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# The Fractionation of Urinary Iodine

## 1. METHOD OF ANALYSIS*


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(*Received* 4 *January* 1957)

The metabolism of radioactive iodine has been the subject of intensive investigation in recent years. This has been concentrated mainly on the production of hormone by the thyroid gland and its subsequent fate in the plasma and tissues, and little has been published about the excretion of the hormone in the urine. It is, however, generally agreed that the greatest proportion of the iodine in urine is present as inorganic iodide, and only a small percentage exists in an organic form. It was to investigate this organic fraction that the present work was undertaken, both to estimate the amount of organically combined iodine and to discover the chemical nature of the products. This present paper describes a method for the investigation of the problem, and subsequent papers will describe the results obtained from the application of this method to the urine of patients receiving large amounts of radioactive iodine for the treatment of disease of the thyroid gland.

Several methods were used in an attempt to separate the organic iodine from the iodide, and most were abandoned. The chief cause of the difficulties encountered was the ease with which iodide is oxidized in an acid solution, leading to the formation of free iodine and the production of artifacts. Acland (1952) has pointed out that biological solutions of iodide can readily produce many artifacts when acidified, and this observation has been amply confirmed. The method finally adopted was the removal of iodide from the urine at an alkaline pH by a column of silver chloride, and the subsequent concentration of the organic iodine by an ion-exchange resin.

### EXPERIMENTAL AND RESULTS

*Paper chromatography.* Throughout this work chromatography was carried out on Whatman 3MM paper. This thick paper was chosen since all the material examined contained

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

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large quantities of extraneous non-radioactive matter, which tended to overload thinner paper.

The chromatograms were developed by the descending method, the upper phase of a mixture of *n*-butanol–dioxan–aq. 2N-$NH_3$ soln. (4:1:5, by vol., Gross, Leblond, Franklin & Quastel, 1950) being used.

*Preparation of radioactive compounds.* Thyroxine and 3:5-di-iodotyrosine were labelled with radioactive $^{131}I$ by a modification of the method of Gross & Leblond (1950). The specific activities obtained were initially about 4 $\mu$C/$\mu$g. Since there is little information about the nature of the iodine-containing compounds of urine, these two compounds were used as models to test the efficacy of the separation procedures.

*Location of radioactive compounds and carriers.* Positions of radioactivity on paper chromatograms were found either by radioautography on Ilford No-Screen X-Ray film, or by scanning the paper strips in an automatic strip counter. The locations of added carrier compounds were found either by spraying with diazotized sulphanilic acid, or, on chromatograms containing much extraneous material, by the method of Bowden, Maclagan & Wilkinson (1955) as modified by Fletcher & Stanley (1955).

*Removal of iodide by silver.* Silver forms an extremely insoluble iodide, the solubility product of which is $0{\cdot}32 \times 10^{-16}$, and therefore precipitation with silver should be a highly efficient means for the removal of iodide. The precipitation of iodide by silver has been used previously by several authors, but some doubt seems to exist about the solubility of the silver salts or complexes of thyroxine and di-iodotyrosine. Thus Albert, Rall, Keating, Power & Williams (1949), following the procedures of Foster & Gutman (1930) and Gross & Leblond (1947), precipitated the iodide from urine with $AgNO_3$ or $Ag_2SO_4$ in an acid solution and found little loss of di-iodotyrosine. Brown & Jackson (1954), on the other hand, fractionated plasma with silver phosphate, and found that thyroxine was unprecipitated whilst still attached to native globulin, but on precipitation and removal of the plasma proteins with methanol the free thyroxine was quantitatively removed with the silver phosphate. Thyroxine is known to form chelated derivatives with many metals (Gemmill & Plunkett, 1952; Lardy, 1955) and it seems possible that silver, which readily forms complexes, might chelate with thyroxine to form an insoluble product.

Preliminary experiments had shown that the direct precipitation of iodide from urine, at an alkaline pH, was impracticable since the silver iodide formed a suspension almost colloidal in nature and impossible to filter clear. It was, however, found that percolation of a solution of iodide through a column of AgCl removed the iodide, and therefore the influence of AgCl upon thyroxine was investigated.

Table 1. *Solubility of silver thyroxine in various media*

For experimental procedure see text.

| Composition of soln. | pH | Solubility of thyroxine ($\mu$g./ml.) Expt. 1 | Expt. 2 |
|---|---|---|---|
| Water | 7 | 5·1 | 7·1 |
| 50% ethanol | 7 | 29·0 | 25·6 |
| 50% ethanol | 9 | — | 81·5 |
| 50% ethanol; NaCl (5 mg./ml.) | 9 | 180·0 | 174·0 |
| 50% ethanol; NaCl (10 mg./ml.) | 7 | 191·0 | — |
| 50% ethanol; NaCl (10 mg./ml.) | 8 | 178·0 | — |
| 50% ethanol; NaCl (10 mg./ml.) | 8·5 | 161·0 | 219·0 |

Labelled thyroxine (225 $\mu$C) was mixed with a solution of 10·8 mg. of unlabelled thyroxine in 0·001N-NaOH. A volume (2 ml.) of 0·1N-$AgNO_3$ was added and the solution kept for 2 hr. The precipitate of silver thyroxine was centrifuged and the supernatant discarded. The precipitate was washed twice with 0·001N-NaOH and four times with water. A small quantity of the precipitate was then suspended in 10 ml. of various media and left overnight with constant shaking. The solutions were centrifuged and portions of the clear supernatants counted. The results of two such experiments are given in Table 1. From these results it would appear that there would be no loss, by the formation of an insoluble silver salt, of thyroxine in the concentrations usually encountered in biological fluids, provided the optimum conditions of Table 1 were used. A similar series of investigations on the solubility of di-iodotyrosine was not performed since experiment showed that 100 ml. of urine would dissolve at least 1 mg. of di-iodotyrosine in the presence of AgCl.

*Preparation of silver chloride column.* Silver chloride was precipitated from $AgNO_3$ by the addition of $HNO_3$ and NaCl. The resulting solid was washed completely free from acid by decantation and poured in a slurry, in a solution of 50% ethanol containing 1% NaCl at pH 8·5, into a glass tube closed by a plug of glass wool and a tap. This was packed to give a flow rate of 0·2–1 ml./min., the slower rate being for a column 1·5 cm. × 15 cm., the faster for a column 2·8 cm. × 22 cm. The column was jacketed with dark translucent paper to reduce photodecomposition of the AgCl.

### *Removal of iodide from urine*

To test the efficacy of the column for the removal of iodide the following solution was made up. Radioactive $^{131}I$ and carrier $^{127}I$ were added to normal urine and the pH was adjusted to 8·5. An equal volume of ethanol was added and the precipitated phosphates were filtered off and washed with 50% ethanol, the washings being added to the filtrate. The total activity of the solution was determined, and the solution was allowed to flow slowly through a column of AgCl prepared as above. When nearly all the solution had run through, a further 50 ml. of 50% ethanol was added to the column to wash the remaining urine down the tube. The activity of the percolate was found. The results of experiments on the recovery of iodide from urine are given in Table 2 (*a*). It can be seen that the removal is almost complete, and examination of the column showed that the iodide was removed in the first few centimetres of the column, as judged by the yellow band of the AgI.

### *Recovery of* [$^{131}$I]*thyroxine and* [$^{131}$I]*di-iodotyrosine*

The recovery of labelled thyroxine and di-iodotyrosine was found in a manner similar to that used for iodide except that no stable iodide was

added and no additional thyroxine or di-iodotyrosine was present, apart from that in the radioactive preparations. The results obtained are given in Table 2 (*b*, *c*). Chromatography of the solutions after passage through the column showed only the added radioactive compound to be present, plus small traces of iodide.

*Separation of [$^{131}$I]iodide, [$^{131}$I]thyroxine and [$^{131}$I]di-iodotyrosine*

Table 3 shows the results obtained when mixtures of radioactive iodide, thyroxine and di-iodotyrosine were passed through the column. The percentages of organic activity in the urine before and after the column treatment are given.

*Concentration of organic iodine from urine*

The urine, after passage through the AgCl column, still contained nearly all the salts and extraneous matter originally present. It was necessary therefore that the organic iodine be concentrated before it was suitable for study. It seemed probable that any metabolites of the thyroid hormone excreted in urine would still retain one or two benzene rings and that they should therefore be capable of separation by adsorption. Experiments with various activated charcoals showed that these materials led to both extensive decomposition and irreversible adsorption of thyroxine and di-iodotyrosine. Use was therefore made of an ion-exchange resin as an adsorbing material.

Table 2. *Recoveries of [$^{131}$I]iodide, [$^{131}$I]thyroxine and [$^{131}$I]di-iodotyrosine after passage through a column of silver chloride*

| Size of columns (cm. × cm.) | Compounds added | Amount added (μc) | Urine volume (ml.) | Recovery (%) |
|---|---|---|---|---|
| 1·5 × 15 | (*a*) Iodide [Each urine contained 1% (v/v) of N-NaI] | 123 | 160 | 0·035 |
| 1·5 × 15 | | 43 | 25 | 0·71 |
| 1·5 × 15 | | 200 | 50 | 0·17 |
| 2·8 × 22 | | 635 | 500 | 0·038 |
| 1·5 × 15 | (*b*) Thyroxine | 3·9 | 40 | 97 |
| 1·5 × 15 | | 2·1 | 48 | 97 |
| 2·5 × 22 | | 71·0 | 450 | 84 |
| 1·5 × 15 | (*c*) Di-iodotyrosine | 2·7 | 45 | 92 |
| 1·5 × 15 | | 12·6 | 100 | 94 |
| 2·5 × 22 | | 16·0 | 350 | 82 |

Table 3. *Separation of [$^{131}$I]thyroxine and [$^{131}$I]di-iodotyrosine from [$^{131}$I]iodide by a column of silver chloride*

Each urine contained 1% (v/v) of N-NaI.

| Size of column (cm. × cm.) | Compounds added | Amount (μc) | Radioactivity of organic iodine (%) Added | Recovered | Urine vol. (ml.) |
|---|---|---|---|---|---|
| 1·5 × 15 | Iodide | 40 | 3·2 | 3·3 | 40 |
| | Thyroxine | 1·3 | | | |
| 1·5 × 15 | Iodide | 8·9 | 8·3 | 7·9 | 120 |
| | Thyroxine | 0·8 | | | |
| 2·8 × 22 | Iodide | 289 | 11·6 | 11·9 | 500 |
| | Di-iodotyrosine | 37·3 | | | |
| | Thyroxine | 0·5 | | | |

Table 4. *Recovery of thyroxine, di-iodotyrosine and iodide in the various fractions obtained by treatment of urine with an ion-exchange resin*

Each column represents a separate experiment. The recovery is given as percentage of the original radioactivity in the urine. For experimental details see text.

| Radioactive compound added to urine | Thyroxine | Di-iodotyrosine | Iodide |
|---|---|---|---|
| Filtrate after adsorption on resin | 2·4 | 22 | 82 |
| Filtrate after acid wash | 0·1 | 5 | 11 |
| Filtrate after alkaline elution | 92·0 | 66 | 4 |
| Filtrate after alkaline wash | 2·3 | 4 | 0·5 |
| Residue left on resin | 3 | 3 | 2 |

Urine (500 ml.) containing either labelled thyroxine or di-iodotyrosine was adjusted to pH 4 with 10N-HCl, and cooled in an ice bath to 0–4°. A settled suspension (100 ml.) of Zeo-Karb 215 (200–400 mesh, Na form) was added and the mixture stirred continuously in the ice bath for 3 hr. The resin was filtered, and again stirred with 250 ml. of water at pH 4 for a further hour, and the washing fluid was filtered off. The resin was then stirred at room temperature for 2 hr. in 500 ml. of a mixture ethanol–aq. 2N-$NH_3$ soln. (1:1, v/v). After filtration the resin was finally washed with a further 250 ml. of ethanol–aq. 2N-$NH_3$ soln. The recovery of thyroxine and di-iodotyrosine in the various fractions is given in Table 4. Chromatography of these fractions showed only the added compound to be present. Table 4 also shows the results obtained when a solution of [$^{131}$I]iodide in urine was subjected to the same procedure.

*Extraction with butanol*

By adsorbing the organic iodine on to the resin, the greater part of the interfering substances in the urine was removed, but the alkaline eluate still contained too much material for paper chromatography of the whole solution. The combined alkaline elution fractions were therefore evaporated *in vacuo* to approx. 15 ml. and extracted four times at pH 2 with equal volumes of *n*-butanol. The pooled butanol extracts were then neutralized with conc. $NH_3$ soln. and evaporated to dryness. The residue proved suitable for the isolation of the organic iodine by paper chromatography.

*Chromatography of the concentrated organic iodine*

The residue after the evaporation of the butanol was dissolved in a minimum quantity of ethanol–aq. 2N-$NH_3$ soln. (1:1, v/v). This solution was then applied in a band along the top of two sheets of chromatography paper 15 cm. × 45 cm. The chromatogram was developed overnight, and, after drying, areas of radioactivity were located by radioautography. The radioactive bands were cut out of the paper and eluted overnight. Portions of these eluates were rechromatographed with added carrier compounds and, by the correspondence of the radioautographic spot and the stained area, were shown to contain only the compounds originally added to the urine.

## DISCUSSION

By using a column of silver chloride, the iodide in urine may be removed whilst still retaining good recoveries of added labelled compounds. Table 2 (*a*) shows that iodide is not completely removed from the urine, and the amount flowing through the column is variable; in no case, however, did the amount of unextracted iodide exceed 0·8 %, and it is felt that this amount is insignificant. Table 2 (*b*, *c*) shows that the recoveries of added labelled compounds are good, although it seems that the recovery diminishes with increased time of passage through the column. No attempt has been made to increase this recovery since it is probably due to decomposition of the compounds, and further washing of the column elutes only insignificant amounts of radioactivity. When the recovery of organic iodine, in the presence of iodide, is considered, it is found that there is agreement between that recovered and the amount originally added, although this is to some extent fortuitous since the small leak-through of iodide will counterbalance the loss of thyroxine or di-iodotyrosine.

Although it has been shown that the method of analysis is valid when applied to thyroxine and di-iodotyrosine dissolved in urine, it is not necessarily so for urine containing various other metabolites. The form in which iodine is excreted in the urine is almost unknown, and it is possible that compounds are excreted whose behaviour with silver is different from thyroxine. The daily excretion of iodine by persons on a normal diet is approximately 100 $\mu$g., and of this it seems that less than 20 % would be in an organic form. The method here described is applicable to a maximum concentration of thyroxine of about 150 $\mu$g./ml. and would only fail if the concentration in the urine were to exceed this, or a compound were excreted whose silver salt was some thousand times less soluble than that of thyroxine. Both of these hypotheses are unlikely. It is possible, however, that a compound might be chemically altered during its passage through the column. Silver chloride in an alkaline solution is an oxidizing agent, and examination of the column after the treatment of urine shows that some of the silver chloride has been reduced to metallic silver, an effect that increases sharply with an increase in the pH of the percolating fluid. Therefore a compound with an easily oxidizable group might emerge from the column in an oxidized form, and comparison with the untreated urine would be necessary to determine the original structure of the compound.

The concentration of the activity after the removal of iodide is in several ways unsatisfactory. While thyroxine is recovered almost completely in the alkaline eluate from the resin, much di-iodotyrosine is lost during the adsorption stage. Chromatographic separation, on ion-exchange resins, of these two substances in urine has been attempted, but with less success than with the method discussed here. The pH of 4 used during the adsorption appears to be optimum, and lengthening the time of adsorption does little to improve the recovery of di-iodotyrosine. Since this part of the separation procedure is intended only to

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.

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# The Fractionation of Urinary Iodine

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

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(*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.

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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,