iodine to the optimal level established for thyroxine formation, and (b) incubation of the iodinated protein at 60 to 70°C. The general procedure is as follows:

> Twenty g. of casein is placed in 700 ml. of distilled water containing 5 g. of sodium bicarbonate, and is dissolved by stirring. The mixture is then placed in a water bath held at 38 to 40° C., and a total of 3.7 g. of finely powdered iodine is added in small portions over a period of 3 to 4 hours, the solution meanwhile being agitated vigorously with a mechanical stirrer. When the requisite amount of iodine has been added the solution is incubated at 70°C., with vigorous stirring, for 18 to 20 hours. After dialysis, the iodinated protein is recovered by isoelectric precipitation, dried and ground to a fine powder.

Individual factors in this basic procedure were varied singly to determine their effect on the end product. The thyroidal potency of the iodinated protein was determined by biological assays on tadpoles and guinea pigs (126) and more recently by chemical determination of its thyroxine content (135).

a. Effect of Extent of Iodination. The necessity of following rather closely defined limits of iodination for the formation of iodinated proteins capable of yielding thyroxine on hydrolysis was stressed by Ludwig and von Mutzenbecher (93), but no information was available as to the effect of varying degrees of iodination on the thyroidal activity of the iodinated protein itself. Muus *et al.* (105) reported that when serum albumin in an ammoniacal medium was treated with progressively increasing amounts of iodine, thyroidal activity, as determined by tests on myxedematous patients, did not begin until 6% of iodine or the equivalent of 2 atoms per mole of tyrosine in the protein had been bound. With increasing iodination the thyroidal activity increased until 3 to 4 atoms had been combined per mole of tyrosine, and thereafter remained at a relatively constant level.

A distinctly different picture, probably because of differences in the medium used, was obtained by Reineke *et al.* (137) when both casein and the mixed proteins of skim milk were iodinated progressively in the sodium bicarbonate medium already described. Beginning at a low level, the thy-

roidal activity of successive preparations increased with increasing iodination until 4.5 to 5.0 atoms had been added per mole of tyrosine in the protein. If it is assumed that one-half the iodine is used in the formation of hydriodic acid and the remainder for substitution, this would be just sufficient for the substitution of 2 atoms per mole of tyrosine in the protein. Iodination beyond this point resulted in a rapid decline in activity.

With the discovery (126) that the formation of active thyroidal substance is increased markedly by incubation of the iodinated protein at an elevated temperature, the effect of progressively increasing increments of iodine on the activity of iodinated casein and soybean protein when incubated at

TABLE II

Effect of Progressive Iodination and High Temperature Incubation on Thyroidal Activity of Iodinated Protein

Series No.	Preparation No.	Iodine added per 100 g. protein	Iodine added per mole tyrosine	Iodine combined	Iodine combined per mole tyrosine	Thyroxine content	Tadpole responses	*Per cent* of thyroxine response	P*
		g.	*atoms*	*per cent*	*atoms*	*per cent*	*per cent*		
I. Iodinated casein	1	7.5	1.89	4.11	1.08	0.67	16.2	4.17	1
	2	12.5	3.16	5.93	1.59	1.31	27.2	5.83	1
	3	19.0	4.80	7.55	2.06	1.85	34.9	8.50	5
	4	25.0	6.31	8.19	2.25	1.72	21.6	5.07	1
	5	32.5	8.21	8.60	2.38	1.35	11.8	3.42	1
	6	38.0	9.60	9.13	2.54	1.04	4.6	2.40	5
II. Iodinated soy bean protein	1	6.0	2.03	3.21	1.12		14.2	3.82	
	2	11.5	3.88	5.20	1.85		21.5	5.07	5
	3	17.5	6.35	6.15	2.22		22.9	5.25	5
	4	23.5	8.95	6.51	2.35		7.7	2.92	1
	5	30.0	10.14	7.40	2.70		2.9	2.35	1
	6	36.0	12.17	7.78	2.85		1.6	2.32	5

*P—the *per cent* probability that the difference from the preceding member of the series is due to chance variation.

(From the *J. Biol. Chem.* (136), with thyroxine analyses (135) added.)

70°C. for 18 to 20 hours was investigated (136). At all levels of iodination the actual thyroidal potency was considerably higher than that of similar preparations incubated at a lower temperature. Just as in the earlier series, however, the thyroidal activity rose to a maximum (Table II) when sufficient iodine had been added to substitute 2 atoms per mole of tyrosine in the protein. Further iodination resulted in a pronounced decline in activity of both the iodinated casein and the soybean protein.

For reasons which will be discussed later, the thyroxine content of iodinated proteins as determined by chemical analysis is considerably lower than that indicated by the tadpole assays. However, the two measures fol-

low a parallel course with respect to the relative potency of succeeding preparations in the iodinated casein series.

Approximately one-half the iodine used is combined with the protein until sufficient has been added to substitute 2 atoms per mole of tyrosine. At this point the Millon reaction becomes negative, indicating full substitution on the 2 carbon atoms *ortho* to the phenolic hydroxyl group of tyrosine.

It is thus apparent that, under the conditions employed, iodine is taken up principally by tyrosine according to the equation,

$$\text{Tyrosine} + 2I_2 \rightarrow \text{Diiodotyrosine} + 2HI$$

After the tyrosine has been fully substituted iodine is combined less readily, with the result that progressively smaller increments are taken up as more is added. The best evidence available indicates that the formation of thyroxine in iodinated proteins is effected by the oxidative coupling of two molecules of diiodotyrosine, with the elimination of one side chain. The addition of iodine in excessive amounts apparently causes further oxidations which result in inactivation of the compound.

b. Relation of pH and Extent of Iodination to Formation of Active Substance. The pH of the medium appears to have a decided influence on the relative reactivity of the iodine-binding groups in proteins, and this is also reflected in the amount of thyroxine formed under various conditions. Investigation of this problem is complicated by the fact that the continuous formation of hydriodic acid with increasing iodination depresses the pH of the medium (137) unless a considerable excess of buffer substance is present.

The effect of the pH on the thyroidal activity of skim milk proteins was studied (126) by making up a series of preparations in which the iodine input was held constant, and the amount of sodium bicarbonate added was increased in progressive order in succeeding samples. As indicated by assays on tadpoles, the formation of active substance was markedly retarded when the amount of buffer present was insufficient to hold the pH of the reaction medium at a value of approximately 7.0 or above. Excess of sodium bicarbonate beyond this amount appeared to have no effect on the result. From the fact that normal amounts of iodine were combined by the protein at all pH values covered, it is believed that substitution of iodine on the tyrosine occurred as usual, but conditions were not such as to permit the formation of appreciable amounts of thyroxine at the lower pH levels.

In further experiments (132) it was found that when the sodium bicarbonate concentration of the medium was increased concurrently with the iodine input in order to prevent decline in pH with excessive iodination, considerably more iodine could be added before the usual decline in thyroxine content occurred. Under these conditions, thyroxine formation increased until 6 to 7 atoms of iodine were added per mole of tyrosine in the

protein. Further addition of iodine caused a decline in thyroxine content even though the pH of the solution was not depressed. The thyroxine content at the optimum level of iodination was also somewhat higher than under the former conditions.

c. Relation between Iodination and Incubation Temperature. In all the earlier experiments on formation of thyroidally-active iodinated proteins the reactions were conducted, where possible, at approximately 38°C., presumably in the expectation that thyroxine formation would be favored at physiological temperatures. von Mutzenbecher (106) reported that when casein was iodinated in ammonia solution in the cold it showed little or no thyroidal activity. Re-suspension of the iodinated casein in sodium bicarbonate solution, and incubation at 37°C., with stirring, for 2 to 3 days resulted in appreciable increases in activity as indicated by tests on guinea pigs. From this it might be expected that the potency of the iodinated protein would be a function of the time of incubation.

In order to determine the effect of long-continued incubation on the formation of thyroidal substance, casein was iodinated by the usual procedure (126), and then placed in a water bath at 37° to 38°C., with continuous stirring, for varying periods up to 39 hours (Table III). When a uniform dosage of iodinated protein from the various lots was injected into groups of tadpoles there was no significant difference in the stimulation of metamorphosis, as indicated in the column headed "Per cent response." This experiment, with slight variations, was repeated several times with essentially the same result. Thus, it was concluded that little or no increase in thyroidal potency could be obtained by use of a long incubation period under the conditions employed.

However, it was discovered (126) that a pronounced increase in thyroidal potency of iodinated protein could be obtained by holding the reaction mixture at the elevated temperature of 60° to 70°C., beginning either before the iodination step or subsequent to it. In two groups of preparations (Table IV), incubation at 39°C. was continued for 28 hours without a demonstrable increase in the thyroidal activity as determined by injection in tadpoles. When the temperature was increased to 65°C. during the last 18 hours of incubation there was a large increase in potency.

When preparations were incubated at various temperatures from 30° to 97°C. the thyroidal potency remained at a uniform level over the range of 30° to 45°C. There was a pronounced rise in activity at 60°.C, with the maximum occurring at 70°C. Further increase in the temperature of incubation to 97°C. resulted in a considerable decline in activity of the resulting product.

In all of these experiments the iodinated protein was incubated at the elevated temperature for 18 to 20 hours subsequent to iodination. The

effect of longer incubation periods under the given conditions has not been reported. In further investigations by the author (unpublished) it has been observed that the thyroxine content of iodinated casein increases progressively with increasing length of incubation up to 24 hours. At this point

TABLE III

The Effect of Length of Incubation Period at 37° to 38°C. on Thyroidal Potency of Iodinated Casein

Preparation No.	No. of Tadpoles	*Per Cent* Response	Hours Incubated
AB26-1 (in glass)	9	12.6	None
2	4	12.5	5.0
3	9	12.1	16.0
4	7	13.8	21.0
5	7	9.7	39.0
AB27-1 (brass stirrer)	6	13.6	None
2	6	16.6	5.0
3	7	17.3	16.0
4	8	17.1	21.0
5	7	14.0	39.0

Dosage 0.025 γ per tadpole.
(From *Agr. Exp. Sta. Mo., Res. Bull.* **355** (126).)

TABLE IV

The Effect on Thyroidal Activity of Elevating the Incubation Temperature Subsequent to Iodination

Preparation No.	No. of Tadpoles	*Per Cent* Response	Hours Incubated	Temperature °C.
AB47-1 (in glass)	10	1.20	4	39
2	10	4.37	16	39
3	8	5.56	28	39
4	6	12.10	46	65
AB48-1 (brass stirrer)	9	12.40	4	39
2	7	10.80	16	39
3	8	6.20	28	39
4	10	19.20	46	65

Dosage 20 γ per tadpole.
(From *Agr. Exp. Sta. Mo., Res. Bull.* **355** (126).)

thyroxine formation appears to continue, but at a constantly diminishing rate.

d. Catalysis of Thyroxine Formation by Manganese Compounds. During the course of the work just described, it was observed that iodinated proteins made up in the presence of a common brass stirrer rather consistently possessed greater thyroidal properties than those prepared exclusively in

TABLE V

Showing the Effect of the Incubation Temperature, Manganese Compounds and Amount of Agitation on the Formation of Thyroxine in Iodinated Protein

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

(From the *J. Biol. Chem.* (132).)

glass equipment. This led to the supposition that one of the metals contained in brass, or perhaps the combination of materials present, catalyzed the formation of thyroxine. In further experiments no augmentation of the thyroidal potency of iodinated proteins prepared in the presence of salts or oxides of copper, iron or cerium was observed. Thyroxine formation was uniformly increased, however, upon the addition of small amounts of manganese compounds (132), including manganese sulfate and a series of manganese oxides.

The effects on thyroxine formation of the incubation temperature, amount of agitation and various manganese compounds are summarized in Table V. It will be noted that the rate of stirring during the incubation period is a factor in the formation of thyroxine. From results obtained in the incubation of diiodotyrosine (133) this is believed to be due to incorporation of atmospheric oxygen in the solution.

With all other factors held constant, the thyroxine content of iodinated casein was increased markedly by incubation in the presence of a small amount of manganese tetroxide (Mn_3O_4). Similar results were obtained by use of the mixed oxides resulting from the reduction of potassium permanganate with glucose. Definite increases in thyroxine formation, but of a smaller magnitude, were obtained with the other manganese compounds tested.

The thyroxine content of a series of preparations formed by combining progressively increasing amounts of iodine with casein in the presence of manganese tetroxide is compared graphically in Fig. 1 with that of a control series prepared without the catalyst. The procedure differed from that reviewed previously in that the amount of sodium bicarbonate added was increased proportionately with the iodine. Under these conditions thyroxine formation continued to increase in the control series until slightly more than 7 atoms of iodine had been added per mol. of tyrosine in the casein. In the presence of manganese oxide approximately 6 atoms of iodine per mol. of tyrosine were required for maximum thyroxine formation. More iodine was required for optimal results than under the former conditions of bicarbonate concentration, but the maximum thyroxine content was also higher. By addition of the catalyst the thyroxine content at the level of optimum iodination increased from 2.8 to 3.37%. The formation of thyroxine from diiodotyrosine was catalyzed by manganese oxide (133) in a similar manner.

In view of the many factors now known to influence the formation of thyroxine in iodinated proteins, it is not surprising that many of the early reports in this field were negative or contradictory. The reaction appears to be quite specific, depending for its successful completion upon the proper balance of the various factors reviewed in this discussion.

Assuming the tyrosine content of casein to be 5.65%, the theoretical yield of thyroxine would be 10.6%. The maximum thyroxine content of

3.37% (Fig. 1) represents slightly more than 30% of the theoretical. In instances where proper attention has been given to all factors now known to influence this reaction, iodinated casein containing 4% or more of

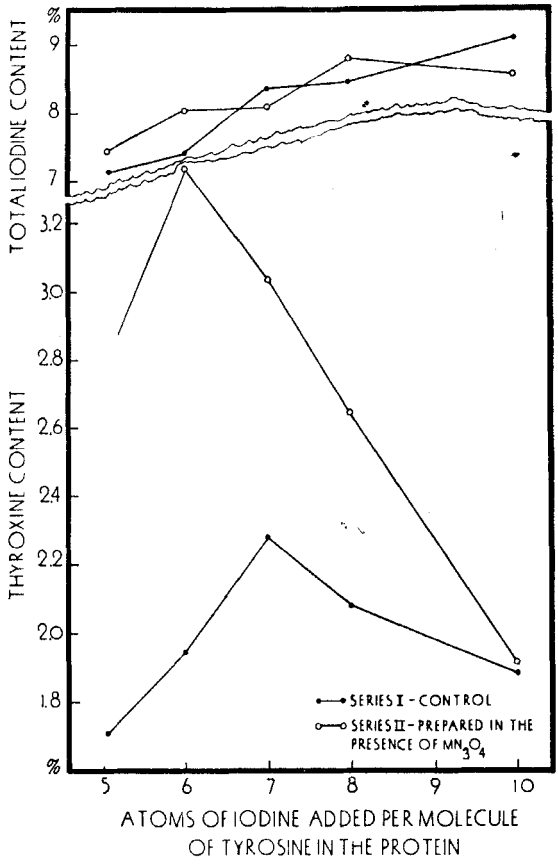


Fig. 1

The Effect of Progressive Iodination in the Presence of Excess Bicarbonate and Manganese Oxide on the Thyroxine Content of Iodinated Casein

(From the *J. Biol. Chem.* (132))

thyroxine, as determined by both biological assay and chemical analysis, has been reported (135).

5. Proteins Suitable for Iodination

Of the many proteins that might be considered for the formation of thyroidally active substances, casein has been most widely used, probably because of its ready availability and the ease with which it can be manipulated. It is well established that by means of the iodination and subsequent

treatment thyroxine is formed from the tyrosine originally present in the protein. Thus, it might be expected that any protein containing tyrosine might be suitable for this purpose unless the position of this amino acid was such in certain proteins as to interfere with the coupling reaction involved in the formation of thyroxine. Although the number of proteins studied from this point of view has been limited, no instance has come to the attention of the author wherein a protein which contains tyrosine has failed to form a thyroidally active substance when iodinated under suitable conditions.

Ludwig and von Mutzenbecher (93) reported that crystalline thyroxine was obtained by hydrolysis in alkali of iodinated casein, iodinated serum albumin, iodinated serum globulin, iodinated silk fibroin, and iodinated edestin. It was believed that all proteins containing tyrosine could be used successfully, but that the best conditions must be established for each protein.

Abelin and Neftel (6) reported that the formation of thyroidally active iodinated proteins depended upon both the type of protein used and the iodination method. Iodination of peptones failed to produce an active product. Because of the paucity of information available at that time on the other factors affecting this reaction, however, the interpretation of these results is doubtful.

Hypothyroidism was corrected by the administration of acid-insoluble substance obtained from hydrolyzates of blood serum proteins (143), by iodinated serum proteins given as such (91), and by iodinated serum albumin (105).

A relatively low order of thyroidal potency was reported by Blaxter (17) for iodinated blood proteins. Higher activity was observed with iodinated ardein, while the highest potencies were obtained with iodinated casein. Data on the preparation of these products were not given, however.

Highly active iodinated proteins were prepared from casein, egg albumin and soybean proteins by Reineke and Turner (126). In comparison with iodinated casein prepared under the same conditions, iodinated soybean protein showed lower thyroidal potency, proportionate with its lower original tyrosine content.

With the factors influencing the formation of iodinated protein now worked out sufficiently well to permit standardization of procedures, it would be of considerable interest to extend these observations to a series of proteins of widely varying tyrosine content.

IV. The Isolation of Thyroxine from Iodinated Protein

1. Isolation of dl-Thyroxine

Once an iodinated protein has been prepared under the proper conditions for thyroxine formation, the pure amino acid can be isolated readily

by use of the principles first established by Kendall (77) and Harington (49) in the isolation of thyroxine from thyroid substance.

The isolation of crystalline thyroxine from iodinated proteins was first reported by Ludwig and von Mutzenbecher (92) in 1936. In a later report (93), details of the isolation procedure used for the recovery of thyroxine, diiodotyrosine and monoiodotyrosine were presented. The iodinated protein was first hydrolyzed in boiling 40% barium hydroxide solution for 20 hours to liberate the thyroxine. The sandy precipitate of barium salts which formed was recovered by filtering the hot solution, and then decomposed with hydrochloric acid to obtain an acid-insoluble precipitate of high iodine content. A second portion of acid-insoluble substance was obtained by acidification of the liquid portion of the hydrolyzate after removing the excess barium hydroxide which crystallized when the solution was cooled. The last traces of barium were removed from the combined acid-insoluble precipitates by treatment with sodium sulfate in boiling $N/10$ sodium hydroxide solution, the barium sulfate formed being removed by centrifuging. After precipitation from the hot sodium hydroxide solution by acidifying with dilute sulfuric acid solution, and washing with dilute acetic acid, the acid-insoluble substance was dissolved in a minimum of boiling $N/10$ potassium carbonate solution. The mono-potassium salt of thyroxine crystallized from this solution when cooled to 0°C. After purifying the compound by recrystallization as the mono-potassium salt, it was dissolved in 70% alkaline alcohol. Upon acidifying the boiling solution with glacial acetic acid, free thyroxine crystallized in the characteristic bundles of microscopic needles. The yield of purified thyroxine obtained amounted to approximately 0.1% of the iodinated casein hydrolyzed. Harington and Pitt Rivers (55) reported that a similar yield of thyroxine was obtained when casein was iodinated and treated in a manner identical with that described by Ludwig and von Mutzenbecher.

By using iodinated casein with high initial thyroidal activity, and a similar isolation procedure, Reineke and Turner (127) obtained a yield of 0.424% of crystalline thyroxine. The increased yield of thyroxine obtained by isolation thus supported the evidence of increased thyroidal activity indicated by biological assays. The actual yield, however, was only 28% of the figure indicated biologically.

The thyroxine obtained from iodinated proteins has been found to be identical with synthetic thyroxine in every respect. Iodine contents ranging from 63.6 to 65.0% (theoretical 65.4%) have been reported (93, 127) When the iodine was removed by reduction (93), thyronine was obtained. The spectrographic absorption curve of thyroxine isolated from iodinated protein is identical with that of synthetic thyroxine (Fig. 2).

Metabolic stimulation equal to that produced by the synthetic compound was observed (127) when thyroxine obtained from iodinated protein was

administered to guinea pigs, thus providing biological proof of its identity. Because of racemization occurring during hydrolysis in alkali, the thyroxine obtained by the barium hydroxide procedure is a racemic mixture.

2. Isolation of l-Thyroxine

From theoretical considerations on the mode of its formation, the thyroxine in iodinated proteins would be expected to be the natural levorota-

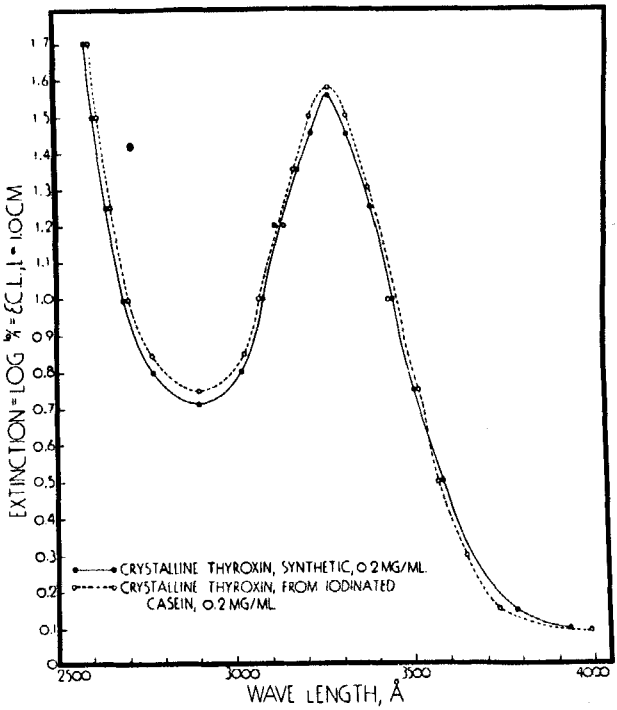


Fig. 2

Spectrographic Absorption Curves of Synthetic Thyroxine and Thyroxine Isolated from a Barium Hydroxide Hydrolyzate of Iodinated Casein

(From the *J. Biol. Chem.* (127))

tory isomer. Optically active amino acids are ordinarily obtained from proteins by hydrolyzing with acids to avoid racemization. Early attempts to concentrate the active principle of thyroid substance after hydrolysis with acid as reviewed by Kendall (78) and Harington (52) resulted in failure, presumably due to destruction of the thyroxine by the acid. Minute yields of *l*-thyroxine were obtained from thyroid by Harington and Salter (58) and Foster *et al.* (41) after hydrolysis with proteolytic enzymes.

Abelin (3) reported that hydrolysis of iodinated casein in 10% sulfuric acid solution failed to yield a thyroidally active product, either in the acid-insoluble portion or in a *n*-butanol extract. Ludwig and von Mutzenbecher (93) stated that attempts to isolate thyroxine from iodinated casein, hydrolyzed with either sulfuric acid or proteolytic enzymes, were unsuccessful. Although Lerman and Salter (91) were able to separate iodinated serum protein into thyroxine and diiodotyrosine fractions by stepwise hydrolysis with enzymes, attempts to isolate thyroxine resulted in failure.

The isolation of pure *l*-thyroxine from iodinated casein was finally accomplished by Reineke and Turner (128). To obtain this result, advantage was taken of the fact that thyroxine is soluble in *n*-butanol even in strongly acid solution. When iodinated casein was hydrolyzed in a mixture consisting of equal parts by volume of 32% sulfuric acid and *n*-butanol, crystalline *l*-thyroxine was obtained on the first attempt. A greatly diminished yield of thyroxine was also recovered after hydrolysis in a *n*-butanol-hydrochloric acid mixture.

Isolation of the thyroxine from the acid hydrolyzate required a procedure modified considerably from that used previously with barium hydroxide hydrolyzates. After 13 hours heating under reflux in a boiling water bath, the *n*-butanol-sulfuric acid digest of the iodinated protein was diluted with 6 volumes of distilled water, whereupon the *n*-butanol, with the dissolved hydrolytic products, formed a separate layer. A considerable amount of dark colored impurity was removed from the *n*-butanol extract by several extractions with 4 *N* sodium hydroxide solution containing 5% sodium carbonate. Removal of the *n*-butanol by vacuum distillation left a residue which still contained considerable tarry material that remained with the acid-insoluble portion despite repeated dissolution and precipitation. This was removed rather easily, however, by dissolving the precipitate in distilled water with the aid of ammonia, heating to 60°C., and adding a slight excess of warm barium hydroxide solution. The barium salts of the tarry substances formed a flocculent precipitate, leaving most of the thyroxine in solution. When acidified, this solution yielded a light-colored precipitate of greatly improved appearance. The traces of barium remaining were removed by centrifuging, after dissolving the precipitate with the aid of ammonium hydroxide and adding ammonium sulfate to the boiling solution. The thyroxine concentrate was recovered by acidifying the hot solution with dilute sulfuric acid, and was finally dissolved in a minimum of boiling sodium carbonate solution. Upon chilling this solution a heavy white precipitate of the monosodium salt of thyroxine settled out. After several recrystallizations from sodium carbonate solution, the thyroxine was dissolved in alkaline 70% alcohol. The free amino acid (Fig. 3) crystallized immediately when a few drops of acetic acid were added to the boiling solu-

tion. The yield of crystalline material was approximately 0.1%, as compared to 0.421% of *dl*-thyroxine that was obtained from the same lot of iodinated casein. The iodine content of the purified *l*-thyroxine was 65.1%, and the

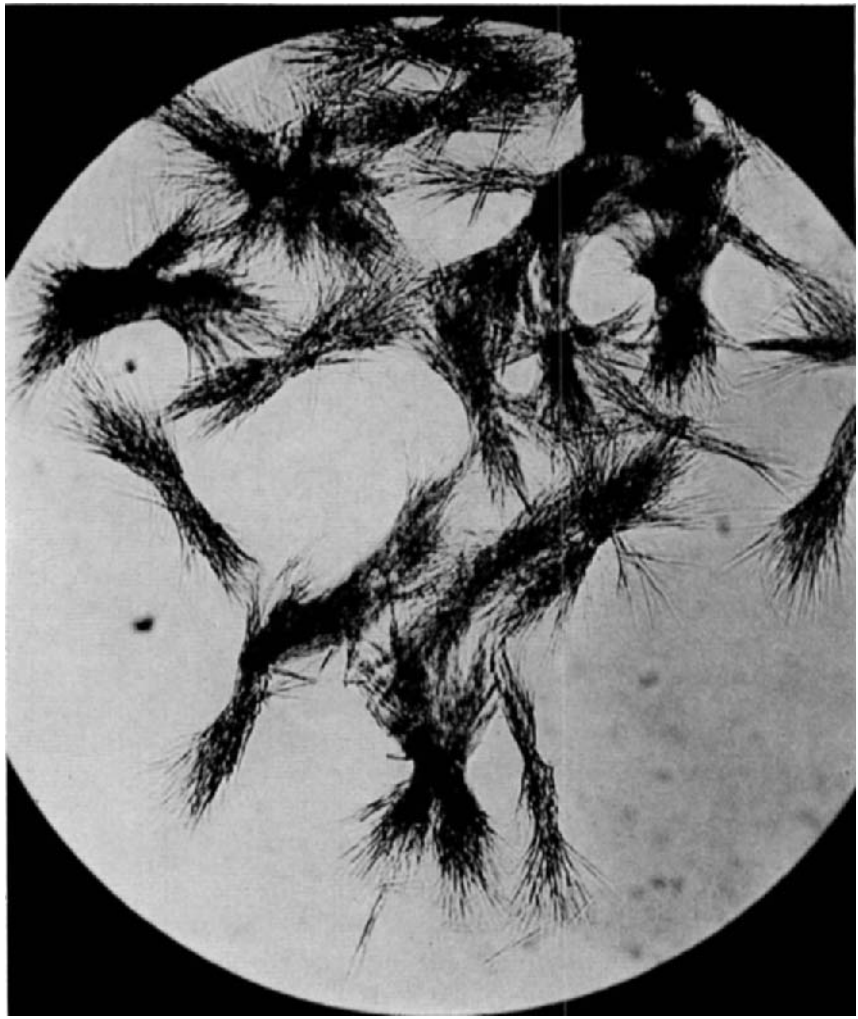


Fig. 3

Crystalline *l*-Thyroxine Isolated from an Acid Hydrolyzate of Iodinated Casein (×400)

melting point was 236–238°C., as compared to a melting point of 230–232°C. obtained with *dl*-thyroxine. The specific rotation was $[\alpha]_D = -4.2$. Metabolic tests on guinea pigs indicated that *l*-thyroxine possesses twice the thyroidal potency of a *dl*-mixture, a point which has an important bearing on the interpretation of biological assays of iodinated proteins to be discussed later.

V. The Quantitative Assay of Thyroxine in Thyroactive Iodinated Proteins

1. Biological Assays

The biological methods developed for the assay of thyroid are all adaptable, with slight modifications in some cases, for estimation of the thyroidal activity of iodinated proteins. While no attempt will be made to cover the extensive literature in this field, the results obtained by various assay methods when applied to iodinated proteins will be reviewed.

The correction of myxedema and elevation of the metabolic rate in man was used by Salter and associates (143, 91, 105) to measure the potency of iodinated serum proteins. For laboratory investigations methods based on the acceleration of metamorphosis in frog tadpoles or elevation of the metabolic rate of small animals such as guinea pigs are the most suitable.

a. Stimulation of Metamorphosis in Frog Tadpoles. The sudden and dramatic metamorphosis of amphibian larvae when fed a small amount of thyroid substance was first described by Gudernatsch (47, 48) in 1913. It was found that frog tadpoles exposed to thyroid, either by feeding or as a solution in the water surrounding the tadpole, showed rapid and precocious differentiation, but no further body growth. These findings were confirmed and extended by Lenhart (89), Romeis (141), Kahn (75), Rogoff (138) and others. Allen (7, 8) and Hoskins and Hoskins (63, 64) reported that when thyroidectomized at an early stage, tadpoles would not metamorphose unless fed thyroid substances.

Gaddum (42) reported that the decrease in body length of tadpoles in response to thyroxine administration was roughly proportional to the dose. Wokes (165) showed that the percentage decrease in body length of tadpoles given thyroid substance bore a straight line relationship to the log of the dosage, and described a detailed procedure for the use of this measure in the assay of thyroid preparations.

As already discussed, the early evidence of the stimulation of metamorphosis by iodinated proteins was discounted as a non-specific response, not indicating true thyroidal properties. However, little or no metamorphosis is induced by the administration of non-thyroxine iodine compounds such as diiotyrosine or potassium iodide (42, 87, 126).

Application of the tadpole method to the quantitative assay of the thyroidal activity of iodinated proteins has been reported by Reineke and Turner (126). Tadpoles will respond to active iodinated protein when it is fed or when a small amount in solution is injected into the body cavity. This material differs from thyroid substance or thyroxine in that it is not absorbed when placed in solution in the water surrounding the tadpole.

Of the many endpoints which could be taken as measures of metamorphosis, such as the decrease in body weight, rate of growth of the limbs and

the time of emergence of the left front limb bud, the percentage decrease in body length is the most convenient measure, and shows the best proportionality with the dosage. *Rana pipiens* larvae were the most satisfactory of the species tested.

The sensitivity of tadpoles to thyroidal stimulation depends upon the species, their stage of development, the environmental temperature and probably other factors. For this reason the best comparisons are obtained by assaying a large number of preparations concurrently on tadpoles of uniform size and development. Comparative assays can be obtained by simply injecting a uniform dosage of each preparation into groups of tadpoles, and taking the average percentage decrease in body length produced by each preparation as a relative measure of its potency. For quantitative results it is necessary to set up a graded dosage series, using thyroxine or a standard preparation in order to establish a response curve from which the potency of the unknowns can be estimated.

Large *Rana pipiens* tadpoles collected in nature and injected at about the 60 mm. stage show pronounced metamorphosis within 4 days after a single injection of iodinated protein (Fig. 4). Laboratory-reared tadpoles obtained by the method of Rugh (142) require from 6 to 10 days after injection to show sufficient response for satisfactory measurement.

When assayed by injection in tadpoles, iodinated casein shows about 2.7 times the thyroidal potency, expressed in terms of a thyroxine standard, indicated by oral assays on guinea pigs (126). While a part of this discrepancy can undoubtedly be explained by the difference in the route of administration, further work should be done to establish more fully the reasons for this difference in response.

b. Assays Based on Elevation of the Metabolic Rate and Decrease in Body Weight. Of the common laboratory animals, the guinea pig is very suitable for thyroidal assays (163, 38, 126) because of its sensitivity to this type of stimulation. The increase in carbon dioxide production of mice in response to thyroid administration was used by Morch (102) and Gaddum (44) in the assay of thyroid preparations. While the elevation of the oxygen consumption of normal rats in response to thyroidal stimulation has been used (43) in studies on thyroxine and related compounds, thyroidectomy has been reported by Meyer and Wertz (100) to increase the sensitivity of rats to such stimulation 25- to 30-fold.

The method of Kreitmair (83), based on the percentage weight loss of guinea pigs induced by administration of the test substance for 6 days was used (93) in developing the procedure for the isolation of thyroxine. This method was compared (126) with a procedure based on the elevation, due to thyroidal stimulation, of the oxygen consumption of guinea pigs. The weight loss method can be criticized seriously for a lack of specificity. However, the results agree fairly well with values obtained by the metabolic

method. A group of iodinated proteins assayed by this method showed thyroidal potencies equivalent to 1.0 to 4.0% of the activity of *dl*-thyroxine, the value obtained depending on the method of formation of the iodinated

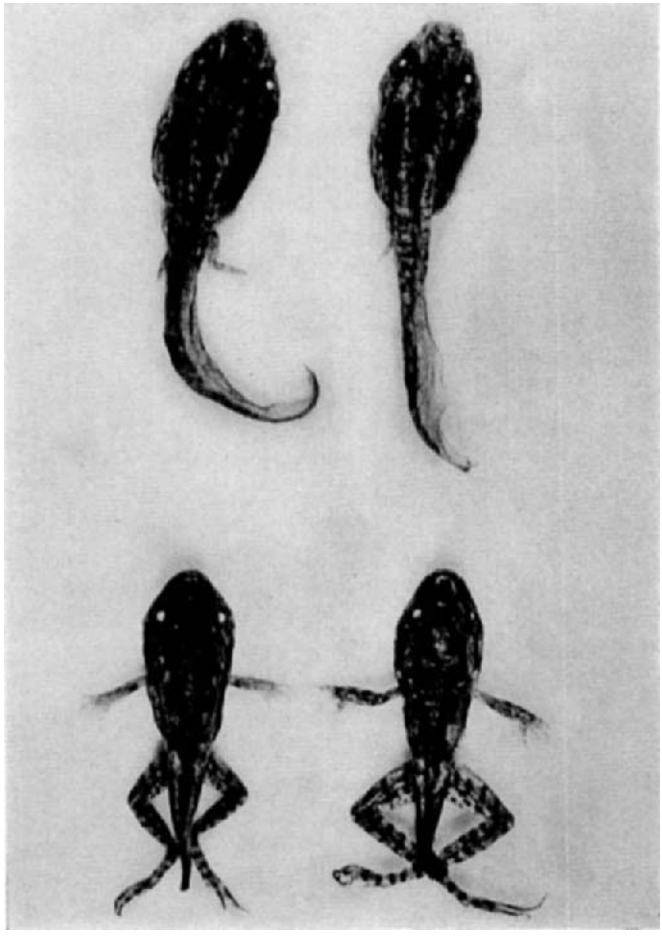


Fig. 4

The Response of Large Frog Tadpoles (60 mm. size) to the Injection of Artificial Thyroproteins

The tadpoles at the top are normal controls. The two at the bottom illustrate the striking degree of metamorphosis occurring within four days after the injection of 0.1 mg. of iodinated casein. (From *Agr. Exp. Sta. Mo., Res. Bull.* **355** (126).)

protein. Estimates of the thyroxine content of iodinated proteins as indicated by biological assay in guinea pigs show excellent agreement with chemical analyses for thyroxine (135).

A recently developed assay method based on the ability of thyroidal substances to reduce the enlarged thyroids of thiouracil-treated chicks (101) and rats (123) may be useful in the biological assay of thyroidally active iodinated proteins. The percentage reduction in thyroid weight of thiouracil-treated animals given graded amounts of thyroxine shows good proportionality with dosage, and agrees well with the results of metabolism measurements. Considerably less labor is involved than in the metabolic procedures. Preliminary experiments by Reineke and Turner (134) indicate that iodinated proteins assayed in chicks by this method produce results which are proportionate with the values obtained by the other biological procedures. The exact quantitative relationship between this and other assay methods, however, remains to be established.

In most of the experiments conducted by the author, the iodinated protein has been administered by either subcutaneous or intraperitoneal injection instead of orally, because it was desired to avoid possible differences in the relative absorption of the various test materials from the gastrointestinal tract. By use of this technique, processes for the formation of iodinated proteins of exceptionally high thyroidal potency when assayed by injection have been developed. These materials are also effective orally, but little information is available on their relative effectiveness by the various routes of administration. Further research on these points is needed to provide the basis for practical use of thyroactive iodinated proteins by oral administration.

c. The Relative Thyroidal Potency of l- and dl-Thyroxine. Experiments dealing with the question as to whether all of the physiological activity of *dl*-thyroxine resides in the natural levorotatory component, or whether the dextrorotatory isomer also contributes some activity will be reviewed at this point because this question has an important bearing on the interpretation of chemical and biological estimates of thyroxine content.

Both *d*- and *l*-thyroxine obtained by Harington (51) by resolution of the racemic mixture were assayed by Gaddum, who reported that in both tadpoles (42) and rats (43) the *d*-form showed about one-third the potency of the *l*-compound. Salter, Lerman and Means (144) reported that no difference in the activity of the two compounds could be discerned as judged by their effects on myxedema in man. Foster, Palmer and Leland (41) reported that *l*-thyroxine obtained by the enzymatic hydrolysis of thyroid substance exerted twice the calorigenic effect of a racemic mixture when administered to guinea pigs. Similarly, *l*-thyroxine isolated from an acid hydrolysate of iodinated casein was reported by Reineke and Turner (128) to produce twice the metabolic stimulation of a racemic mixture when tested on guinea pigs. These observations on the same compounds were extended to three additional species (131). As determined by its ability to reduce the weights

of the thyroids of thiouracil-treated chicks (Fig. 5), *l*-thyroxine again showed fully twice the potency of a *dl*-mixture. Closely similar results were obtained with tadpoles and thiouracil-treated rats. From the close agreement of results in four species of animals it was concluded that all of the activity of racemic thyroxine can be accounted for by its *l*-component and that the *d*-compound must have little or no activity. From the fact that the

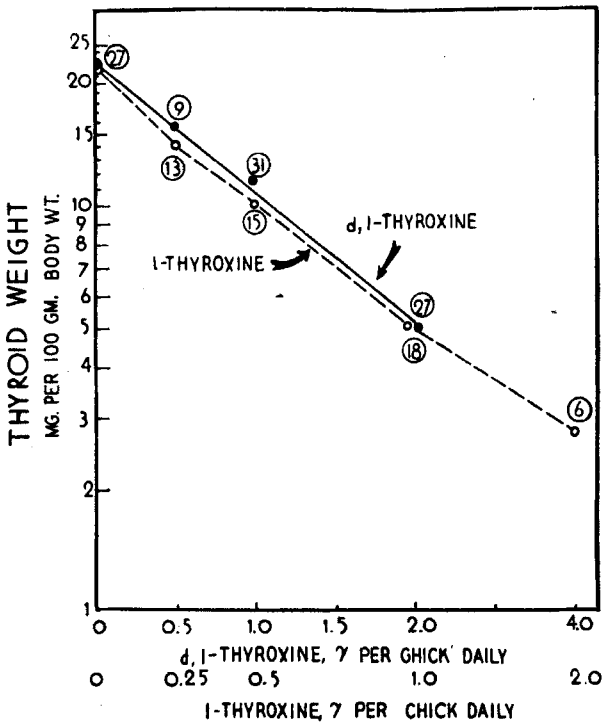


Fig. 5

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, 131.)

l-thyroxine isolated for the more recent assays (41, 128, 13) showed a higher specific rotation than did the compounds separated by Harington (51) it appears that the discrepancy in the biological results can be explained by somewhat incomplete resolution of the latter compounds. This is a possibility which was recognized by Harington in his original report.

In comparing biological assay data with chemical determinations of

thyroxine content it is necessary to take into account the fact that the thyroxine formed in the iodinated proteins is the pure *l*-compound, which has twice the potency of the racemic mixture ordinarily used as a standard.

2. Chemical Determination of the Thyroxine Content of Thyroactive Iodinated Proteins

Although a number of chemical methods have been devised for the estimation of thyroxine in thyroid substance, none of these methods has been used with iodinated proteins until quite recently because of serious questions as to their specificity when applied to such materials. Ludwig and von Mutzenbecher (93) expressed doubt that the iodine of the acid-insoluble substance obtained after alkaline hydrolysis of iodinated protein could all be considered to be thyroxine iodine as in the method of Harington and Randall (56). It was concluded by Abelin and Neftel (6) that the method of Leland and Foster (88) yielded only qualitative results when applied to iodinated proteins because a direct relation between the thyroxine iodine as determined by this method and the physiological effects produced could not be demonstrated.

TABLE VI

Data Demonstrating the Correlation Between the Chemical and Biological Assay Methods for Thyroxine

Preparation No.	Iodine Added per Mole Tyrosine in Protein	Iodinated Protein Injected	Increase in CO_2 Output	Thyroxine Found		Difference
				Bioassay*	Chemical Analysis	
	atoms	*(γ/100 g. body wt.)*	*per cent*	*per cent*	*per cent*	*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 reponse curve for intraperitoneally injected *l*-thyroxine. (From the *J. Biol. Chem.* (135).)

Excellent agreement between the results of biological assays and a chemical extraction procedure for thyroxine modified from the method of Blau (15, 16) have been obtained recently by Reineke *et al.* (135). Preliminary work indicated that Blau's method as described for thyroid substance yielded values which were considerably too high when compared with biological assays on the same preparations. The method also appeared to be non-specific when applied to preparations that had been iodinated excessively. By hydrolyzing the iodinated casein in 40% barium hydroxide

TABLE VII

Data Showing the Thyroxine Content of Samples of Iodinated Casein Carried through Different Stages of the Chemical Assay Procedure

Hydrolysate Injected* (γ/100 g. body weight)	Increase in CO_2 Output per cent	Thyroxine found Bioassay** per cent	Thyroxine found Chemical Analysis per cent	Difference per cent
I. Acid *n*-butanol extract				
500	24.0	2.04	2.06	− 1.0
150	14.8	3.93	3.88	+ 1.3
II. Acid butanol extract after washing with alkali				
360	23.5	2.76	3.00	− 8.0
412	22.3	2.28	2.62	−13.0
545	23.9	1.88	1.98	− 5.1
294	24.6	3.57	3.67	− 2.7
Average		2.74	2.87	− 4.36

* The figures given indicate the amount of original iodinated casein represented.

** Estimated from standard response curve for intraperitoneally injected *d,l*-thyroxine. (From the *J. Biol. Chem.* (135).)

instead of in the 8% solution employed in the original method, values agreeing well with the biological data were obtained. Apparently the more drastic hydrolysis liberates the thyroxine from combination with non-thyroxine compounds otherwise carried through with the thyroxine in the extraction with *n*-butanol.

In the modified method the iodinated casein is first hydrolyzed with 40% barium hydroxide by heating in a boiling water bath for 18 to 20 hours. After dilution of the hydrolyzates and decomposition of the barium salts, aliquots are acidified with dilute hydrochloric acid and then extracted with an equal volume of *n*-butanol. The *n*-butanol extract is washed in turn with an equal volume and a half-volume of 4 *N* sodium hydroxide, containing 5% sodium carbonate. Finally the *n*-butanol is removed by evaporation, and the iodine content of the residue determined.

For comparison with the chemical extraction values, the metabolic stimulation produced by a group of iodinated proteins when administered by intraperitoneal injection was determined, and the apparent thyroxine content estimated from a standard response curve based on *l*-thyroxine.

In a group of 15 iodinated casein preparations formed under varying conditions, the guinea pig assays indicated an average thyroxine content of 2.79% as compared to 3.03% thyroxine obtained by the chemical method (Table VI).

Biological assays of the *n*-butanol extract at two stages in the procedure (Table VII) showed that all of the thyroidally active substance of the original iodinated protein is recovered by the extraction. By comparison of the chemical and biological data, it is evident that if an active compound other than thyroxine is present it must be very similar to thyroxine in both iodine content and thyroidal potency.

From these results it is believed that the chemical method as modified for iodinated protein is highly specific for thyroxine, and it supports the early evidence of high thyroxine content provided principally by biological assays. As indicated earlier, however, further research is needed on the relative utilization of the thyroxine in iodinated proteins when administered orally.

VI. The Formation of Thyroxine from Diiodotyrosine

The formation of thyroxine directly from diidotyrosine was first reported by von Mutzenbecher (106). By incubating diiodotyrosine in mildly alkaline solution at 37°C. for a period of two weeks, a yield of crystalline thyroxine equivalent to about 0.1% of the diiodotyrosine taken initially was obtained. By use of the same incubation procedure, Block (20) formed thyroxine from synthetic diiodotyrosine, thus ruling out the possibility that the thyroxine might have had its origin from preformed thyronine occurring in tyrosine obtained from natural sources. By use of the same conditions very similar yields of thyroxine were obtained by Johnson and Tewkesbury (73) and Barkdoll and Ross (13). Harington (53) stated that a slight increase in thyroxine formation was obtained by oxidation with hydrogen peroxide at 37°C. By the addition of hydrogen peroxide at steam bath temperatures, the solution meanwhile being shaken constantly with *n*-butanol to extract the thyroxine as it was formed, a yield of 1.36% thyroxine was obtained.

The formation of thyroxine from diiodotyrosine has been found by Reincke and Turner (133) to be influenced by the same conditions established previously for iodinated proteins. Diiodotyrosine dissolved in *N*/10 sodium hydroxide at a pH of approximately 9.5 was incubated for 18 to 20 hours under various conditions. The yield of thyroxine obtained

by isolation, after incubation at a given temperature, was increased by either stirring or aeration, or by the addition of manganese oxide as a catalyst (Table VIII). The catalyst was ineffective, however, in the absence of added oxygen introduced either by stirring or aeration.

The temperature of incubation is highly critical (Fig. 7), having its optimum at 60°C. instead of at 37°C., the temperature employed in the earlier investigations. Manganese oxide increases the amount of thyroxine formation throughout the effective temperature range. With all conditions optimum an overall yield of 0.85% and a net yield of 2.8% of crystalline thyroxine was obtained.

TABLE VIII

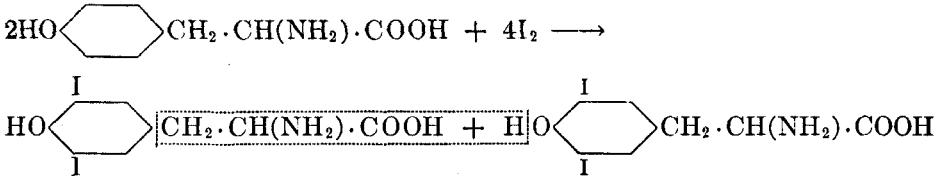
The Effect of Stirring and Aeration on the Formation of Thyroxine from Diiodotyrosine

Incubation Temperature °C.	Thyroxine yield *per cent*	Treatment
40	0.04	Mn_3O_4, 2 g.; stirred at 600 RPM
40	0.04	Mn_3O_4, 2 g.; aerated vigorously
50	0.38	Mn_3O_4, 2 g.; stirred at 600 RPM
50	0.36	Mn_3O_4, 2 g.; aerated vigorously
60	0.85	Mn_3O_4, 2 g.; stirred at 600 RPM
60	0.52	No catalyst; stirred at 600 RPM
60	0.02	Mn_3O_4, 2 g.; no stirring or aeration
70	0.27	No catalyst; stirred at 600 RPM
70	0.01	No catalyst; no stirring or aeration

(From the *J. Biol. Chem.* (133).)

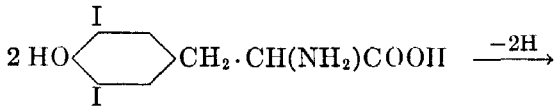
VII. Mechanism of Thyroxine Formation

In connection with their classical experiments on the constitution and synthesis of thyroxine (54), Harington and Barger first advanced the theory that thyroxine is synthesized biologically in the thyroid by iodination of tyrosine, followed by oxidative coupling of two molecules of diiodotyrosine, and the elimination of one side chain, as shown below.



With the discovery that thyroxine can be isolated from proteins iodinated artificially under the proper conditions, this oxidative mechanism appeared to provide the most plausible explanation for formation of the compound in

such materials (93, 55). Because of the complexity of protein systems, however, it was necessary to turn to experiments with diiodotyrosine itself for further development of the theoretical background.



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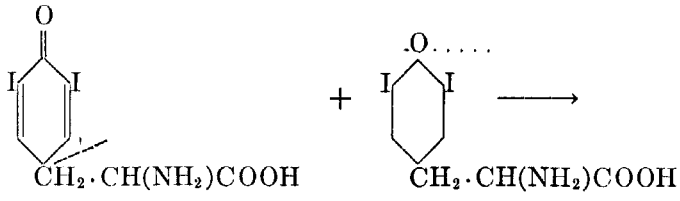

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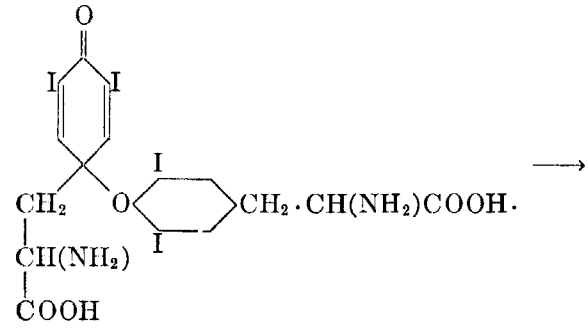

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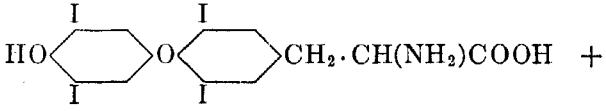

IV

$$CH_2 = C(NH_2)COOH \longrightarrow CH_3C(:NH)COOH \xrightarrow{H_2O} CH_3CO\cdot COOH + NH_3$$

Fig. 6.

A Mechanism for the Conversion of Diiodotyrosine to Thyroxine
(From *Proc. Nat. Acad. Sci., U. S.* (73))

By analogy with the results reported by Pummerer *et al.* (115) on the oxidation of *p*-cresol with potassium ferricyanide, Johnson and Tewkesbury (73) proposed a detailed reaction mechanism (Fig. 6) which would account

for the oxidative formation of thyroxine from diiodotyrosine. The first steps in the reaction would result in the oxidative coupling of two molecules of diiodotyrosine to form the intermediate compound **III.** Compound **III** must follow one of two courses, namely, (a) molecular dissociation with loss of one alanine side chain and formation of thyroxine, **IV,** and iminopyruvic acid, or (b) hydrolysis, with production of serine.

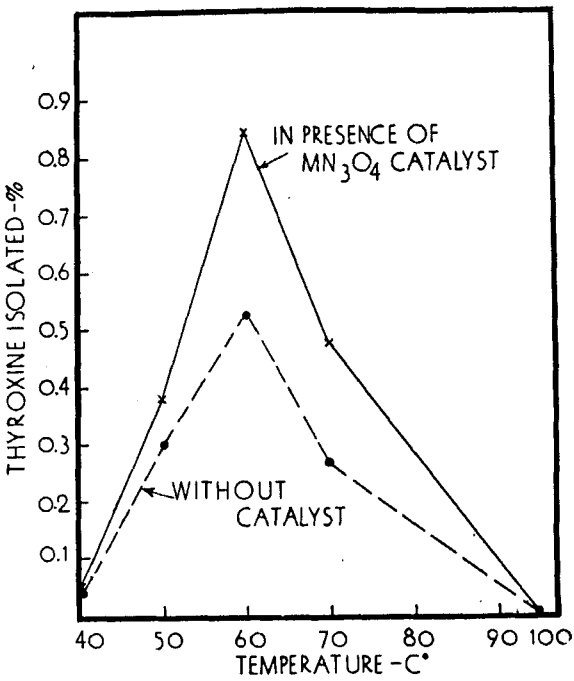

Fig. 7

Gross Yield of Thyroxine Isolated after Incubation of Diiodotyrosine at Various Temperatures

(From the *J. Biol. Chem.* (133))

In support of the theoretical considerations, pyruvic acid and ammonia, but not serine, were identified as secondary products in the reaction mixture. The results support the view that the formation of thyroxine is an oxidative process. In fact, it was stated that the yield of thyroxine obtained was increased slightly by adding hypoiodous acid to the reaction mixture in theoretical amounts. Harington (53) concurred with the general outline of the proposed reaction mechanism, and developed the theoretical background supporting it still further.

It has generally been assumed that the coupling reaction believed to be

involved in the formation of thyroxine in iodinated proteins, as well as from diiodotyrosine, is brought about by the mild oxidative action of hypoiodite (93, 55, 106, 73, 53). It has been reported by Barkdoll and Ross (13), however, that diiodotyrosine incubated in an oxygen-free system yielded no thyroxine and, conversely, the yield of thyroxine was increased by bubbling air through the solution. In recent experiments by Reineke and Turner (133), greatly increased yields of thyroxine were obtained by stirring or aeration of the solutions. Manganese oxide was effective as a catalyst in the presence, but not in the absence, of added oxygen introduced either by stirring or aeration, suggesting that manganese can act as a carrier for oxygen involved in the coupling reaction (See Fig. 7). From these results it appears that the oxidation previously attributed to hypoiodite is actually brought about by atmospheric oxidation.

It is of considerable interest that the results obtained with iodinated proteins *in vitro* support and supplement the recent investigations on the mechanism of thyroxine formation *in vivo*.

Chapman (32) reported that extra iodine administered to rats on a low-iodine diet produced thyroidal effects in thyroidectomized but not in intact animals. It was concluded that iodine may play a role in body metabolism in the absence of the thyroid, possibly by production of a thyroxine-like substance in the tissues. Morton *et al.* (104) administered radioactive iodine to thyroidectomized rats, and separated the thyroxine-like and diiodotyrosine-like iodine from hydrolysates of the tissues, by extraction with *n*-butanol. It was reported that 96 hours after its injection 30% of the radioiodine obtained from the liver and small intestines was organically bound, 20% as diiodotyrosine and as much as 8% as thyroxine.

Analyses of purified thyroglobulin (31) indicated that the protein from goitrous thyroids contained less thyroxine and non-thyroxine iodine than that from normal glands. Colloid from goitrous glands, although otherwise quite constant in amino acid composition was deficient in the iodine-containing amino acids; the tyrosine content showed a proportionate increase (Cavett, 30). McClendon, Foster and Cavett (94) reported that the thyroglobulin from colloid goiters was subnormal in both its thyroxine content and calorigenic effect. This was believed to indicate that thyroglobulin is first secreted as a colloid, and that the thyroxine radicle may then be synthesized within the protein molecule in a manner similar to that occurring in iodinated protein *in vitro*.

Mann *et al.* (95) injected radioiodine into dogs and compared the activity of thyroid iodine fractions. The data supported the belief that diiodotyrosine is the natural precursor of thyroxine and, further, that the iodination of tyrosine occurs outside of the thyroid cell. Further studies of histological sections of thyroid glands by Leblonde (86) after the administration of trace

doses of radioiodine indicated that the iodine is located almost exclusively in the colloid.

Salter and McKay (145) reported that in thyroids in which the formation of hormone was inhibited by the administration of thiouracil or thiocyanate the synthesis of thyroid protein can proceed independently of endocrine potency. All of these findings are in harmony with the idea that thyroxine is formed within the thyroglobulin molecule by an iodination process.

The influence of anaerobiosis and enzyme inhibitors on the formation of thyroxine and diiodotyrosine was studied by Schachner *et al.* (146) by means of radioiodine. Under the conditions cited, the formation of both substances was inhibited leading to the conclusion that the formation of both thyroxine and diiodotyrosine by the thyroid gland is linked with aerobic oxidations in which the cytochrome-cytochrome-oxidase system is involved.

Paschkis *et al.* (113) reported that the oxidase activity of thyroid tissue is decreased by adding thiouracil; inhibition of oxidase may be a factor, therefore, in the suppression of thyroid function by this drug.

Ray and Deysach (117) reported that the thyroid has a special capacity for the storage of manganese and, further, that the injection of small amounts of manganese chloride caused an increase in the oxygen consumption of guinea pigs. This, together with the finding that manganese catalyzes the formation of thyroxine in iodinated proteins and also from diiodotyrosine, prompted Reineke and Turner (132) to suggest that manganese may act *in vivo* as well as *in vitro* in promoting the oxidative formation of thyroxine.

VIII. The Effect of Iodination on Physico-Chemical Properties of Protein

1. Spectrographic Absorption

Most proteins exhibit specific light absorption in the ultraviolet region between 2500 and 3000 Ångström units. This property is due to the presence in proteins of the aromatic amino acids. In common with other cyclic compounds thyroxine shows a typical absorption curve, with a maximum at approximately 3250 Å (Fig. 2). Tyrosine, phenylalanine, tryptophane and indole show maxima in the range of about 2600 to 2900 Å (150, 39). Spectrographic measurements on proteins are thus complicated by the fact that the influence of the various amino acids cannot be differentiated because of their overlapping spectra. Certain changes due to iodination have been reported, however.

By measurements on thyroxine and related compounds, Marenzi and Villalonga (96) determined that iodination of the phenolic nucleus ortho to the hydroxyl group shifts the absorption maximum toward slightly longer wave lengths and also increased the difference in molecular extinction be-

tween the minimum and maximum. Iodinated casein had its absorption maximum shifted toward longer wave lengths as compared with normal casein (97).

Progressive iodination of casein (126) caused a shift in the absorption maxima to longer wave lengths and a simultaneous increase in the intensity of absorption which, in the earlier samples of the series, appeared to be correlated with the increase in thyroidal activity. The trend toward high intensities continued in excessively iodinated preparations reaching the maximum value in samples of declining thyroidal potency. This was believed to indicate the formation of a compound with excessive iodination, physiologically inert but thyroxine-like in structure and absorptive properties.

2. X-ray Diffraction Pattern of Iodinated Amino Acids

By study of the X-ray diffraction patterns of tyrosine, diiodotyrosine and thyroxine, Spiegel-Adolph *et al.* (152) concluded that iodination causes some structural rearrangement of amino acids. No difference was found in the X-ray diffraction pattern of thyroglobulin preparations of varying thyroxine content. Likewise, the diffraction pattern of iodinated casein was the same as that of the iodine-free protein.

3. The Effect of Iodination on the Dissociation Constant of Tyrosine

Cohn (33) pointed out that tyrosine has three pK values, of which two are due to the dissociation of the amino and carboxyl groups, and the third to the phenolic hydroxyl group. Iodination of tyrosine increased the dissociation of the phenolic hydroxyl group about a thousand-fold, and the amino group also dissociated at a somewhat more acid reaction.

When iodine was added to zein in an amount sufficient to iodinate completely the tyrosine present (107), the total acid- and base-binding capacity of the iodozein was identical with that of the original protein. However, the portion of the curve corresponding to the titration of the phenolic group of tyrosine was shifted to a lower pH range, as expected from the lower pK (OH) value of diiodotyrosine. Cohn, Salter and Ferry (34) reported that when iodine sufficient to iodinate the tyrosine radicals was combined with globin the base combined was diminished by an amount approximately equivalent to the amount of iodine taken up. In other words, the phenolic hydroxyl groups apparently disappeared completely from the reaction. It is interesting to note that this would be expected with the formation of the intermediate compounds postulated in the mechanism for thyroxine formation (Fig. 6) proposed by Johnson and Tewkesbury (73).

IX. Effect of Thyroactive Iodinated Proteins on Physiological Processes of Domestic Animals

With a large supply of thyroidally active material now potentially available by means of controlled methods of iodination, investigations have been inaugurated to determine the possibility of influencing various productive processes in domestic animals by the administration of these substances. Although this is a comparatively new field of study, a number of discoveries of considerable physiological interest and some of possible economic importance have been made. Experiments of this type are based on the concept that through its influence on general metabolism, and possibly on endocrine systems interrelated with the thyroid, the administration of small amounts of thyroidal substance will favorably influence certain physiological functions such as milk production, egg production, or growth. Obviously, regulation of the dosage to avoid overstimulation is an all-important factor.

1. Effect on Milk Secretion

The fact that the administration of thyroid substance (45) or thyroxine (46) to normal cows will cause a rapid increase in both milk yield and the percentage of fat in the milk was first reported by Graham in 1934. A greater effect was observed on milk fat production than on milk yield. This increase in both milk yield and milk fat production has been confirmed by all of the subsequent investigators. A less consistent effect has been observed on the constituents of milk other than the fat.

Slight increases in the solids-not-fat content of milk following the administration of thyroid powder or thyroxine for varying periods of approximately three days to four weeks were reported by Herman *et al.* (60, 61), Folley and White (40) and Ralston *et al.* (116), No change was observed in the solids-not-fat fraction by Jack and Bechdel (71) and Smith and Dastur (151), although the usual rise in milk yield and fat percentage occurred. Jones (72) reported that when eight cows were injected with 10 mg. of thyroxine daily for 14 days, the milk yield and pulse rate increased 28%, and 23%, respectively. The blood sugar increased 10% and then returned to normal, while the lactose content of the milk increased an average of 9% Sharp increases of as much as 38% in milk and 60% in fat production were reported by Hurst (65, 66) when 10 to 15 mg. of thyroxine were injected daily in lactating cows for a period of 4 weeks. In one instance the persistency of production was increased during a 9 month injection period.

In most of these experiments the dosage given was 1 to 2 oz. of desiccated thyroid or 10 to 15 mg. of thyroxine daily. Although quite variable, the average increase in milk production was approximately 13 to 18%, with

an increase in the yield of milk fat of 22 to 24% during limited periods of thyroidal stimulation.

Turner (155) called attention to the possibility of stimulating milk secretion in dairy cattle by the use of thyroactive iodinated proteins. Data on the actual stimulation of lactation by feeding iodinated proteins were first reported by Reineke and Turner (125). In 14 individual feeding trials in which active iodinated protein was fed to cows in advanced lactation for a 3 day period a rise in milk production ranging from 6 to 22% was observed in all except two cases. Due to the increase in fat percentage of the milk, the total yield of milk fat increased as much as 28%. Similar results were obtained in experiments with goats.

Further investigation resulted in the development of methods for preparing iodinated proteins of greatly increased thyroidal potency (120, 121). The administration of these preparations to lactating cows at the rate of 1.5 to 2.5 g. per 100 lbs. body weight daily resulted in an average increase of 18.6% in milk production in a group of 27 animals. Increases in the milk fat yield of more than 50% occurred due to the concurrent increase in milk fat percentage. After the initial rise in milk production, lactation again declined, but at a retarded rate as compared with the normal.

Van Landingham *et al.* (160, 161) in similar experiments reported that the feeding of 15 g. of iodinated casein daily to lactating cows caused an increase of 5 to 20% in milk yield and 25 to 50% in the yield of milk fat. The solids-not-fat of the milk increased slightly, but there was a 33% decrease in the ascorbic acid content.

Most of the results obtained by the administration of thyroidal substances to dairy cows indicate that there is a greater percentage increase in the milk fat percentage than in milk yield. With high dosages the increase is obtained at the expense of some loss in body weight. Reece (118) reported that when a moderate amount of iodinated casein was fed, the milk fat percentage increased from 3.6 to 4.1%, with little increase in milk yield, and only slight losses in body weight. The continuous feeding of thyroidally active iodinated protein for periods of 3 to 16 months (116) resulted in substantial increases in total production of milk and milk fat, with no harmful effects on the cows being noted.

The effects of a broad range of iodinated protein dosage on the milk production and physiologic well-being of dairy cows was investigated by Reineke *et al.* (122). Expressed in terms of an iodinated protein standardized to 3% thyroxine content as determined by chemical analysis, a dosage of 0.5 g. per 100 lbs. body weight daily produced a slight, but questionable rise in milk fat test with little or no effect on milk yield. A dosage of 1.0 to 1.5 g. caused an average increase over paired controls of about 10% in milk yield and 15 to 25% in milk fat yield, the increase being maintained for five

months, while the treatment was continued. Higher dosages, up to 4.75 g. per 100 lbs. body weight daily (Fig. 8) caused a higher initial rise in production, but this was accompanied by severe losses in body weight, and elevation of the pulse rate and body temperature.

Although some increase in milk production and milk fat test were noted when iodinated casein was fed, Seath *et al.* (148, 149) questioned the advisability of its use under Louisiana conditions because of possible injury

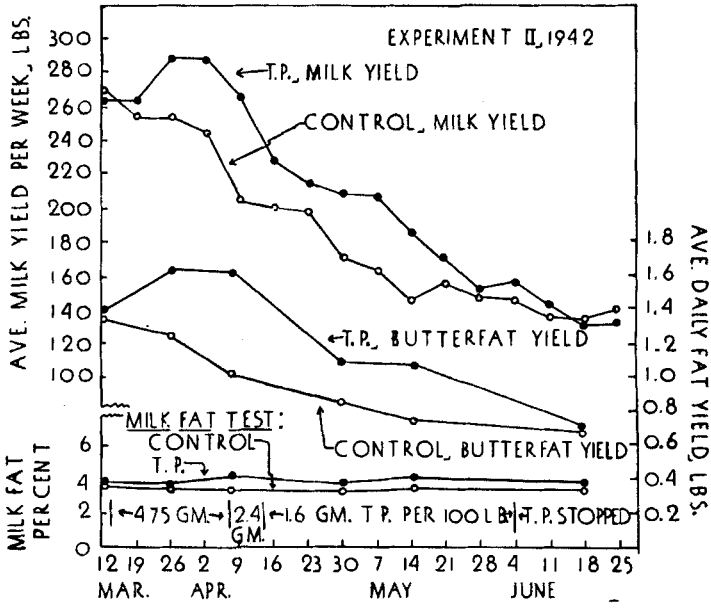

Fig. 8

The Lactational Response of Cows when Fed Heavy to Moderate Amounts of Thyroidally Active Iodinated Casein

The letters T.P. refer to the treatment with this material. The records of four experimental and three control cows are averaged in data for the respective groups.

to the cows due to slight losses of body weight and increases in body temperature during hot weather.

The results of extensive experiments conducted in England on the feeding of iodinated proteins to lactating cows have been reported by Blaxter (17). Iodinated casein, whole blood protein and ardein were all effective in stimulating milk production. Fifteen grams of iodinated casein daily increased production by about 16%, and 30 g. daily caused an increase of 33%, indicating that within this range the response is directly proportionate to the dosage. The heart rate increase was nearly trebled by doubling the

dosage, which was believed to indicate that at low dose levels the increased metabolism is probably not reflected by an increased heart rate. The increase in pounds of milk per day was greater the higher the initial milk production, but the percentage response declined with increasing initial yield. A favorable outlook for increasing milk production under practical conditions by feeding iodinated protein is given in the recent report by Blaxter (18).

In view of the possibility of feeding thyroidally active substances to increase milk production it is important to know whether or not some of this material would be secreted in the milk. No clear-cut evidence for the detection of thyroid hormone in milk could be found in the literature (129), although a number of reports to the contrary have appeared. In further experiments on this question, guinea pigs were given daily approximately 100 ml. of milk obtained from cows receiving a high dosage of active iodinated protein. Frequent determinations of their metabolism during this period failed to show any significant differences between these animals and paired controls receiving a milk diet, or normal guinea pigs on a stock diet. Similar results were obtained in trials with thyroidectomized goats. Thus, the amount of thyroid hormone passing into the milk, if this occurs at all, is too small to be detected by the biological methods used.

Comparisons of the amount of thyroidally active iodinated protein required to produce a lactation response in dairy cattle with the dosage of thyroxine required to produce the same effect when injected, indicate that iodinated protein is utilized quite inefficiently in ruminants. As judged by the dosage of iodinated protein required to cause a standard weight reduction in sheep (159) only 5% as much iodinated protein was required by subcutaneous injection as when given orally. When the iodinated protein was placed directly in the abomasum through a permanent cannula in order to bypass the rumen, no increase in utilization was observed. Thus the rumen was ruled out as a possible site of inactivation of the active principle. The oral utilization was increased by preliminary hydrolysis of the iodinated protein with acid, indicating that the poor utilization obtained with the whole protein may be due to incomplete digestion.

2. Effect on Body Growth

It is well established that hypothyroidism, whether induced or spontaneous, is detrimental to growth. Complete thyroidectomy in immature animals results in growth stasis and symptoms of cretinism; replacement therapy with thyroidal substance corrects these deficiencies.

The oral administration of graded doses of thyroidally active iodinated protein to young thyroidectomized goats arrested the symptoms of cretinism, and stimulated growth approaching the normal (124). Within the

range covered, the growth was roughly proportional to the dosage. When the iodinated protein therapy was begun immediately after thyroidectomy, and the dosage was gradually increased to keep pace with increasing body size, young goats developed normally in every respect during nearly a year of treatment (126). An animal in which pronounced cretinism was allowed to develop made a complete recovery when given iodinated protein.

From the marked improvement in growth which results from the administration of small amounts of thyroidal substance to hypothyroid individuals, it might be suspected that the induction of a slightly hyperthyroid condition would cause some acceleration of the growth rate above the normal. Although the literture on this subject, as reviewed by Turner and Koger (82), is quite controversial, there is some evidence that a properly regulated dosage of thyroid substance will cause an increase in the growth rate, at least in some species.

Parker (112) reported that Rhode Island Red chicks raised to the age of twelve weeks on diets containing graduated dosage levels from 0.025 to 0.2% of active iodinated protein made slightly greater gains in body weight than did the control chicks. A slight increase in the body weight of White Plymouth Rock chicks when fed a ration containing 0.08% of thyroactive iodinated casein was reported by Irwin *et al.* (70). Lower dosages had no effect on body weight, while amounts greater than 0.08% of the ration caused retardation of growth. The iodinated casein used in this instance showed 3.1% of the potency of *dl*-thyroxine by the guinea pig assay method. The same preparation fed at the level of 0.1% of the ration caused a slight decrease in growth, to twelve weeks of age, of Barred Plymouth Rock cockerels (156). Experiments by Schultze and Turner (147) indicate that a ration containing 0.009% of an iodinated casein preparation of a potency similar to that used in the work cited above will replace the thyroid hormone secretion of thiouracil-treated chicks. The most favorable effects on growth could be expected to occur with amounts slightly above this figure. The broad tolerance range to this type of treatment is indicated by the fact that chickens receiving iodinated protein equivalent to ten times the normal thyroid hormone secretion rate showed little growth retardation.

A significant increase in body weight gains of growing mice injected with small amounts of thyroxine was reported by Koger *et al.* (80). The carcasses of the experimental mice contained more protein and water but less fat than those of the control mice. The total energy stored by the two groups was the same. A similar increase in body weight gains and also in skeletal growth was observed in mice fed suitable amounts of thyroactive iodocasein (81). Oral administration of iodinated casein over broad ranges of dosage produced no acceleration of the growth rate of rats, rabbits or guinea pigs (82),

except for slight increase in growth of female rats of the Missouri strain. The growth rate of mice was increased by iodinated casein administered either orally or by injection.

3. Effect on Feather Growth

The administration of large doses of thyroid substance or thyroxine to mature chickens (168–173, 98, 69) causes abrupt moulting of old feathers, and depigmentation of the new feathers which appear.

When more nearly physiologic doses of thyroactive iodinated protein were given continuously to growing chicks (112, 70, 156) there was a significant stimulation of feather growth above that of normal controls. The rate of feathering in various groups was in direct proportion to the dosage of iodinated protein, the most rapid feather growth being obtained with dosage levels high enough to depress the growth rate. Quite pronounced stimulation of feather growth (Fig. 9) was obtained on a dosage which permitted approximately normal gains in body weight.

Further evidence that the thyroid is concerned with feathering is provided by the reports that feather development is retarded by thyroidectomy (19) or thiouracil administration (36).

4. Effect on Egg Production

In a famous paper published in 1925, Crew (35) reported the rejuvenation of aged fowls by the administration of desiccated thyroid. In addition to the development of a younger type of plumage in both hens and cocks, there was an increase in egg production. Zawadowsky *et al.* (171) claimed that the egg production of certain hens was increased when 0.01 to 0.05 g. of desiccated thyroid was fed daily. Asmundson and Pinsky (9) found no increase in the egg production of hens fed 0.33 mg. of desiccated thyroid daily, although some changes in egg composition were reported.

Egg production was reduced markedly by thyroidectomy (154, 164).

It is known that egg production of hens is at a maximum during their first years of life, and declines thereafter at a rate of approximately 15% per year. It was believed possible that this decrease in production might be due to a diminishing rate of thyroid hormone secretion. In an attempt to arrest the decline in egg production with increasing age, Turner *et al.* (157) fed rations containing 5, 10 and 20 g. of thyroactive iodinated protein per 100 lbs. of feed to White Leghorn hens in their second year of production. On the 5 and 10 g. levels there was some increase in egg production above that of controls. Of particular interest, however, was the fact that the egg production of the experimental groups was maintained at the winter level during the hot weather of summer, while the controls showed the usual seasonal decline. Rhode Island Red pullets, in their first year of egg pro-

duction, receiving the optimal dosage of iodinated protein showed no increase above the controls during the winter (158). With the onset of hot

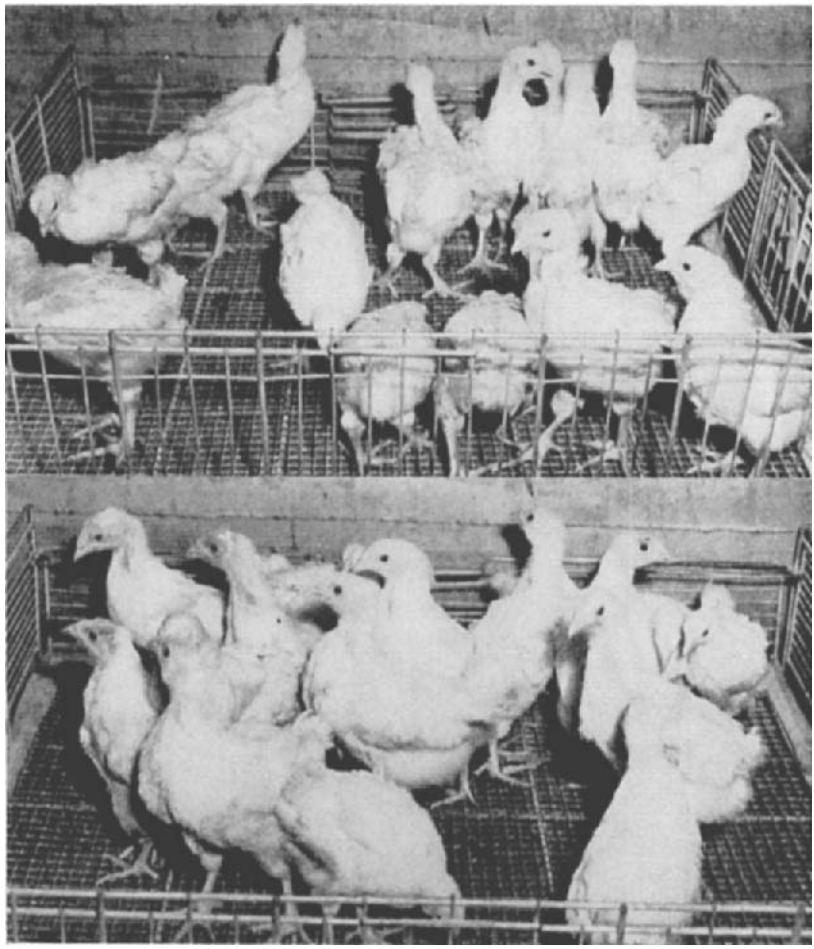

Fig. 9

The Effect of Thyroactive Iodinated Protein on Feathering in Young Chickens

Upper: Normal controls.

Lower: White Plymouth Rock chicks receiving 0.1% iodinated protein in their ration at the age of six weeks.

weather, the egg production of the controls declined while that of the experimental group was maintained for a time (Fig. 10).

The seasonal decline in normal egg production is believed to be associated with a diminished rate of thyroid hormone secretion during the summer

months. While seasonal variations in thyroid hormone output of hens have not actually been determined, a seasonal rhythm in the thyroid secretion rate of chicks has been observed (130).

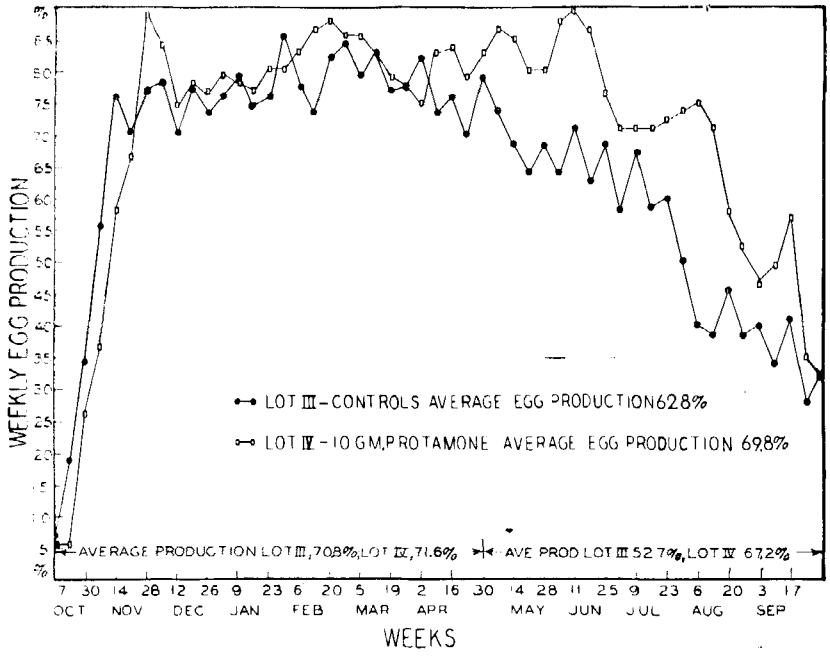

Fig. 10

Effect of Thyroactive Iodinated Protein on The Egg Production of Rhode Island Red Pullets

(From *Poultry Sci.*, (158))

X. Discussion and Summary

With the background provided by a half century of investigation on the iodination of proteins together with the continually broadening knowledge of the natural thyroid secretion, many of the early discrepancies and apparent contradictions can now be fitted into a fairly orderly pattern. Because of the many factors affecting both the combination of iodine with protein and the formation of thyroxine within the iodinated protein it is not surprising that the early attempts to produce an active substance resulted in failure.

By control of the factors now known to influence the formation of thyroxine in iodinated proteins it is possible to produce consistently preparations containing from 3 to 4% thyroxine as indicated by either chemical

analysis or biological assay (135). Although further investigation is needed to establish unequivocally that all of the material indicated by these measures is actually thyroxine, comparisons of results obtained by the two methods indicate a very close similarity to thyroxine in both chemical characteristics and biological activity. Subsequent to hydrolysis of thyroactive iodinated protein, yields of crystalline thyroxine far in excess of that actually present in U.S.P. thyroid can be obtained.

These artificial preparations are active when administered either orally or parenterally. No protein sensitization has been observed after parenteral administration of these nondescript iodinated proteins, probably because iodination causes a loss of antigenic specificity (166, 28, 79). Research is much needed on the digestion and absorption of these preparations, and also on their metabolism in the body tissues.

Although Abelin (4) as recently as 1942 still denied that intact iodinated proteins possessed complete thyroid activity, the bulk of the evidence now available indicates that preparations formed under properly controlled conditions will provide full replacement for the thyroid.

With the exception of the early work by Lerman and Salter (91, 143) no reports on the use of thyroactive iodinated proteins in clinical therapy have appeared. Clinical investigations with the more active products now available would be of great interest. In fact, such preparations have been reported to be free of the heart-stimulating factor claimed to be present in thyroid (99), and in this respect might afford some advantages over thyroid substance.

Only a few of the possible applications of thyroactive iodinated proteins to some of the problems of agriculture have been indicated in this review. It is fully established that lactation can be stimulated by the administration of a properly regulated amount of such material. The growth rate in certain species of animals is accelerated slightly, and the rate of feather growth in chickens is stimulated markedly by such treatment. Although the results still remain to be confirmed by other laboratories, it appears that the summer decline in egg production can be prevented in large part by feeding optimal amounts of iodinated protein. Although at present only in its beginning, the application of the thyroactive iodinated protein now available to the problems of animal physiology promises to be a profitable field for future investigation.

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