THE IODINE COMPOUNDS OF THYROID AND PLASMA STUDIED BY COLUMN CHROMATOGRAPHY

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Summary

A system of column chromatography suitable for the separation of the biologically important iodine compounds is described. Analysis of hydrolysed thyroid tissue shows the presence of thyroxine, 3,5,3′-tri-iodothyronine (TIT), 3,5-di-iodotyrosine (DIT), mono-iodotyrosine (MIT), and other unidentified iodine-containing compounds. The relative abundance of thyroxine and TIT present as free amino acids in the thyroid is different from that of the same amino acids in protein combination. The amount of free thyroxine is less than the free TIT, whereas in the combined amino acids thyroxine is in excess of TIT.

TIT was not found in the plasma of the rat except when radiation damage to the thyroid gland had occurred. Under these circumstances it was accompanied by MIT and DIT and an iodine-containing protein presumed to be thyroglobulin.

I. INTRODUCTION

Recent knowledge of the iodine compounds of the thyroid has been reviewed by Roche and Michel (1954) and Gross (1954). Under normal conditions most of the iodine in the thyroid gland is in the form of thyroglobulin, which contains the iodinated amino acids thyroxine, 3,5,3′-tri-iodothyronine (TIT), 3,5-di-iodotyrosine (DIT), and (MIT). These same four compounds also occur as free amino acids in the thyroid gland presumably arising from proteolytic breakdown of thyroglobulin.

Barker (1955) has reviewed the literature on the nature of the iodine compounds of plasma. Thyroxine loosely bound to protein is the major organic iodine compound in the plasma. TIT has been reported as a plasma constituent but is not a consistent finding. Neither DIT nor MIT appear to be normal constituents of plasma according to recent investigations. Iodine compounds not normally present in plasma may be released from the thyroid when this structure has suffered radiation damage from excessive doses of ^{131}I.

An iodine-containing protein was found in the plasma of rats after destructive doses of ^{131}I by Tong, Taurog, and Chaikoff (1952), and similar material appearing in human plasma was shown to be thyroglobulin by Robbins, Peterman, and Rall (1954) using physicochemical methods. Benua and Dobyns (1955) found DIT and MIT in the plasma of patients receiving large doses of ^{131}I and ascribed their presence to radiation damage.

The appearance of thyroglobulin in plasma after radiation damage to the thyroid makes it likely that any thyroid constituent could appear in plasma under the same circumstances. We have therefore investigated the nature of the iodine compounds present in thyroid and plasma and the time course of the changes induced by thyroid-damaging doses of ^{131}I in the rat. A preliminary account of this work has been published (Kennedy 1953).

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II. Materials and Methods

The iodine-containing compounds were separated by column chromatography with kieselguhr-supported sodium hydroxide solution as the stationary phase and *tert.*-butanol–*cyclo*hexane mixtures, previously equilibrated with 2N sodium hydroxide, as the mobile phase. For most separations, 5 g of kieselguhr were mixed with 3·5 ml of 2N sodium hydroxide solution and packed into a column 12 mm in diameter.

The column was started with a 70:30 (v/v) mixture of *tert.*-butanol and *cyclo*hexane and run until the thyroxine had emerged, when an 80:20 (v/v) mixture of the same solvents was applied to elute the TIT. A further change to a 90:10 (v/v) mixture eluted the iodide, after which DIT and MIT were eluted with a 90:10 (v/v) *n*-propanol–*cyclo*hexane mixture and *n*-propanol respectively. Approximately 75-ml quantities of each solvent were required.

The rats used were a Wistar strain, weight 150–200 g, and were fed with a low iodine diet. Rats maintained on this diet always have hyperplastic thyroids. This increased activity of the thyroid can be prevented by the addition of enough potassium iodide to the drinking water to supply the animals with 1–2 μg iodide daily. ^{131}I was administered in the form of iodide to produce *in vivo* labelling of the iodine compounds of the thyroid gland and hence of thyroid secretion. Preliminary tests with 5 μc doses of ^{131}I showed a 60–80 per cent. uptake in the animals used. Blood samples were taken by heart puncture and the blood centrifuged immediately. The plasma was kept frozen until required.

Plasma, 0·2–0·3 ml, was mixed with 0·1 ml of 10N sodium hydroxide, 0·5 g of kieselguhr added and made into a slurry with 3–5 ml of the mobile phase and packed on top of a column prepared as already described. The free amino acids of the thyroid were examined by grinding the thyroid with 0·2 ml of 3N NaOH, or heating the mixture in a boiling water-bath for 1 min and placing the solution on the column as for plasma.

The effluent from the column was run through an F10 liquid-flow counter (Twentieth Century Electronics) and collected in approximately 3-ml fractions. The radioactivity was measured with a rate-meter and recorded on a recording potentiometer.

Where necessary, carrier amino acids and iodide, about 1 mg of each, were added to the material to be analysed before it was placed on the column. The carrier amino acids were found in the effluent by measuring the absorption at 300 mμ. The iodide was located by addition of a few drops of a reagent made by mixing equal volumes of 6 per cent. hydrogen peroxide and 2N sulphuric acid, to which a small crystal of sodium tungstate was added. The iodine formed was estimated colorimetrically. The commercially-available grades of *tert.*-butanol, *n*-propanol, and *cyclo*hexane were suitable for absorption measurements without preliminary purification.

The radioactivity of the separated substances was measured by gamma counting after evaporation of the pooled fractions to a volume of 3–4 ml. Sufficient counts were made to ensure an accuracy of $\pm$1·8 per cent. or less.

III. Results

The chromatographic system gave an adequate separation of a mixture of thyroxine, TIT, iodide, DIT, and MIT which emerged in that order as shown in Figure 1. 3,5-Di-iodothyronine was not completely separated from iodide by this system.

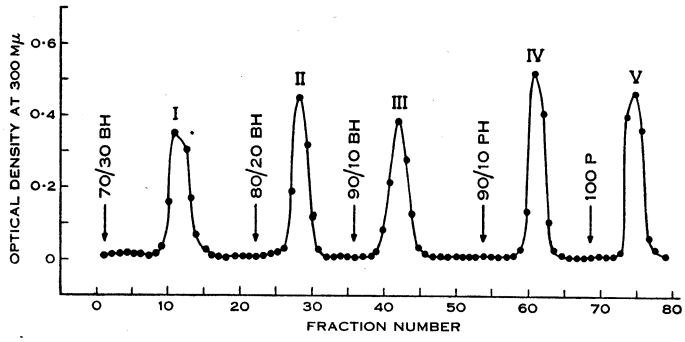

Fig. 1.—Separation of a mixture of thyroxine (I), tri-iodothyronine (II), iodide (III), di-iodotyrosine (IV), and mono-iodotyrosine (V). Solvent changes were made at the points indicated. BH, *tert.*-butanol–*cyclo*hexane (v/v) mixture; PH, *n*-propanol–*cyclo*hexane (v/v) mixture; P, *n*-propanol.

(*a*) *Total Iodo-amino Acids of the Thyroid*

When applied to the separation of the labelled iodine compounds of hydrolysed rat thyroid tissue, a satisfactory separation of the above compounds was obtained by this method. The thyroids were removed 24 hr after the administration of radioactive iodine and were hydrolysed with 2N NaOH for 16 hr at 100°C. Chromatography showed the presence of thyroxine, TIT, iodide, DIT, and MIT, and smaller amounts of a number of unidentified compounds. A fast-running substance appeared with the solvent front ahead of the thyroxine, and further unknowns appeared between thyroxine and TIT, between TIT and iodide, and between iodide and DIT. Analysis of such a hydrolysate gave the following figures: thyroxine 12·4, TIT 2·6, iodide 18, DIT 23·4, and MIT 11·4, all expressed as a percentage of the total radioactivity. No measurements of specific activity were made. A fraction which was washed out of the column with water after the MIT amounted to 13 per cent. and may be incompletely degraded material. Unknowns and intermediate background made up the remaining 19 per cent.

(*b*) *Free Iodo-amino Acids of the Thyroid*

When a solution of unhydrolysed thyroid was placed on the column a fast-running unknown appeared, followed by thyroxine, TIT, and iodide. After the emergence of the iodide the background level of radioactivity steadily increased with the DIT and MIT peaks clearly superimposed. These two fractions were run on paper with *n*-butanol–acetic acid and showed the presence of the two iodinated tyrosines, together with some material near the origin which may be peptide in nature. The major part of the radioactivity, presumably thyroglobulin, was washed out of the column with water.

In all animals examined, except one, the free thyroxine was less than the free TIT, from about one-half to one-fifth as much. Cold 2–3N NaOH would not be expected to produce extensive hydrolysis of thyroglobulin in the 6–8 hr which were required to run the thyroxine and TIT out of the column. The fact that the carrier amino acid and the radioactivity ran parallel provides evidence against the liberation of significant amounts of free thyroxine and TIT during the separation. The presence of labile linkages rapidly split on solution of the thyroglobulin in alkali cannot be excluded. Free DIT was always less than free MIT.

(*c*) *Comparison of Free and Combined Iodo-amino Acids*

The thyroids of four rats, removed 24 hr after receiving 25 μc each of ^{131}I, were dissolved in 0·5 ml of 2N NaOH. An 0·3 ml aliquot of this solution was run on the column for estimation of free thyroxine and TIT, and another 0·1 ml run after hydrolysis for 16 hr at 100°C with a further 0·1 ml 2N NaOH. The free thyroxine was 0·09 per cent. and the free TIT was 0·16 per cent. of the total ^{131}I. In the hydrolysed sample thyroxine was 2 per cent. and the TIT was 0·65 per cent. of the total ^{131}I.

(*d*) *Free Iodo-amino Acids of the Thyroid after Radiation Damage*

In one animal given 10 μg of thyroxine to inhibit thyroid secretion 24 hr before the administration of 300 μc of ^{131}I, and 5 μg of thyroxine daily thereafter, the thyroid radioactivity measured *in vivo* fell sharply after 48 hr. At 72 hr it had fallen to one-third of the 48 hr value, and at 96 hr it had fallen to one-third of the 72 hr value. At this stage the gland was removed and examined for free amino acids. A trace of thyroxine was found and no TIT. Iodide, DIT, and MIT were present, the amount of DIT being at least twice the MIT, as estimated from the area under the curve on the recorder.

(*e*) *Iodine Compounds of the Plasma*

When plasma was placed on the column, the radioactive thyroxine was usually spread more than when the pure substance alone was run. It appeared earlier, and finished later, but in all cases of spreading, added thyroxine carrier behaved like the radioactivity. The other substances behaved normally in the presence of plasma.

In two animals the distribution of radioactive compounds in the plasma was examined at 24-hr intervals after the administration of ^{131}I. The results are shown in Table 1.

In the animal which received 1 mc ^{131}I (Table 1), thyroglobulin was found at 24 hr and iodinated tyrosines at 72 hr. The latter were not estimated separately at this time. At 96 hr, DIT and MIT were 2·7 and 4·7 per cent. respectively of the total plasma iodine. The large amount of iodide present in the plasma of this animal was probably due to the fact that on this occasion the ^{131}I injected contained considerable amounts of ^{127}I. At this time the ^{131}I contained about 5 μg of ^{127}I per mc on the date of shipment, and the material had been kept in the laboratory for some time so that the dose injected might have contained as much as 40 μg of iodide, a small proportion only of which would be concentrated by the thyroid. In the animal which received

200 μc of ^{131}I (Table 1), thyroglobulin appeared at 96 hr. No iodinated tyrosines were found in the plasma of this rat. In neither animal was TIT found in the 0·2 ml of plasma used for direct chromatography.

A larger amount of plasma (2·3 ml) from the rat receiving 1 mc ^{131}I was obtained by pooling the residues of the daily samples of the first to the fifth days. The radioactivity was extracted by making the plasma twice normal with respect to sodium hydroxide, absorbing on 10 g of kieselguhr, and eluting with *n*-propanol. The alcoholic solution was concentrated under reduced pressure at room temperature and the concentrate run on a column after the addition of thyroxine and TIT carriers. A small radioactive peak, coincident with the TIT was found and amounted to 1·4 per cent of the thyroxine present or about 0·01 per cent. of the plasma ^{131}I. Similar treatment of 2·3 ml of pooled plasma from the animal receiving 200 μc showed no detectable radioactivity with the carrier TIT.

TABLE 1

DISTRIBUTION OF LABELLED COMPOUNDS IN PLASMA AFTER ^{131}I ADMINISTRATION

Dose	Time after ^{131}I Administration (hr)	Total ^{131}I in 0·2 ml Plasma (counts/min)	Components in 0·2 ml Plasma (counts/min)				Components as % of Total Plasma ^{131}I			
			Thyroxine	Iodide	DIT and MIT	Protein	Thyroxine	Iodide	DIT and MIT	Protein
1 mc	24	3650	163	3250	32	191	4·5	89·5	0·9	5·3
	48	3090	193	2665	16	191	6·3	86·9	0·5	6·2
	72	5660	365	2370	585	2530	6·2	40·5	10·0	43·2
	96	4712	352	2740	348	1260	7·5	58·3	7·4	26·8
200 μc	24	400	304	96	—	—	76	24	—	—
	48	400	228	172	—	—	57	43	—	—
	72	460	208	253	—	—	45	55	—	—
	96	640	205	282	—	153	32	44	—	24
	120	750	270	374	—	105	36	50	—	14

Reference has been made above to the examination of the free amino acids in the thyroid of a rat which received thyroxine injections and 300 μc of ^{131}I, and showed extensive loss of thyroid iodine after 48 hr due to radiation damage. Plasma from this animal taken at 72 hr showed the presence of considerable amounts of thyroxine, iodide, DIT, and thyroglobulin, together with traces of TIT, and MIT.

In a further experiment five rats were given 100 μc of ^{131}I each and were bled 24 hr later. One ml of the pooled plasma was extracted with *n*-butanol after being made alkaline with sodium hydroxide. The concentrated butanol extract on analysis showed the presence of thyroxine (62 per cent.), TIT (2·5 per cent.), iodide (28 per cent.) and combined MIT and DIT (8 per cent.).

IV. Discussion

The chromatographic system used in these investigations has certain advantages over most of the systems which have been used hitherto for this type of investigation. Small amounts of plasma can be applied directly to the column and the radioactivity can be quantitatively accounted for in the effluent. This obviates the incomplete recovery found when attempts are made to isolate the iodine compounds by extraction and concentration. However, this direct chromatography of the unextracted plasma is only possible when the specific activity is high. The examination of plasma with weak radioactivity which requires preliminary extraction of large volumes of plasma is less satisfactory, because spreading of the peaks is encountered.

Use of ultraviolet absorption of the fractions as indicator of the position of added carrier substances facilitates comparison of the distributions of the radioactive compounds and the carriers. The recovery of separate fractions from the column can be easily achieved by evaporation and is more satisfactory than the elution of spots from paper chromatograms.

The examination of the free amino acids of the thyroid shows that thyroxine, TIT, DIT, and MIT can be easily demonstrated in every case. It is striking that, in general, the amount of free TIT is in excess of the amount of free thyroxine and that the amount of free MIT is in excess of the amount of DIT. The relatively small amount of free thyroxine found in thyroid tissue is ascribed to the effect of selective secretion of thyroxine by the gland and the relative accumulation of free TIT is considered to indicate that this compound is secreted less readily than thyroxine. The relative preponderance of free MIT, as compared with free DIT, does not appear to be due to any secretion of DIT by the thyroid since we have not found any DIT in plasma except under conditions when severe radiation damage has caused a leakage of thyroid constituents, including thyroglobulin, into the plasma. Free DIT and MIT in the thyroid are presumably disposed of by de-iodination (Roche *et al.* 1953). It would appear that DIT is disposed of more rapidly than MIT.

From the results of alkaline hydrolysis of thyroid tissue, it is clear that TIT, like thyroxine, is present in thyroglobulin since the absolute amount of TIT increased during hydrolysis. The amount of TIT, however, in the thyroglobulin was considerably less than the amount of thyroxine, in contrast to the ratio found for the free amino acids.

Some comment is called for by the low percentage of thyroxine found in one of the thyroids examined after hydrolysis. We believe that this low value is associated with a low concentration of iodine in the gland. In some especially hyperplastic thyroid glands we have found less than 1 per cent. of the radioactivity present in the form of thyroxine after hydrolysis. Similar observations have been made on transplanted thyroid tumours in the rat in which the iodine content was much lower than that of normal thyroid tissue. The low iodine content of the thyroids may also be responsible for the relatively large amounts of MIT found. In the one animal treated with thyroxine, in which an increased thyroid iodine content would be expected, free DIT was found in excess of MIT.

Our investigations on the iodine compounds of plasma indicate that under normal conditions the major component is in the form of thyroxine. Iodide is always

present. Both these substances are present in the plasma before radiation damage effects appear, as signalled by the appearance of thyroglobulin, MIT, and DIT in the plasma.

Since our animals were iodine-deficient and their thyroids contained easily demonstrable amounts of free TIT we considered it likely that they would be especially favourable subjects for the demonstration of TIT as a product of thyroid secretion. It also seemed reasonable to assume that in these animals the plasma level of TIT would be raised by radiation-induced leakage from the thyroid. Despite this, TIT could be found only with difficulty in some of the animals suffering from the effects of radiation damage and not at all in others. It has not yet been determined to what extent radiation-induced leakage from the thyroid gland is responsible for the TIT found in the plasma in these experiments. With ^{131}I doses which do not produce radiation damage, the total radioactivity level of the plasma is low, and more sensitive methods than we have used here for the measurement of radioactivity would be needed to detect traces of TIT under these conditions.*

V. Acknowledgment

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* Since this paper was completed, TIT has been found in the plasma of rats given small non-damaging amounts of ^{131}I. The amount present was about 1 per cent. of the thyroxine.