LV. THE ISOLATION OF L-THYROXINE FROM THE THYROID GLAND BY THE ACTION OF PROTEOLYTIC ENZYMES.

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IN previous communications by one of us [Harington, 1926, 1928] reference has been made to experiments with the object of isolating thyroxine from the thyroid gland by means of the action of enzymes alone. All these attempts had hitherto proved abortive, but the problem remained of such interest that it was determined to make a renewed attack upon it in a more systematic manner and upon a larger scale than had so far been attempted.

The problem seemed to us to present three main points of interest; in the first place the isolation of thyroxine through the unaided action of proteolytic enzymes would supply the final proof that the compound was present in the gland in peptide combination, provided that the possible objection that the isolation was rendered possible by the digestion of protein containing the thyroxine either in simple admixture or adsorbed could be dealt with on analytical grounds. Secondly, during the isolation of thyroxine by the action of enzymes alone no racemisation was to be anticipated, so that the determination of the optical rotation of the product would provide us with the information which we required as to the completeness of the resolution of *dl*-thyroxine already described [Harington, 1928]. In the third place the physiological investigation of the intermediate thyroxine-containing digestion products of the thyroid offered an opportunity of testing the correctness of the suggestion recently advanced [Harington and Randall, 1929] that any remaining discrepancy which appears to exist between the physiological activity of thyroid gland and that of an equivalent amount of thyroxine may be accounted for by an enhancement of the activity of the latter compound when it is in peptide combination.

One of the main difficulties which we anticipated was the loss of iodine as iodide which has been reported to occur in attempts to digest the thyroid protein with trypsin. Oswald [1908] has stated that in one experiment the whole of the iodine of a preparation of iodothyreoglobulin was thus liberated during digestion with an extract of pancreas for 3 weeks, and he has also

published experiments [1909] to show that a similar liberation of iodine occurs when 3 : 5-diiodotyrosine is subjected to the action of trypsin. One of us [Harrington, 1926] in the course of his earlier experiments on the isolation of thyroxine met with the phenomenon under discussion, although the liberation of iodine did not appear to be so extensive as was indicated by the results of Oswald.

As a preliminary step, therefore, we undertook some experiments concerning the action of various preparations of trypsin on diiodotyrosine, since the possibility was present to our minds that the liberation of iodine might not be due to the trypsin as such, but to some accompanying enzyme; we hoped in fact that it might prove possible to procure or to make a preparation of trypsin which, whilst retaining its proteolytic activity, would be free from the disadvantage that it also liberated iodine from compounds of this type.

To our surprise, however, with none of the preparations of trypsin which were at our disposal were we able to repeat Oswald's experiments. After 2-3 weeks of exposure to the action of trypsin under the conditions specified by him there was scarcely a trace of the effect which he describes, and, although on keeping the solutions for a period of months there was detectable a certain amount of liberation of iodine as iodide, this amount remained small, and we are inclined to ascribe the phenomenon to the effect of prolonged keeping in slightly alkaline solution, rather than to any action of the enzyme. We found, however, in the course of our experiments, that the technique of analysis was not so simple as at first appears. In the presence of nitric acid and silver nitrate even in the cold, the iodine of diiodotyrosine is liberated with comparative ease, and precipitated as silver iodide. In the experiments under discussion samples of the solutions were taken from time to time, and the state of affairs determined by treatment with nitric acid and silver nitrate to remove inorganic iodide, followed by filtration and determination of the organic iodine remaining in the filtrate. At times the filtration of the precipitate produced by silver nitrate in acid solution would be slow, and it was noticed that in such experiments the liberation of iodine appeared to have been greater; it was then found that by keeping the solution for different periods of time at the stage of precipitation with silver nitrate and nitric acid the result of the analysis could be varied almost at will. It is evident, therefore, that unless the analysis be carried out with the utmost care and rapidity the results obtained are of little value, and it seems to us not unlikely that the findings of the earlier investigator may be ascribed to an unsuspected error of technique of this description.

These experiments at any rate relieved us of a large part of our fear that the action of trypsin would be inevitably associated with serious losses of material through liberation of iodine, so long as the duration of the experiment were not unduly great. We could not indeed be certain that the conditions would be the same during the actual hydrolysis of a protein containing thyroxine (or diiodotyrosine) in peptide combination, or in the presence of

oxidases which might be contained in the gland itself; there was, however, no indication of the necessity of attempts to purify the proteolytic enzyme further, and it was decided to begin work on the enzymic hydrolysis of the thyroid protein, the general plan being to make the enzymic attack of as intensive a nature and of as short duration as possible.

About 30 years ago Hutchison [1896, 1898] described experiments in which he was able, as the result of the action of pepsin on "colloid" (presumably crude iodothyroglobulin) to obtain an acid-insoluble precipitate containing a considerably higher concentration of iodine than the starting material. The preparation of this product of peptic digestion appeared to us to offer a promising starting point for our experiments, more especially as preliminary peptic digestion is known to lay a protein more open to subsequent attack by trypsin.

As a source of material we have used, throughout the greater part of this work, chilled thyroid glands. These were obtained for us by the British Drug Houses in sealed tins, and were conveyed direct from the cold store to the National Institute for Medical Research, where, by the courtesy of Dr H. H. Dale, F.R.S., they were stored at -5° . The material was taken from the cold store as required, rapidly minced, and extracted with a slightly alkaline solution containing 1 % of sodium chloride; the extract, which contained practically the whole of the iodine, was either adjusted to p_H 1.5 and submitted directly to intensive peptic digestion, or the globulin was first separated by dilution or by heat-coagulation, and a suspension of the precipitate in dilute hydrochloric acid at p_H 1.5 was digested with pepsin. In the latter case the action of the pepsin brought about rapid solution of the greater part of the precipitate, and in all cases after 48–96 hours the peptic action was complete. At this stage, the reaction of the solution having been maintained at p_H 1.5 by periodic additions of hydrochloric acid, the amount of precipitate was very small; on reducing the acidity, however, a considerable increase in precipitation occurred, and indeed a sharp optimum precipitation point was observed at p_H 5.0. The precipitate so obtained was large in amount and contained about 40 % of the total iodine at a concentration of about 1 % of the dry weight; it was, however, heavily contaminated with fat. Attempts to remove the fat at this stage were unsuccessful owing to the almost permanent emulsions which were formed with ether or other fat solvents. The material was therefore dissolved as completely as possible in dilute sodium hydroxide, the reaction being finally adjusted to p_H 8.0, and was subjected to digestion with trypsin. In spite of our previously mentioned failure to observe any liberation of iodine as the result of the action of trypsin on diiodotyrosine, we had nevertheless in mind, for reasons already mentioned, the possibility that during the actual digestion of the protein conditions might be different. We therefore conducted the digestion as intensively as possible, employing large amounts of an active preparation of trypsin and arresting the reaction as soon as the curve representing the increase of amino-nitrogen

began to flatten out. This occurred after 72–96 hours, at which point the amino-nitrogen represented about 50 % of the total; the solution was diluted with water and adjusted to p_H 5.0 and the precipitate separated by filtration. It was found that at this stage a considerable amount of material of negligible iodine content could be rejected by extracting the precipitate with 70 % acetone containing some hydrochloric acid, nearly all the iodine passing into solution in this solvent and the larger part of the material being left undissolved. By adjustment of the reaction of the filtrate to p_H 5.0 and removal of the acetone by distillation the iodine-containing material was recovered. A fairly satisfactory removal of lipoids could now be effected by solution of the precipitate in dilute alkali followed by reprecipitation with simultaneous extraction with ether, and the residual material could be readily dried to a dark brown powder which usually contained about 15 % of iodine and 7 % of nitrogen.

It was resolved at this point to attempt to make use of the fact observed by Kendall [1919] that the pigmented substances of high molecular weight which are formed during the hydrolysis of the thyroid have a very low solubility in barium hydroxide. We therefore dissolved our material in a large volume of boiling water with the aid of the minimal amount of ammonia, and added barium hydroxide (hydrate) to make a concentration of 5 % of the latter; this produced a bulky precipitate, which was immediately filtered off from the hot solution; the filtrate, which was of a light brown colour, was adjusted to p_H 5.0 with hydrochloric acid. This caused an immediate precipitation, and further separation of material occurred on standing; after 48 hours the solid material was filtered off and dried, when it formed a light brown-coloured powder containing usually a little over 30 % of iodine and giving a very strong nitrous acid reaction for thyroxine.

Attempts to crystallise this material by dissolving it in 80 % alcohol containing sodium hydroxide and acidifying the hot solution with acetic acid resulted in no separation. When acetone was added to the acid solution, however, separation of a considerable amount of pigmented oily material of low iodine content occurred; after removal of the latter the clear liquid was poured into a large volume of water whereupon there slowly separated a cream-coloured product which, after drying, had an iodine content of 45–50 %, and a nitrogen content of about 5 %; since, as the result of a number of experiments, we arrived constantly at a product of approximately the same composition, in spite of the complexity of the process, we were at first inclined to believe that this substance, which, from the analytical figures, appeared to be a tri- or tetra-peptide containing thyroxine, represented a true end-product of tryptic digestion. It was possible to isolate thyroxine after hydrolysis of the material with a mixture of hydrochloric and acetic acids or with alkali, but we were unable to effect any hydrolysis by means of active erepsin preparations. In solubilities the substance differed markedly from thyroxine. Whereas thyroxine is practically insoluble in acid, the digestion product was

precipitated from its alkaline solution sharply and completely on adjustment of the reaction to p_H 5.0 but only incompletely on addition of excess of acid. It was soluble in glacial acetic acid and partly so in 50 % acetic acid, particularly when hot. It failed to separate on addition of acetic acid to its solution in alkaline 80 % alcohol. In 0.1 *N* sodium carbonate the material was moderately soluble, but when a hot concentrated solution was allowed to cool slowly a separation of a colourless sodium salt occurred; the latter was, however, not crystalline and did not have the characteristic appearance of the sodium salt of thyroxine.

Table I. *Course of purification.*

Yields and atomic nitrogen : iodine ratios at various stages.

Starting material	Peptic product	After trypsin and ether	After baryta	After acetone-alcohol purification
A. Iodothyreoglobulin 2.17 g. iodine	0.95 g. iodine N : I 49	0.2 g. iodine N : I 3.4	—	—
B. Iodothyreoglobulin 1.455 g. iodine	0.88 g. iodine N : I 57	0.34 g. iodine N : I 6.3	0.071 g. iodine N : I 1.8	—
C. Iodothyreoglobulin 1.81 g. iodine	0.91 g. iodine N : I 60	0.30 g. iodine N : I 5.3	0.068 g. iodine N : I 2.5	—
D. Iodothyreoglobulin 4.65 g. iodine	Not analysed	0.85 g. iodine N : I 4.4	0.42 g. iodine N : I 1.9	0.145 g. iodine N : I 1.0
E. Desiccated thyroid 3.34 g. iodine	1.44 g. iodine N : I 55	0.65 g. iodine N : I 4.2	0.4 g. iodine N : I 1.0	0.17 g. iodine N : I 0.8
F. Desiccated thyroid 3.77 g. iodine	1.78 g. iodine N : I 76	1.07 g. iodine N : I 6.0	0.57 g. iodine N : I 1.3	0.25 g. iodine N : I 1.15
G. Iodothyreoglobulin 11.3 g. iodine	4.8 g. iodine N : I 57	(a) 1.15 g. iodine N : I 5.6 (b) 0.78 g. iodine N : I 6.5	(a) 0.47 g. iodine N : I 1.9 (b) 0.31 g. iodine N : I 2.8	0.32 g. iodine N : I 1.1
H. Iodothyreoglobulin 11.0 g. iodine	8.2 g. iodine N : I 109	1.7 g. iodine N : I 11.4	0.9 g. iodine N : I 2.2	(After 2nd trypsin) (a) 0.22 g. iodine N : I 0.98 (b) 0.45 g. iodine N : I 1.1
K. Iodothyreoglobulin 2.06 g. iodine	No pepsin	0.2 g. iodine N : I 4.3	0.075 g. iodine N : I 1.3	0.028 g. iodine N : I 0.6
L. Iodothyreoglobulin 12.6 g. iodine	No pepsin	2.4 g. iodine N : I 4.1	1.5 g. iodine N : I 1.0	0.63 g. iodine N : I 0.6

Much time was spent in attempts to separate a pure peptide from this digestion product, until the experiments described in detail in the experimental part led to the isolation from it, as the result of simple methods of purification, of a small amount of crystalline thyroxine. The character of the digestion product was thus revealed in its true light as a mixture of free thyroxine with a preponderance of thyroxine still in peptide combination.

In Table I are given figures for the yields of iodine and for the atomic nitrogen : iodine ratios of the thyroxine-containing digestion product at various stages of three incomplete and seven complete preparations carried out by the method described above. The nitrogen : iodine ratios give a useful indication of the extent of digestion at the various stages, and it will be seen that, except in preparation H where the early stages of the digestion were

peculiarly inefficient, the figures show a remarkable degree of constancy. If we multiply these ratios by 4 we obtain the nitrogen : thyroxine ratios, and it may then be seen that the product after trypsin and ether treatment has the approximate composition of a thyroxine peptide containing 19 other amino-acids: after purification with baryta the product gives approximate figures for a hepta-peptide, and after the acetone-alcohol purification for a tri- or tetra-peptide containing thyroxine. All these deductions are naturally based on the assumption that no diamino-acids are present; the markedly acidic properties of the product indicate that this assumption, for the more highly purified material at least, is probably a fair one.

Table II shows the percentage of iodine in the thyroxine-containing digestion product at three significant stages of purification in the various preparations.

Table II.

Percentage of iodine in thyroxine-containing fraction at various stages of digestion and purification.

Preparation	After trypsin and ether	After baryta purification	After acetone-alcohol purification
A	15.3	—	—
B	—	29.1	—
C	2.2*	23.5	—
D	15.0	32.4	42.0
E	17.1	41.7	47.6
F	12.4	40.6	49.8
G	(a) 13.8	(a) 32.0	43.0
	(b) 11.9	(b) 29.0	
H	8.1	30.2	(a) 45.3† (b) 42.0†
K	15.7	38.1	49.8
L	10.2	43.8	56.5

* No ether.

† After 2nd tryptic digestion.

In Table III are recorded the figures for the percentage of iodine and of total and amino-nitrogen in the more highly purified fractions of two preparations. The figures to which we wish to call special attention here are those in the last column, which represent the ratio of amino-nitrogen to thyroxine in the product. The determinations of amino-nitrogen were made by the colorimetric method of Folin [1922] on small amounts of material, and no very high degree of accuracy is claimed for them; it is, however, sufficiently obvious that the ratio approximates to unity; that is to say, that there is in the product one free amino-group for every molecule of thyroxine. Such an analytical result is consistent with the supposition that the material is composed exclusively of peptides containing thyroxine, or of thyroxine mixed with thyroxine-containing peptides; it could not, however, be obtained from a mixture of free thyroxine with peptide material of a different character. As collateral evidence in favour of the hypothesis that thyroxine is present in the thyroid in actual combination these analyses are therefore of value. More direct evidence in this direction is afforded by the fact that on prolonged acid hydrolysis of the digestion product, practically the whole of the nitrogen was

obtained in the amino-condition. It appears therefore that there is no longer any room for doubt that thyroxine does exist in the thyroid gland in peptide combination—as a constituent amino-acid, in fact, of the characteristic thyroid protein, iodothyreoglobulin.

Table III.

Detailed analysis of partly purified digestion products.					
Material		Iodine %	Total nitrogen %	Amino-nitrogen %	<u>Amino-nitrogen thyroxine</u>
Preparation H. After baryta purification		30.2	7.42	0.97	1.2
Preparation H. After acetone purification	(a)	45.3	4.92	1.26	1.01
Ditto	(b)	42.0	5.2	1.01	0.88
Preparation K. After baryta purification		38.1	4.8	1.3	1.3
Preparation K. After acetone purification		49.8	3.4	1.34	0.97

The amount of crystalline thyroxine which could be separated from the digestion product was very small, and since the digestion product itself, as the result of large losses in the course of preparation, represented only a minute fraction of the original iodine, it was not possible to obtain more than the smallest amounts of thyroxine by this method. Sufficient was, however, isolated to permit of satisfactory determinations of optical activity with the aid of a Fischer micropolarimeter tube; these determinations indicated that the thyroxine was laevorotatory and had in fact a specific rotation of approximately $[\alpha]_{5461} - 3.8^\circ$ as the sodium salt in 60 % alcoholic solution; this figure is in close agreement with that obtained for the *l*-thyroxine previously prepared [Harington, 1928] by the resolution of *dl*-3 : 5-diiodothyronine and subsequent iodination of the product. The resolution at that time described must therefore be assumed to have been moderately complete, and the suggestion, made on the basis of the relative physiological activity of the two enantiomorphs, that *l*-thyroxine would prove to be the naturally occurring variety, is confirmed. At the same time this result indicates, in view of the observations of Gaddum [1930] regarding the relative physiological activity of the two enantiomorphs, that no very great discrepancy between the activity of thyroid gland and that of thyroxine isolated therefrom by alkaline hydrolysis can be accounted for by racemisation alone.

This brings us back to the third point of interest of the present work, namely the physiological comparison of the digestion product, containing thyroxine for the most part still in peptide combination and containing no iodine compound other than thyroxine, with free thyroxine itself. This comparison was kindly carried out for us by Dr J. H. Gaddum. The substances were tested on rats and their effects were compared after administration both by the oral and the subcutaneous route. The oral administration of 1 mg. *per diem* for 6 days of pure *l*-thyroxine had practically no effect on the oxygen

consumption of a rat, whilst an equivalent dose of the digestion product (1.3 mg. *per diem* for 6 days of a preparation containing 50 % of iodine) produced an increase in the oxygen consumption of 85 %. By the subcutaneous route, on the other hand, single doses of 1 mg. of *l*-thyroxine and 1.3 mg. of the digestion product caused increases in the oxygen consumption of 32.5 % and 37.5 % respectively; that is to say, in this case the physiological difference between the two substances was very much less marked.

This experiment appears to us to afford an admirable demonstration of the causes which have led various workers to form what we believe to be a false conclusion, namely that the physiological activity of the thyroid gland cannot be entirely accounted for by the thyroxine which it contains. It is indeed quite unjustifiable to deduce any such discrepancy from an apparent difference between the effects of thyroxine and thyroid gland when given by mouth or otherwise. The physical properties of thyroxine are such as to make it highly probable that the absorption of this substance after oral administration would be inefficient and erratic; the digestion product, on the other hand, possessing as it does a much wider range of solubility, might well be absorbed almost quantitatively; such a state of affairs is precisely what is indicated by the first part of the experiment of Dr Gaddum which has just been described. For a similar reason it is equally unjustifiable to attempt to make a comparison between the effect of thyroxine and that of thyroid gland or iodothyreoglobulin when both are injected subcutaneously. In view of the slowness with which the physiological effect of thyroxine develops, it is almost a foregone conclusion that after an injection of a massive dose of the substance a portion will be lost by excretion before it has time to exercise its action on the organism. On the other hand, it is known that the processes involving the removal of a foreign protein from the body after its subcutaneous injection may take a number of days; after the subcutaneous injection of iodothyreoglobulin therefore we should anticipate a more or less prolonged period during which the active principle of the protein would be slowly liberated, thus attaining a therapeutic efficiency greatly in advance of that achieved by injection of a single equivalent dose of free thyroxine. In the last sentence we have used the term "active principle" of the iodothyreoglobulin rather than thyroxine because it appears to us to be probable that the actual substance which the thyroid secretes into the blood may not be free thyroxine but a relatively simple peptide containing this compound—a substance in fact analogous to our final digestion product. This conclusion is supported by the results of recent experiments of Dr Reid Hunt in Boston in which a sample of the digestion product containing approximately 50 % of iodine, from which as much free thyroxine as possible had already been separated, has shown itself to be certainly 250 and possibly even 500 times as active as U.S.P. standardised desiccated thyroid, both substances being administered orally. The digestion product when administered intravenously was approximately as active as when given by mouth. These results were obtained

in mice by the acetonitrile method. Dr J. H. Means of Boston has also studied the calorogenic action of this digestion product when given by mouth to patients with a sub-normal basal metabolic rate; although his controls, which consist in observing the effect of U.S.P. standardised thyroid in the same patients, have not yet been completed, it is already apparent that the digestion product is fully as effective as an equivalent dose of thyroid and very likely more so. The U.S.P. standardised thyroid contains about 0.2 % of iodine, so that the results which have just been quoted mean that, in order to produce a given physiological effect, at least as much and probably more iodine must be given in the form of thyroid than in the form of the digestion product which contains no iodine compound other than thyroxine. This is precisely what our recently acquired knowledge of the chemistry of the thyroid would lead us to expect. There remains, therefore, in our opinion no reason whatever to presume the existence in the thyroid gland of an active principle other than thyroxine, or that the thyroxine in the thyroid exists in an "activated" form except in so far as owing to its optical activity and possibly also to the fact that it is in peptide combination, it can produce an effect greater than that of the free racemic compound. Indeed, taking into account the errors of the physiological method and the essential difficulties of obtaining a therapeutic comparison to which we have called attention, it is a matter for surprise that even an approximate parallelism can be observed between the action of thyroid gland or iodothyreoglobulin or an equivalent amount of free *l*-thyroxine.

EXPERIMENTAL.

A. *Action of trypsin on diiodotyrosine.*

These experiments were undertaken to test the statements of Oswald regarding the hydrolysis of the iodine of diiodotyrosine by trypsin, and to obtain an indication of the extent to which this reaction was likely to prove a serious obstacle in the way of the isolation of thyroxine by enzymic action.

A solution of diiodotyrosine (prepared from *l*-tyrosine) containing 0.5 mg. of iodine per cc. was buffered with phosphate and adjusted to p_H 8.0; 100 cc. samples of this solution were treated with active preparations of trypsin (two commercial samples and one fresh pancreatic extract), toluene being added to inhibit bacterial action, and were placed in an incubator at 40°. Portions of the solutions were withdrawn at intervals and analysed as follows: cold boiled-out nitric acid and silver nitrate were added, and, after 15 minutes, the solution was filtered through asbestos; the filtrate and washings were freed from silver by addition of hydrochloric acid; after removal of silver chloride the solution was made alkaline, evaporated, and analysed for iodine by the method of Kendall [1914]; the figure obtained was taken to represent the iodine remaining in organic combination, any iodide which had been formed having been removed by the preliminary treatment with silver nitrate and nitric acid.

Results of analyses carried out in this manner indicated no significant alteration in the organic iodine after 1, 2 and 3 weeks' digestion respectively. The solutions were then left undisturbed for 5 months and again analysed. The organic iodine was now found to be diminished to about 80 % of the total iodine, but this diminution was just as marked in the control solution, to which no enzyme had been added, as in those containing active trypsin. It was evident, therefore, that no liberation of iodine as iodide could be ascribed to the action of the trypsin as such. In the effort to account for the discrepancy between our results and those of Oswald, we studied the conditions of the analysis more closely, and found, in fact, that increase either in the amount of nitric acid employed (even though the latter were freshly boiled out) or in the length of time allowed for the precipitation in presence of nitric acid, caused an apparent diminution in the proportion of organic iodine. It is known that the iodine of diiodotyrosine is removed with extreme ease when the compound is warmed with silver nitrate and nitric acid; there is little doubt in our minds but that this reaction takes place also to some extent in the cold, and that an error of this type accounts at least in part for the results obtained by Oswald. There exists the further possibility that the enzyme preparations employed by him contained oxidases which were absent in our experiments, and which may have brought about some destruction of the diiodotyrosine.

B. *Enzymic hydrolysis of thyroid gland.*

The early stages of the digestion (leading to the material containing about 15 % of iodine) were carried out by a variety of methods, of which typical examples are described.

(a) *Successive action of pepsin and trypsin on iodothyreoglobulin.* Frozen thyroid gland (5400 g.) was passed rapidly through a mincer and suspended in 5400 cc. of 0.02 % sodium hydroxide containing 1 % of sodium chloride; the suspension was shaken for 3 hours and allowed to stand overnight; it was then strained through butter-muslin and the solid residue was submitted to a second similar extraction. The combined reddish-coloured extracts were chilled and as much fat as possible was removed mechanically. The solution was adjusted to p_H 5.0, treated with sodium sulphate to make 1 % concentration of the latter, and raised rapidly to boiling-point by introduction of a vigorous current of steam with simultaneous external heating. The coagulum of crude iodothyreoglobulin, which contained about 97 % of the total iodine of the extract, was filtered off hot through large Büchner funnels, and the cheesy cakes of material so obtained were suspended in 10 litres of water containing 0.5 % of an active pepsin preparation and sufficient concentrated hydrochloric acid (in this case 240 cc.) to bring the reaction of the solution to p_H 1.6. The mixture was transferred to large bottles which were placed in an incubator at 40°, the contents being frequently shaken during the early stages of the digestion. After 48 hours fresh pepsin (the same amount as

originally employed) was added, the reaction being adjusted to p_H 1.6 by the further addition of hydrochloric acid if necessary. After a further 48 hours the solution was diluted with an equal volume of water and the reaction was adjusted by the cautious addition of sodium hydroxide, vigorous mechanical stirring being employed to obtain efficient admixture; at p_H 5.0 a sharp flocculation was obtained. After standing for a few hours the mixture was poured on to large pleated filters and allowed to drain; filtration was slow and no pretence at efficient washing could be made. The bulky greasy precipitate, containing about 1 % of iodine reckoned on the dry weight and about 40 % of the total iodine of the iodothyreoglobulin, was scraped off the filters and suspended in water (2370 cc.); sodium hydroxide was added until the reaction was p_H 8.0 and the greater part of the material had passed into solution; 0.2 % of an active preparation of trypsin was then added and the material was transferred to the incubator. The reaction was readjusted to p_H 8.0 twice daily by addition of the necessary amount of sodium hydroxide; daily determinations of the amino-nitrogen concentration were made by the Van Slyke method. After 72 hours a second addition of 0.2 % of trypsin was made. At the end of 96 hours from the beginning of the digestion, the rate of increase of the amino-nitrogen (which had by this time reached about 50 % of the total nitrogen) had become very low; as we were anxious so far as possible to avoid the chance of loss of iodine, the digestion was interrupted at this stage by bringing the solution to p_H 5.0 by the addition of hydrochloric acid. The precipitate was again filtered off on pleated filters and washed as well as possible with water; it was then extracted three times in succession with 70 % acetone to which was added enough hydrochloric acid to keep the reaction about p_H 1.6; the bulky insoluble matter was filtered off and the filtrates were immediately brought back to p_H 5.0 with sodium hydroxide. The acetone was then removed by distillation, diminished pressure being used in the last stages. The residual aqueous liquid contained a dark-coloured precipitate of somewhat oily consistency; on addition of sodium hydroxide all passed into solution, and the alkaline liquor was transferred to a beaker and covered with a layer of ether. While the two layers were kept thoroughly mixed by vigorous mechanical stirring, hydrochloric acid was cautiously added until the p_H was again 5.0; the mixture was allowed to stand, when it separated into a brown aqueous layer and an almost black ethereal layer with a dark precipitate at the interface; the latter was filtered off and washed with ether, when it amounted to 5.66 g. of a dark brown powder containing 15.0 % of iodine and 7.28 % of nitrogen.

(b) *Successive action of pepsin and trypsin on alkaline sodium chloride extract of thyroid gland.* In some experiments the isolation of the crude iodothyreoglobulin by heat coagulation was omitted, and the solution obtained by extraction of the gland material with 0.02 % sodium hydroxide containing 1 % sodium chloride, after freeing as far as possible from fat, was adjusted to p_H 1.6 by addition of hydrochloric acid and submitted directly to peptic

digestion, followed by the treatment described above. In one such experiment, 9.5 kg. of frozen thyroid gave 16 g. of material containing 13 % of iodine and 8.5 % of nitrogen.

(c) *Successive action of pepsin and trypsin on desiccated thyroid gland.* Desiccated and finely powdered thyroid gland (725 g.), which had been defatted with light petroleum, was suspended in 7600 cc. of water to which had been added 0.5 % of pepsin and 155 cc. of concentrated hydrochloric acid; the pepsin was renewed after 36 hours and the digestion was arrested at the end of 72 hours; the reaction of the solution was brought to p_H 5.0 and the resulting precipitate was submitted to tryptic digestion and worked up as described above; there was obtained 3.8 g. of material containing 17.1 % of iodine and 8.0 % of nitrogen.

(d) *Action of trypsin only on alkaline sodium chloride extract of thyroid gland.* The extract obtained in the usual manner from 7840 g. of frozen thyroid was submitted directly to digestion with 0.2 % of trypsin, toluene being added to inhibit bacterial action; the trypsin was renewed after 48 hours, the reaction being maintained at p_H 8.5 by periodic additions of sodium hydroxide and at the end of 76 hours the solution was brought to p_H 5.0; the precipitate was then extracted with 70 % acid acetone and the extracted material reprecipitated in presence of ether as already described; there was obtained a precipitate, a sample of which showed an iodine content of 8.3 % and nitrogen 7.8 %; this was subjected to a second similar tryptic digestion, the volume being at first 1200 cc. and being raised after 24 hours to 2200 cc.; after the second addition of trypsin the p_H of this digest, which had fallen spontaneously to 7.6, was allowed to remain at this figure for a day, with the intention of favouring the action of any dipeptidase which might be present in the enzyme preparation; the p_H was then raised to 8.0 again at which it remained unchanged despite the further addition of trypsin; after a total digestion period of 84 hours the material was again precipitated at p_H 5.0, and now contained 10.2 % of iodine and 4.7 % of nitrogen. As might be expected the ethereal layer in all the above-described experiments, being heavily loaded with lipoidal substances, carried a certain amount of iodine-containing material into solution; a large proportion of this separated out on keeping the solution. Thus in one experiment the ethereal layer contained, immediately after separation, 130 mg. of iodine; three weeks later a further precipitate had separated and the iodine content of the solution was 74 mg.; still more material continued to separate after this.

The digestion product obtained by any of the methods described above formed a dark brown or almost black powder which was readily soluble in alkali and much less so in acid; it had a well defined point of maximum flocculation at p_H 5.0; it contained usually 12–17 % of iodine and 5–8 % of nitrogen; that is to say, it had approximately four atoms of nitrogen for every one atom of iodine, or 16 nitrogen atoms for every molecule of thyroxine.

The next stage in the purification of the product was the removal of the

pigmented impurities of relatively high molecular weight by making use of the insolubility of their barium salts. The conditions for this experiment were selected with a view to attainment of the maximum salting-out effect of the barium ions consistent with the exclusion of the possibility of hydrolysis. With this object the concentration of barium hydroxide was not raised above 5 % and the time of exposure even to this small concentration was cut down to a minimum. An example of the procedure may be given as follows. A sample of the tryptic digestion product containing 14 % of iodine and 8.5 % of nitrogen, and representing in all 1160 mg. of iodine, was dissolved in 1200 cc. of water with the aid of the minimum quantity of ammonia; the solution was brought to the boil and treated with 60 g. of crystalline barium hydroxide dissolved in 120 cc. of hot water; boiling was continued for 2 minutes, and the heavy precipitate was filtered off. Filtration was rapid, and the filtrate, whilst still hot, was acidified with concentrated hydrochloric acid; addition of excess of the latter caused partial re-solution of the precipitate; the reaction was therefore brought back to p_H 5.0 by the addition of sodium acetate. After standing for 48 hours at the ordinary temperature the precipitate was filtered off and dried. It contained 468 mg. of iodine at a concentration of 32 % and the nitrogen content was 6.3 %, corresponding to a ratio of 1.86 atoms of nitrogen to 1 atom of iodine. Although therefore the loss of iodine at this stage was heavy, the improvement in the quality of the product far outweighed this disadvantage. The product at this stage was a buff-coloured powder which gave the nitrous acid reaction for thyroxine with intensity. It was easily soluble in alkali, but insoluble in water at p_H 5.0 and only partly soluble at more acid reactions. When it was dissolved in 80 % alcohol with the aid of sodium hydroxide and when the solution was acidified with acetic acid no separation took place; acetone was added to such a solution in the hope of inducing the separation of any free thyroxine which might be present, and it was found that this treatment produced a precipitate of gummy material which contained relatively little iodine and much nitrogen. This observation led to the development of the next stage in the purification. Thus in one experiment 1.48 g. of material resulting from a treatment with barium hydroxide as already described, containing 40.7 % of iodine and 6.0 % of nitrogen (nitrogen : iodine = 1.34), was dissolved in 10 cc. of *N* sodium hydroxide and the solution was then made 80 % with respect to alcohol; a small precipitate was removed by centrifuging and the mother liquor was acidified by the addition of 4.5 cc. of 33 % acetic acid; 4 volumes of acetone were then added and the solution was rapidly centrifuged; the combined clear and light yellow mother liquor was diluted with 1500 cc. of water. Within a few minutes the opalescent solution began to deposit a light flocculent precipitate which gradually increased in amount. After 24 hours this was filtered off and amounted to 0.46 g. of material containing 49.8 % of iodine and 6.3 % of nitrogen; the nitrogen : iodine ratio was therefore 1.15 and the nitrogen : thyroxine ratio 4.6. After standing for

3 days longer a further crop of 71 mg. of material containing 46 % of iodine separated.

The fact that the product at this stage still exhibited marked differences in solubility from thyroxine, although, judging by the iodine content, it contained over 75 % of the latter, led us for a long time to suppose that we were dealing with a somewhat impure compound of thyroxine (probably a tripeptide) rather than with a mixture of free thyroxine with impurities; we were supported in this view by the constancy with which we arrived at a closely similar end-product in spite of the complexity of the preceding manipulation. Finding ourselves unable to achieve any further hydrolysis either by the aid of animal erepsin preparations of proved activity or with the help of yeast peptidases, we had recourse to acid hydrolysis of this product and also of material at an earlier stage of purification in the attempt to isolate thyroxine. We were in fact successful in obtaining thyroxine by hydrolysis with a mixture of hydrochloric and acetic acids at 100°, and we were able to show that during this process the whole of the nitrogen was converted into the amino-condition. Nevertheless the losses involved by the liberation of iodine were severe and, as will be shown later, the thyroxine obtained by this method was partly racemised.

Much effort was then expended in the attempt to obtain our supposed tripeptide in a pure condition, and it was during these experiments that we stumbled on the true explanation of the situation, namely that the purified digestion product consisted of a mixture of free thyroxine with thyroxine peptides.

The substance approximated more closely than the earlier products to thyroxine in that it had almost completely lost its solubility in excess of mineral acid. It still failed to separate, however, when acetic acid was added to its solution in alkaline 80 % alcohol. It was soluble in glacial acetic acid from which it could be precipitated with ether, without, however, effecting any significant purification; in 50 % acetic acid it could be dissolved by boiling and a part separated on cooling; on some occasions this less soluble portion seemed to show signs of commencing crystallisation, but a second separation from acetic acid failed to improve it to any significant extent, although fractions could be obtained in this manner which showed a somewhat higher iodine : nitrogen ratio. It was then decided to try and utilise the low degree of solubility of the product in cold 0.1 *N* sodium carbonate. This property, which has been found to be a general characteristic of the acidic compounds of the thyroxine series, appeared in the substance under discussion for the first time among the various products of digestion of the thyroid, the thyroxine-containing fraction at all earlier stages showing no tendency to separate from a concentrated solution in sodium carbonate. Very concentrated solutions of the material containing 45–50 % of iodine could be prepared in boiling 0.1 *N* sodium carbonate, and if such solutions were rapidly cooled they not infrequently formed stiff gels. By slow cooling, however, the separation of a sodium salt could be induced, and although the concentrated solution was

rather dark in colour, the sodium salt, whilst not yet crystalline, was almost colourless. Moreover the material recovered after separation of the sodium salt on the centrifuge, and acidification of its solution in hot water, was definitely less soluble in acetic acid. A second separation from sodium carbonate at this stage involved heavy loss of material without corresponding improvement in quality; we therefore made use of the fact, which we had observed, that the substance could be precipitated from its solution in pyridine by dilution with water. A fairly concentrated solution in pyridine was prepared, and was diluted with boiling water to permanent turbidity; on cooling, a precipitate separated which showed a tendency to adhere to the walls of the tube where the latter was scratched with a glass rod, and which, under the high power of the microscope, showed commencing crystallisation; this material was separated on the centrifuge and dissolved in boiling 0.1 *N* potassium carbonate; the white precipitate which separated when the solution was kept for some hours at 0° was decomposed with acetic acid, and the product, after being washed with water on the centrifuge, was again dissolved in boiling dilute sodium carbonate solution; this time, as the solution cooled, precipitation of a heavy white microcrystalline substance occurred, which had the characteristic appearance of the mono-sodium salt of thyroxine. This salt was separated on the centrifuge, and dissolved in 80 % alcohol with the aid of sodium hydroxide; the boiling solution was acidified with 33 % acetic acid when rapid separation of typical crystals of thyroxine occurred.

The thyroxine so obtained melted at 235° with decomposition; this melting point was not changed by admixture with synthetic *l*-thyroxine which also melted at 235°. A mixture of synthetic *d*- and *l*-thyroxine sintered at 228° and melted at 231–232°.

The amount of thyroxine isolated from the digestion product was so small as to necessitate the use of a Fischer micropolarimeter tube for the determination of its specific rotation. A tube manufactured by Messrs Schmidt and Häntzsch was employed and considerable care was taken, by preliminary experimentation with known substances, to acquire a reliable technique. No difficulty was experienced in obtaining consistent readings, although there was a constant small difference between the readings of two observers. In order so far as possible to control this source of variation, determinations were made by the two observers on synthetic *l*-thyroxine as well as on the product obtained from the thyroid, the thyroxine in both cases being dissolved at approximately 5 % concentration in a mixture of 2 volumes of alcohol and 1 volume of *N* sodium hydroxide. The results are given in Table IV, from which it will be seen that, within the limits of error of the experiment, no difference could be detected between the synthetic and natural product; moreover, the mean of the four determinations is in close agreement with the value previously obtained, using a large polarimeter tube, for the synthetic *l*-thyroxine, viz. — 3.5°¹ [Harington, 1928].

¹ Cf. note to Table IV.

Table IV.

	Natural thyroxine ($c=4.4$)	Synthetic L-thyroxine ($c=4.45$)
Observer	$[\alpha]_{5461}$	$[\alpha]_{5461}$
C.R.H.	-4.45°	-4.5° (-3.5°)*
W.T.S.	-3.2°	-3.15°
Mean	-3.8°	-3.8°

* The results previously recorded [Harington, 1928], for the specific rotations of *d*- and *L*-thyroxine were erroneously calculated on the concentration by *weight* instead of by volume. The figure given here in brackets represents the earlier value corrected for the specific gravity of the solution in order to make it comparable with the present results.

Isolation of thyroxine by acid hydrolysis of digestion product.

Before we had realised the possibility of separating free thyroxine from the digestion product by ordinary methods of purification, we attempted to obtain optically active thyroxine by acid hydrolysis. The material was dissolved in 90 % acetic acid which was saturated with hydrogen chloride at 0° , and the mixture was heated on the steam-bath in a sealed tube for 6 hours. The solution was evaporated to dryness under diminished pressure and the residue was dissolved in 60 % alcohol with the aid of a little hydrochloric acid. Sodium hydroxide solution was added to bring the reaction to p_H 5.0, and, after standing overnight, the precipitate was filtered off, washed with water, and dissolved in the minimum amount of boiling 0.5 % sodium carbonate. The sodium salt of thyroxine, which separated on cooling, was converted into the free amino-acid in the usual manner. The thyroxine so obtained had $[\alpha]_{5461} - 1.3^\circ$ ($c = 4.88$ in 60 % alcohol containing sodium hydroxide); considerable racemisation had therefore taken place. The hydrolysis was accompanied with some decomposition, free iodine being present at the end of the reaction and the yield of thyroxine was therefore poor. In the solution left after separation of the thyroxine, 66 % of the nitrogen was in the amino-condition; after further hydrolysis the proportion of amino-nitrogen was increased to 84 % of the total; there seems therefore no reason to doubt the peptide nature of the digestion product.

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