concentrate the urinary iodine for chemical study, it is not necessary that it should be quantitative, but an increase in the recovery of di-iodotyrosine and a better separation of the activity from non-radioactive matter are desirable. In its present form, however, the method has been used in a study of the organic iodine compounds of urine, the results of which are to be published later.

SUMMARY

1. A method for the quantitative estimation of small amounts of ^{131}I -labelled thyroxine and di-iodotyrosine in the presence of large amounts of ^{131}I iodide in urine is presented.

2. Iodide is removed as insoluble silver iodide by the use of a column of silver chloride.

3. Under certain conditions thyroxine and di-iodotyrosine are not removed from the urine by the column.

4. Thyroxine and di-iodotyrosine are concentrated from the urine by adsorption on an ion-exchange resin.

5. The eluate from the resin, after extraction into butanol, is suitable for paper chromatography.

I should like to acknowledge the constant co-operation and helpful criticism of E. E. Pochin, M.D., F.R.C.P., during the course of this work.

REFERENCES

- Acland, J. D. (1952). *Nature, Lond.*, **170**, 32.
 Albert, A., Rall, J. E., Keating, F. R., Power, M. H. & Williams, M. M. D. (1949). *J. clin. Endocrin.* **9**, 1392.
 Bowden, C. H., MacLagan, N. F. & Wilkinson, J. H. (1955). *Biochem. J.* **59**, 93.
 Brown, F. & Jackson, H. (1954). *Biochem. J.* **56**, 399.
 Fletcher, K. & Stanley, P. G. (1955). *Nature, Lond.*, **175**, 730.
 Foster, G. L. & Gutman, A. B. (1930). *J. biol. Chem.* **87**, 289.
 Gemmill, C. L. & Plunkett, R. L. (1952). *Arch. Biochem. Biophys.* **36**, 434.
 Gross, J. & Leblond, C. P. (1947). *J. biol. Chem.* **171**, 309.
 Gross, J. & Leblond, C. P. (1950). *J. biol. Chem.* **184**, 489.
 Gross, J., Leblond, C. P., Franklin, A. E. & Quastel, J. H. (1950). *Science*, **111**, 605.
 Lardy, H. (1955). *The Thyroid, Brookhaven Symposia in Biology*, no. 7, pp. 94-95. Upton, New York: Brookhaven National Laboratory.

The Fractionation of Urinary Iodine

2. METABOLITES EXCRETED DURING TREATMENT OF CARCINOMA OF THE THYROID*

By K. FLETCHER†

Medical Research Council Department of Clinical Research, University College Hospital Medical School, London

(Received 4 January 1957)

In the literature it is generally agreed that the major proportion of the iodine excreted in the urine is in the form of inorganic iodide. Albert & Keating (1949) fractionated the urine of patients receiving labelled thyroxine orally. They found that 85% of the urinary activity behaved as iodide, the remainder as 3:5-di-iodotyrosine. No chemical identification was attempted apart from the separation procedure. Later Benua, Albert & Keating (1952) repeated this work after giving ^{131}I thyroxine intravenously. Their findings were similar, save that shortly after the injection they found a definite proportion of the activity, from 0 to 20%, in the thyroxine fraction. This proportion diminished with time, and they concluded that 24 hr. after the dose about 90% of the activity of the urine was present as iodide.

* This work forms part of a Ph.D. Thesis submitted to the University of London.

† Present address: Medical Research Council, Experimental Radiopathology Research Unit, Hammersmith Hospital, London, W. 12.

Taurog (1955), in a review of the excretion of the thyroid hormone, attaches little importance to urinary iodine, but in a paper (Taurog, Briggs & Chaikoff, 1952) on the hepatic formation of the glucuronide of thyroxine by thyroidectomized rats, he regards urinary excretion as 95% iodide and possibly 5% glucuronide. On the other hand Roche, Michel & Tata (1954*a*), giving very large amounts of radioactive thyroxine (approx. 3 mg.) to rats, claim that 40% of the radioactivity present in the urine is in the form of tetraiodothyropyruvic acid, i.e. thyroxine which has been oxidatively deaminated. In contrast with this the same workers (Roche, Michel & Tata, 1954*b*) state that the corresponding keto acid derived from tri-iodothyronine appeared in only minute quantities in the urine, the faeces being the main excretory path for this compound. Tong, Taurog & Chaikoff (1954) found that liver and kidney slices could produce 3:5-di-iodo-4-hydroxyphenylpyruvic acid and 3:5-di-iodo-4-hydroxyphenyl-lactic acid from di-iodotyrosine,

although in urine they found that the iodine from di-iodotyrosine was 90 % present as iodide, the remaining 10 % not being investigated. Foster & Gutman (1930) fed large amounts of di-iodotyrosine to rabbits and isolated from their urine a compound identified as 3:5-di-iodo-4-hydroxyphenyl-lactic acid. Stanbury and his collaborators (Stanbury, Kassenaar, Meijer & Terpstra, 1956*a*; Stanbury, Meijer & Kassenaar, 1956*b*; Querido, Stanbury, Kassenaar & Meijer, 1956) have investigated the metabolism of di-iodotyrosine and 3-monoiodotyrosine in a series of patients. In most of these they found that di-iodotyrosine was rapidly deiodinated, the iodine appearing in the urine as iodide with only a small fraction, about 10 % of the urinary iodine, in the form of di-iodotyrosine; these results were similar to those obtained by Johnson & Albert (1951) in the rat. Monoiodotyrosine was even more rapidly deiodinated, with no monoiodotyrosine appearing in the urine. In a group of people with goitre, however, they found that both monoiodotyrosine and di-iodotyrosine appeared in the urine after the injection of these substances, accompanied by a substance tentatively identified as 3:5-di-iodo-4-hydroxyphenyl-lactic acid and traces of other unknown compounds. They suggested as an explanation of their results that in this second group a deiodinase for di-iodotyrosine and monoiodotyrosine was lacking, and that this led to the appearance of these compounds and their metabolites in the urine.

There is thus little agreement about the form of iodine metabolites in the urine, except that a large proportion is iodide. Quantitatively, however, more evidence is available, and several studies have been made on the rate of excretion of tracer doses of various iodinated compounds. Albert, Rall, Keating, Power & Williams (1949) gave labelled rat thyroglobulin and thyroxine orally to two myxoedematous patients, both before and after therapy with thyroxine. When thyroglobulin was given, 20 % of the radioactive iodine was excreted in 12 hr. and 50 % in 60 hr. When thyroxine was given only 25 % was excreted in 72 hr. by the untreated patient and 45 % by the treated patient. The initial phase of the excretion in the latter was also slower than in the patient receiving thyroglobulin. It is probable that, since the thyroglobulin was given by mouth, it was hydrolysed in the gut to the constituent amino acids, and it was these which were being absorbed. If so, this treatment was equivalent to giving labelled thyroxine plus mono- and di-iodotyrosine, and, in view of the rapid breakdown and excretion of the two latter compounds, the differences between the initial excretion rates of thyroglobulin and thyroxine can be explained. Another point is, however, pertinent here. The thyroglobulin used was rat thyroglobulin, obtained

from a rat given a dose of radioactive iodide, and the thyroxine contained in it would presumably be labelled equally on each of the benzene rings. The radioactive thyroxine, on the other hand, was prepared by iodination of 3:5-di-iodothyronine and would therefore be labelled only on the ring distal to the side chain. This difference is important when the excretion rates of labelled compounds are considered. Roche, Michel & Tata (1952) injected radioactive thyroxine labelled (*a*) in the 3:5 positions and (*b*) in the 3':5' positions, into thyroidectomized rats, and measured the rate of excretion of radioactivity. They found that excretion was faster from the 3':5'-labelled thyroxine than from the 3:5-labelled. In view of this work, it must be expected that endogenously labelled hormone, labelled equally in both rings, will appear to be metabolized differently from hormone labelled either by synthesis from 3:5-di-iodothyronine or by exchange. [From Harington's (1927) work on the extreme difficulty or impossibility of iodinating the thyronine molecule in the 3:5 positions, and from the failure of experiments designed to label 3:5-di-iodothyronine by exchange, it is probable that the iodine atoms in the 3:5 positions are not exchangeable with free iodine.] Furthermore, it is possible that a search for products of thyroxine degradation would be rendered useless if the only label on the molecule were in the 3':5' position and was lost during metabolism. Many workers (Gross & Leblond, 1951; Flock & Bollman, 1954; Pitt-Rivers, Stanbury & Rapp, 1955) have shown that thyroxine can be deiodinated to tri-iodothyronine by various tissues, and it has been postulated (Gross & Pitt-Rivers, 1953; Kalant, Lee & Sellers, 1955; Thibault & Pitt-Rivers, 1955) that some deiodinated form of thyroxine is in fact the active constituent of the thyroid hormone.

From the foregoing it seems probable that, during the metabolism of thyroxine, the iodine atoms are lost, those in the distal ring disappearing first. There is, however, practically no further information on the fate of the thyroid hormone after it has exerted its metabolic effect. The present study was undertaken to discover if any fragments of the various iodinated molecules present in the plasma were excreted in the urine, and also to estimate the quantities of such fragments.

EXPERIMENTAL

The method of analysis used has been described in the previous paper (Fletcher, 1957). The column for this work measured 2.8 cm. $\times$ 42 cm., and was packed with AgCl to give a column approximately 22 cm. high, with a flow rate of about 1 ml./min.

Collection and preservation of urine. Material used for the investigation was the urine of patients receiving radioactive-iodine treatment for carcinoma of the thyroid gland. The

course of such treatment may be divided into two stages. In the first the normal thyroid tissue is destroyed by an 'ablation' dose of radioactive iodine, usually of about 80 mc. After destruction of the normal tissue there is often enhanced uptake of radioactive iodine by the abnormal carcinomatous cells, and treatment is continued by succeeding 'therapy' doses of radioactive iodine of 150 mc. The daily excretion of radioactivity after each treatment is shown in Fig. 1. Fig. 1 (a) shows the excretion after an ablation dose of radioactive iodine. There are two phases: (1) an initial high output of activity decreasing daily, due to the excretion of unchanged iodide; and (2) a second rise and fall. This latter peak of activity is consequent upon a release of large quantities of organically bound iodine, from the thyroid cells breaking down under irradiation, and may be observed at various time intervals after the dose, usually 5-7 days. Fig. 1 (b) illustrates the excretion of activity from a patient with little functioning tissue. The initial fall is more sustained and finally only a small quantity of activity is excreted, this small quantity presumably being derived from a correspondingly low amount of organically bound iodine in the plasma. The two graphs cited are extreme examples of the two modes of excretion. The secondary peak in Fig. 1 (a) is not necessarily so marked, and, on the other hand, the urine from a patient with large amounts of functioning neoplastic tissue may contain far less of the dose in the early phase than is shown in Fig. 1 (b), and may even show the later peak typical of patients undergoing thyroid ablation. Urine therefore has usually been collected on the sixth day after a dose of radioactive iodine, since it is on about this day that the highest quantity of iodine, from organic iodine in the plasma, will be present. Urine collected on other days has, however, also been investigated. The plasmas of many of the patients have been examined chromatographically and electrophoretically on the sixth day after a dose.

Fig. 1. Daily excretion of radioactive iodine, expressed as a percentage of the dose in patients receiving radioactive iodine for (a) ablation of the thyroid gland, (b) destruction of residual neoplastic tissue.

A 24 hr. specimen of urine was collected. To avoid possible decomposition of any iodine metabolites or the production of artifacts by the oxidation of iodide, the urine immediately after being passed was poured into a 2.5 l. bottle containing 10 ml. of 40% NaOH, 10 ml. of $N-NaI$ and 1 g. of Na_2SO_3 , which was kept in a refrigerator. Initially the pH of the solution in the bottle was probably high (pH 12), but after the 24 hr. specimen had been collected the pH was usually about 10.

Estimation of organic iodine. The 24 hr. specimen was shaken to suspend the precipitated phosphates and 500 ml. was taken for analysis. A small portion was put aside for counting, to determine the initial activity of the urine. To the 500 ml. were added 500 ml. of ethanol and sufficient HCl or NaOH to bring the pH to 8.5. The solution was warmed to about 40° to coagulate the precipitate, and filtered. The residue was washed with 50% ethanol, the washings being added to the filtrate. The precipitate contained a negligible quantity of radioactivity. The clear solution was then put into a large separating funnel attached to the AgCl column and allowed to percolate through the column overnight. When all the solution had drained through, the funnel was washed with 100 ml. of 50% ethanol at pH 8.5, the washings being passed through the column to elute the remaining urine held by the column. The total volume of the percolate was measured, and its activity determined by counting a portion of it. This activity has been taken to be the organic activity of the urine, and, after correction for radioactive decay, has been expressed as a percentage of the original activity of the urine.

Recovery of silver chloride. After the column had been used it was extruded from the tube and, after removal of the top few centimetres containing the bulk of the radioactive AgI (about 50 g.), dissolved in sodium thiosulphate and filtered. To the filtrate was added 100 g. of glucose and 100 ml. of 40% NaOH. On gentle warming for 24 hr. silver was precipitated, and collected by filtration and washed. The silver was dissolved in 33% HNO_3 and filtered, and the resulting solution used to prepare pure AgCl for another column by the addition of excess of a solution of NaCl.

Isolation of organic iodine. The effluent from the column was evaporated, *in vacuo*, at less than 40°, to about 100 ml. to remove the ethanol, and then diluted to approx. 400 ml. It was then subjected to the resin treatment (Fletcher, 1957). The final concentrate was extracted with *n*-butanol, and the butanol extract chromatographed on two sheets of Whatman 3MM paper 15 cm. $\times$ 45 cm.

Chromatography. Portions of all the fractions obtained during the separation procedure were chromatographed with *n*-butanol-dioxan-aq. $2N-NH_3$ soln. (4:1:5, by vol.) and radioautographed to check that no component was being lost completely. The chromatograms of the final concentrated extract were radioautographed to determine the positions of the various radioactive compounds. The required compound was eluted from the chromatogram and rechromatographed in *n*-butanol-ethanol-water (5:1:4, by vol.). Elution of the bands produced in this second chromatogram gave material which was sufficiently pure for chromatographic comparison with known compounds.

Identification of compounds. A portion of the isolated radioactive compounds was mixed with approx. 100 μ g. of a known compound and chromatographed in *n*-butanol-dioxan-aq. NH_3 soln. The pure compound was also run on the same sheet. The chromatogram was radioautographed,

and the position of the reference compound found by spraying with diazotized sulphanilic acid. Coincidence of the radioautographic image and the stained spot was taken to indicate tentative identity of the compounds. To confirm this, the unknown and the suspected known compounds were chromatographed in four different solvents. Coincidence in all four solvents was taken as evidence for the identity of the unknown with the known compound.

Reference compounds. Thyroxine, 3:5:3'-tri-iodothyronine and 3:5-di-iodothyronine were obtained from Glaxo Laboratories Ltd., and were recrystallized before use. 3:5-Di-iodotyrosine was obtained from B.D.H. Laboratories Ltd.

3:5-Di-iodo-4-(3-iodo-4-hydroxyphenoxy)phenylacetic acid, 3:5-di-iodo-4-(3:5-di-iodo-4-hydroxyphenoxy)phenylacetic acid, 3:5-di-iodo-4-(3-iodo-4-hydroxyphenoxy)-phenylpyruvic acid and 3:5-di-iodo-4-(3:5-di-iodo-4-hydroxyphenoxy)phenylpyruvic acid were the gift of Dr R. Pitt-Rivers.

3:5-Di-iodo-4-hydroxyphenyl-lactic acid and 3:5-di-iodo-4-(3:5-di-iodo-4-hydroxyphenoxy)phenyl-lactic acid were synthesized by the method of Saul & Trikojus (1948).

3:5-Di-iodo-4-hydroxyphenylpyruvic acid was synthesized by the method of Tong *et al.* (1954). (This compound was not obtained pure despite repeated recrystallization. However, only one of the spots on a chromatogram of it gave the reactions of an α -keto acid and this was taken as the reference spot.)

3-Monoiodotyrosine was synthesized by the iodination of tyrosine.

RESULTS

Percentage of organic iodine of urine. Table 1 shows the percentage of organic activity found in the urine of patients undergoing ablation of the thyroid gland. Table 2 shows the percentage of organic activity found in the urine of patients with no thyroid, but with uptake in carcinomata.

Isolation of urinary iodine compounds. Fig. 2 shows three chromatograms of the final concentrate of 500 ml. of urine from three different patients. It can be seen that there is a broad similarity between these and they are in fact typical of the chromato-

Table 1. *Percentage of the total iodine as organic iodine in the urine of patients undergoing ablation of the thyroid gland*

Numbers in parentheses refer to the day after the ablation dose on which the urine was collected.

Patient	Total activity of 500 ml. of urine (μ C)	Organic activity (μ C)	Organic percentage
Cr. (2)	3290	81.7	2.5
Cr. (5)	964	84.6	8.8
Cr. (6)	865	62.7	7.3
Ta. (4)	919	29.4	3.2
Ta. (5)	531	34.6	6.5
Ta. (6)	169	12.9	7.6
Ev. (4)	485	13.9	2.8
Mi. (6)	185	15.0	8.1
Be. (6)	255	23.1	9.1

grams obtained. The greatest part of the organic activity is found in two compounds, A and B, running very close together near the origin. By counting a chromatogram of a small portion of the concentrate, it has been found that 80-90 % of the

Table 2. *Percentage of the total iodine as organic iodine in the urine of patients with no thyroid but with uptake in carcinomata*

Numbers in parentheses refer to the day after the dose on which the urine was collected.

Patient	Total activity of 500 ml. of urine (μ C)	Organic activity (μ C)	Organic percentage
Ma. (2)	5090	50.6	1.0
Ma. (3)	2320	19.2	0.83
Ma. (4)	432	4.8	1.4
Ke. (2)	4720	2.0	0.04
Ke. (6)	52	1.9	3.5
Ju. (3)	1426	3.4	0.23
To. (5)	3190	484	15.1
Ev. (7)	1081	73.8	6.8

Fig. 2. Final chromatograms of the organic fraction obtained from the urine of three different patients. O, Origin; SF, solvent front. Bands A and B, containing the bulk of the radioactivity, were identified as 3:5-di-iodotyrosine and 3:5-di-iodo-4-hydroxyphenyl-lactic acid.

Table 3. R_F values of reference compounds

The descending method with Whatman no. 3MM paper was used. Solvents: I, *n*-butanol-dioxan-aq. 2*N*-NH₃ soln. (4:1:5, by vol.); II, *n*-butanol-ethanol-water (4:1:5, by vol.); III, *n*-butanol-2*N*-acetic acid (1:1, v/v); IV, collidine-water (1:1, v/v). Time of run, 16 hr.

Compound	R_F value in solvent			
	I	II	III	IV
3-Monoiodotyrosine	0.20	0.42	0.50	0.63
3:5-Di-iodotyrosine	0.18	0.26	0.60	0.49
3:5-Di-iodo-4-hydroxyphenylpyruvic acid	0.21	0.29	0.86	0.78
3:5-Di-iodo-4-hydroxyphenyl-lactic acid	0.21	0.43	0.91	0.76
Iodide	0.30	0.29	0.21	0.71
3:5:3'-Tri-iodothyronine	0.70	0.95	0.89	0.93
Thyroxine	0.60	0.93	0.92	0.84
3:5-Di-iodo-4-(3-iodo-4-hydroxyphenoxy)phenylacetic acid	0.74	0.84	0.96	0.91
3:5-Di-iodo-4-(3:5-di-iodo-4-hydroxyphenoxy)phenylacetic acid	0.65	0.84	0.97	0.92
3:5-Di-iodo-4-(3:5-di-iodo-4-hydroxyphenoxy)phenyl-lactic acid	0.61	0.80	0.96	0.93
3:5-Di-iodo-4-(3-iodo-4-hydroxyphenoxy)phenylpyruvic acid	0.76	0.85	0.95	0.96
3:5-Di-iodo-4-(3:5-di-iodo-4-hydroxyphenoxy)phenylpyruvic acid	0.66	0.87	0.96	0.98

total activity resides in these two compounds, the remaining activity being distributed between the various compounds separating along the length of the chromatogram. Initially the investigation of the nature of the urinary iodine was devoted to the two compounds constituting the bulk of the organic radioactivity. After purification, these two compounds were run with known compounds. Table 3 gives the R_F values of the reference compounds after chromatography in four solvents. It can be seen that in solvent I the compounds separate into two distinct groups: the single-ring compounds having R_F values of about 0.2 and the two-ring compounds with R_F values of about 0.6. Since the two main compounds from the urinary chromatograms gave R_F values of 0.0-0.2 in solvent I, they were purified and re-run with carrier amounts of all the single-ring compounds in solvents I-IV. It was found that compound A, the slower of the two unknowns in solvent I, was chromatographically identical with di-iodotyrosine, and compound B with 3:5-di-iodo-4-hydroxyphenyl-lactic acid.

Similar experiments with the other unknowns from the urine chromatograms did not yield such unequivocal information. In these only small amounts of activity were available, and comparison between different chromatograms was difficult since there was no certainty that the same compound was being studied. In no case, however, was thyroxine, tri-iodothyronine, tri-iodothyroacetic acid or tetraiodothyroacetic acid found. Identity of some of the spots with other reference compounds was equivocal, and further work is needed to confirm this. Experiments were also carried out to hydrolyse the various components, and to treat them chemically in other ways, but again no useful information was obtained. From this work it appears that most of the minor iodine-containing compounds of urine are not recognized thyroid metabolites.

DISCUSSION

The results in Tables 1 and 2 show that the amount of organically combined iodine in urine reflects the uptake by functioning tissue of the patient. Thus in the group with no normal thyroid tissue (Table 2), patients Ev. and To. were known to have large uptakes in various metastases, the latter especially having total metastatic tissue which could be equivalent to three or four normal thyroid glands in the rate of turnover of iodine; this is reflected in the high excretion of organic iodine, the highest in fact found in any group. Where analyses have been done on consecutive days after the dose, on the same patient, there is an increase in the percentage of organic iodine excreted with succeeding days, this probably indicating a greater output of iodine by tissue which is being destroyed by radiation.

The chromatographic evidence for the presence of di-iodotyrosine and 3:5-di-iodo-4-hydroxyphenyl-lactic acid is not conclusive proof of their presence in urine, although identity in four different solvents is strong evidence in its favour. Further evidence could be obtained by adding unlabelled di-iodotyrosine and 3:5-di-iodo-4-hydroxyphenyl-lactic acid to urine, isolating these compounds, and recrystallizing them to a constant specific activity. Assuming these identities to be valid, it is pertinent to inquire the source of these compounds. 3:5-Di-iodophenyl-lactic acid can arise by the metabolism of di-iodotyrosine, as shown originally by Foster & Gutman (1930) and confirmed by Stanbury *et al.* (1956*b*), and hence if di-iodotyrosine is present in the circulation 3:5-di-iodo-4-hydroxyphenyl-lactic acid may well appear in the urine. The di-iodotyrosine found in these experiments could be conceived as arising in one of the following ways: (1) as an artifact during the separation; (2) as an artifact in the kidney or bladder; (3) from circulating di-iodotyrosine; (4) from circulating thyro-

globulin, by breakdown in the kidney; (5) from circulating thyroxine, by breakdown in the kidney.

Possibility (1) is extremely unlikely, since the urine is made alkaline soon after it is passed and relatively enormous quantities of carrier iodide are added, and since radioiodination of tyrosine would depend on the presence of free radioactive iodine the possibility of radioiodination is remote. Furthermore, as is shown in Tables 1 and 2, the amount of organic iodine in the urine, and, to a close approximation, the amount of di-iodotyrosine, is dependent on the amount of functioning thyroid tissue and not on the amount of iodide present. If the di-iodotyrosine were an external artifact the quantity of it should parallel the total activity.

Similar arguments may be applied to possibility (2). Iodination in the kidney or bladder, where urine is usually at an acid pH of 5-6, could conceivably occur, but, again, it would then presumably be proportional to the total activity of the urine. Also the amount of iodide excreted per day (about 100 μg .) is totally inadequate for a double iodination of the daily excretion of tyrosine, and hence if iodination occurred, monoiodotyrosine and not di-iodotyrosine would probably be formed.

Urinary di-iodotyrosine could be derived from free di-iodotyrosine in the plasma as (a) free di-iodotyrosine released from the thyroid gland; (b) di-iodotyrosine produced by the breakdown of thyroxine in the tissues; (c) di-iodotyrosine produced by the breakdown of thyroglobulin in the tissues. (a) is unlikely since studies of the thyroid have shown that free di-iodotyrosine is present in only small amounts (Tishkoff, Bennett, Bennett & Miller, 1949; Tong, Taurog & Chaikoff, 1951). Since di-iodotyrosine is rapidly removed from the circulation and excreted, and most of it is de-iodinated before excretion, any di-iodotyrosine in the urine would reflect an amount in the plasma about 10 times this (Tong *et al.* 1954). It is improbable that such amounts could be released by the thyroid.

Little is known about possibility (b). Although there is no evidence that thyroxine is metabolized by tissues to give di-iodotyrosine, a consideration of the thyroxine molecule shows that hydrolytic or reductive cleavage at the ether oxygen would result in the formation of di-iodotyrosine. This reaction could only give labelled di-iodotyrosine from thyroxine labelled in the 3 or 5 position. Only if a molecule of alanine were involved in the cleavage of the ether bond could di-iodotyrosine be formed from the distal ring of thyroxine. This would in fact be a reversal of synthesis, and would appear to be rather unlikely. However, Albert *et al.* (1949) found a recovery of 15% of the urinary activity in the di-iodotyrosine fraction after an oral dose of thyroxine labelled in the 3' position. This result is difficult to interpret. No positive identification of

the di-iodotyrosine was made and the analytical method was crude.

The breakdown of thyroglobulin in the plasma (c) is another possible source of di-iodotyrosine in the urine. In all the cases studied where simultaneous plasma and urine analyses were carried out, thyroglobulin was present in the plasma. The distribution of the iodine in thyroglobulin is given by Taurog, Tong & Chaikoff (1950) as monoiodotyrosine 15%, di-iodotyrosine 30%, thyroxine > 22%, and by Roche, Jutisz, Lissitsky & Michel (1951) as monoiodotyrosine 33%, di-iodotyrosine 33% and thyroxine 27%. Although reports of the composition of thyroglobulin differ, it seems that it contains about 33% of the iodine in the form of di-iodotyrosine. The excretion of the breakdown products of thyroglobulin is unknown, but it seems at least possible that the di-iodotyrosine present in it would be excreted eventually in a manner similar to free di-iodotyrosine. From the work of Stanbury *et al.* (1956a) the iodine of monoiodotyrosine in thyroglobulin would not be expected to appear in organic combination in the urine.

It is possible from a knowledge of the di-iodotyrosine content of the urine and of the thyroglobulin content of the plasma, to find very approximately the amount of plasma cleared of thyroglobulin in a day.

Thus for patient Be. the total di-iodotyrosine excreted in one day was 43 μC . If it is assumed that 90% of the di-iodotyrosine in plasma is degraded to iodide before excretion, and only 10% appears as di-iodotyrosine or 3:5-di-iodo-4-hydroxyphenyl-lactic acid in the urine (Tong *et al.* 1954), then 43 μC of di-iodotyrosine in the urine represents 430 μC of di-iodotyrosine in the plasma. Assuming that the di-iodotyrosine was derived from thyroglobulin in the plasma, then, since the iodine of di-iodotyrosine is approximately one-third of the iodine of thyroglobulin, 430 μC of di-iodotyrosine is equivalent to 1290 μC of thyroglobulin. An analysis of the plasma on the same day showed that it contained 0.04 μC of thyroglobulin/ml. Therefore the amount of di-iodotyrosine found in the urine would be derived from the thyroglobulin contained in 1290/0.04 ml., or 32 l., of plasma.

Therefore the di-iodotyrosine in the urine represents 32 l. of plasma cleared of thyroglobulin. Other results obtained from other patients were 12 and 18 l. of plasma cleared/day.

From these figures it would seem that thyroglobulin is removed from the circulation rapidly. Brown & Jackson (1954, 1956) injected homologous labelled thyroglobulin into several species and found that with the exception of rabbit thyroglobulin, after 24 hr. only a few per cent. of the activity remained in the plasma, and this result is evidence in favour of the figures obtained from the urinary excretion.

These last three sources of urinary di-iodotyrosine presuppose that free di-iodotyrosine is circulating in the plasma. Simultaneous analysis of plasma has

in no case shown the presence of free di-iodotyrosine, but since the analysis was performed on only 50 μ l. of plasma, any di-iodotyrosine present may have been too low in amount for detection, but might have been revealed by extracting larger quantities of material.

Di-iodotyrosine could appear in urine without being in the plasma by the breakdown of thyroxine or thyroglobulin in the kidney tissue with the elimination of the di-iodotyrosine thus formed in the urine. There is, however, no evidence on this point and elucidation must await the results of future work. At the moment it would appear probable that the di-iodotyrosine and 3:5-di-iodo-4-hydroxyphenyl-lactic acid of the urine are produced by the breakdown of thyroglobulin.

SUMMARY

1. The urine of patients receiving large doses of radioactive iodine for treatment of thyroid carcinoma has been investigated.
2. The amount of the iodine present in organic combination has been found to be proportional to the known uptake of radioactive iodine by the thyroid or neoplastic tissue.
3. The organic fraction has been studied by paper chromatography.
4. Two main compounds in the organic fraction have been shown to be chromatographically identical with 3:5-di-iodotyrosine and 3:5-di-iodo-4-hydroxyphenyl-lactic acid.
5. Several compounds of unknown structure have also been shown to be present.
6. The origin of the urinary di-iodotyrosine is discussed. It is considered probable that this is derived from circulating thyroglobulin.

REFERENCES

- Albert, A. & Keating, F. R. (1949). *J. clin. Endocrin.* **9**, 1406.
- Albert, A., Rall, J. E., Keating, F. R., Power, M. H. & Williams, M. M. D. (1949). *J. clin. Endocrin.* **9**, 1392.
- Benua, R. S., Albert, A. & Keating, F. R. (1952). *J. clin. Endocrin.* **12**, 1461.
- Brown, F. & Jackson, H. (1954). *Biochem. J.* **56**, 399.
- Brown, F. & Jackson, H. (1956). *Biochem. J.* **62**, 295.
- Fletcher, K. (1957). *Biochem. J.* **67**, 136.
- Flock, E. V. & Bollman, J. L. (1954). *Fed. Proc.* **13**, 209.
- Foster, G. L. & Gutman, A. B. (1930). *J. biol. Chem.* **87**, 289.
- Gross, J. & Leblond, C. P. (1951). *Proc. Soc. exp. Biol., N.Y.*, **76**, 686.
- Gross, J. & Pitt-Rivers, R. (1953). *Biochem. J.* **53**, 645, 652.
- Harington, C. R. (1927). *Biochem. J.* **21**, 169.
- Johnson, H. W. & Albert, A. (1951). *Endocrinology*, **48**, 669.
- Kalant, H., Lee, R. & Sellers, E. A. (1955). *Endocrinology*, **56**, 127.
- Pitt-Rivers, R., Stanbury, J. B. & Rapp, B. (1955). *J. clin. Endocrin.* **15**, 616.
- Querido, A., Stanbury, J. B., Kassenaar, A. A. H. & Meijer, J. W. A. (1956). *J. clin. Endocrin.* **16**, 1096.
- Roche, J., Jutisz, M., Lissitsky, S. & Michel, R. (1951). *Biochim. biophys. Acta*, **7**, 257.
- Roche, J., Michel, R. & Tata, J. (1952). *C.R. Soc. Biol., Paris*, **146**, 1003.
- Roche, J., Michel, R. & Tata, J. (1954a). *C.R. Soc. Biol., Paris*, **148**, 1036.
- Roche, J., Michel, R. & Tata, J. (1954b). *C.R. Soc. Biol., Paris*, **148**, 642.
- Saul, J. A. & Trikojus, V. M. (1948). *Biochem. J.* **42**, 80.
- Stanbury, J. B., Kassenaar, A. A. H., Meijer, J. W. A. & Terpstra, J. (1956a). *J. clin. Endocrin.* **16**, 735.
- Stanbury, J. B., Meijer, J. W. A. & Kassenaar, A. A. H. (1956b). *J. clin. Endocrin.* **16**, 848.
- Taurog, A. (1955). *The Thyroid, Brookhaven Symposia in Biology*, no. 7, p. 111. Upton, New York: Brookhaven National Laboratory.
- Taurog, A., Briggs, F. N. & Chaikoff, I. L. (1952). *J. biol. Chem.* **194**, 655.
- Taurog, A., Tong, W. & Chaikoff, I. L. (1950). *J. biol. Chem.* **184**, 83.
- Thibault, O. & Pitt-Rivers, R. (1955). *Lancet*, **268**, 285.
- Tishkoff, G. H., Bennett, R., Bennett, V. & Miller, L. L. (1949). *Science*, **110**, 452.
- Tong, W., Taurog, A. & Chaikoff, I. L. (1951). *J. biol. Chem.* **191**, 665.
- Tong, W., Taurog, A. & Chaikoff, I. L. (1954). *J. biol. Chem.* **207**, 59.

Preparation and Properties of Highly Purified Diaphorase

By NERINA SAVAGE

Department of Biochemistry, University of Cambridge

(Received 20 February 1957)

In 1937 an enzyme catalysing the reaction between reduced coenzyme I (Co I-H₂) and methylene blue (or other suitable carriers) was found to be present in the plant and animal kingdoms and was named at the time 'coenzyme factor' or diaphorase by two groups of workers who independently discovered it (Green, Dewan & Leloir, 1937; Dewan & Green,

1937; Adler, Euler & Hellström, 1937; Adler, Euler & Günther, 1938). It was subsequently found widely distributed in animal tissues (Green, Needham & Dewan, 1937; Dewan & Green, 1938; Green & Dewan, 1937; Euler & Hasse, 1938; Euler & Günther, 1938a); in yeast (Euler & Günther, 1938b; Dewan & Green, 1938); and in plant tissues (Lockhart, 1939).