- McGirr, E. M., Hutchison, J. H. & Clement, W. E. (1956). *Lancet*, ii, 906.
- Mosier, H. D., Blizzard, R. M. & Wilkins, L. (1958). *Pediatrics, Springfield*, 21, 248.
- Ruegamer, W. R. & Chodos, R. B. (1956). *Fed. Proc.* 15, 343.
- Ruegamer, W. R. & Chodos, R. B. (1958). *Arch. Biochem. Biophys.* 77, 403.
- Stanbury, J. B., Kassenaar, A. H., Meijer, J. W. & Terpstra, J. (1956). *J. clin. Endocrin.* 16, 735.
- Stanbury, J. B. & Litvak, J. (1957). *J. clin. Endocrin.* 17, 654.
- Stanbury, J. B., Meijer, J. W. & Kassenaar, A. H. (1956). *J. clin. Endocrin.* 16, 848.
- Tong, W., Taurog, A. & Chaikoff, I. L. (1954). *J. biol. Chem.* 207, 59.

Radioiodine Compounds of Human Urine after ^{131}I Therapy

1. FRACTIONATION

By CHRISTINA CAMERON

Medical Research Council, Department of Clinical Research, University College Hospital Medical School, London, W.C. 1

(Received 4 May 1959)

After administration of radioiodine (^{131}I) in therapeutic amounts for the treatment of thyrotoxicosis and thyroid carcinoma, organic ^{131}I -labelled compounds appear in the urine, due in part presumably to the destruction of thyroid tissue by radiation from ^{131}I taken up by the gland. Concentration of these compounds for identification is difficult, since the total quantity of iodine excreted per day as iodide and as organic iodine compounds is small in comparison with the other constituents of urine.

Rall (1950) made quantitative estimates of the organic radioiodine compounds in such urine by precipitation with zinc hydroxide according to the Somogyi technique. The organic iodine fraction was also concentrated by butanol extraction and fractionated by phenol-water chromatography. Horst & Heuwieser (1957) employed butanol extraction followed by chromatography in butanol-dioxan-ammonia for the identification and quantitation of radioiodine compounds in urine.

Such methods were found by Fletcher (1956, 1957*a*) to be of limited use as the degree of separation achieved was poor, and in most cases too little of any one organic compound could be isolated to permit a detailed study and conclusive identification. A method was therefore developed in which the organic iodine fraction was concentrated on ion-exchange resin (Zeo-Karb 215) after removal of inorganic iodide on a column of silver chloride. Extraction of the organic fraction into butanol and chromatography of the extract on large sheets of chromatography paper (6 in. $\times$ 18 in., Whatman no. 3MM) effected partial separation of the mixture of iodine compounds. Each component of the original mixture thus fractionated had then to be subjected to further paper chromatography to complete the separation of radioiodine compounds.

The method was applied to the study of the urines of the fifth day after administration of 80 mc of ^{131}I for destruction of normal thyroid tissue and of 150 mc for treatment of thyroid carcinoma, at which time the proportion of radioiodine in organic combination in the urine was maximal, comprising 5–10% of the total. The major components of the organic radioiodine fraction were identified as 3:5-di-iodotyrosine and 3:5-di-iodo-4-hydroxy-phenyl-lactic acid (Fletcher, 1957*b*).

In order to study the other minor components observed by Fletcher (1957*b*) and to isolate the organic iodine fraction from urines excreted 24–72 hr. after therapy, in which the proportion of radioactivity in organic combination is usually only 1–2%, a modification was introduced to avoid the losses caused by deiodination and by decay of activity during the stages of paper chromatography. Kieselguhr-column chromatography was employed (Gross & Pitt-Rivers, 1952, 1953) in the isolation of 3:5:3'-tri-iodothyronine from rat thyroid and in the study of the ^{131}I -labelled compounds excreted in the urine after administration of $[3':5':^{131}\text{I}]_2$ thyroxine and $[3':^{131}\text{I}]$ -3:5-tri-iodothyronine to dogs (Flock, Bollman, Grindlay & Orvis, 1956; Flock, Bollman & Grindlay, 1957). The methods described were applied to the separation of mixtures of iodine compounds of known structure, but the recovery of the iodotyrosines was poor. Similarly, the recovery of organic radioiodine compounds was poor when the methods were used to fractionate the mixture concentrated from urine after ^{131}I therapy. The method described here, which gave an improved recovery of organic radioiodine compounds, was developed by fractionation of aqueous mixtures of iodine compounds of known structure on kieselguhr columns packed in aqueous solvents.

EXPERIMENTAL

The method employed by Gross & Pitt-Rivers (1952, 1953) to obtain chromatographically purified 3:5:3'-tri-iodothyronine from rat thyroid was employed to fractionate mixtures of mono- and di-iodotyrosine. After mixing 10 g. of acid-washed kieselguhr with 8 ml. of 0.5N-NaOH, CHCl_3 saturated with 0.5N-NaOH was added, forming a slurry which was packed into a column (1 cm. $\times$ 30 cm.) with the aid of a glass rammer. A solvent consisting of 20% (v/v) of chloroform in butan-1-ol saturated with 0.5N-NaOH was passed through the column to replace the packing solvent. About 0.5 cm. of asbestos was packed on the top of the column and the mixture of compounds to be fractionated was applied in a minimum volume of 0.5N-NaOH. More of the slurry used for packing was poured on to the column to inhibit the removal of the stationary phase as the eluting solvent passed through the column.

Mixtures of 0.5–200 mg. of mono- and di-iodotyrosine were used, in some cases with addition of [^{131}I]di-iodotyrosine prepared according to the method of Tong, Taurog & Chaikoff (1954). The fractions containing iodotyrosine were detected chemically by the addition of 0.25 ml. of 5% (w/v) sodium arsenite in $\text{N-H}_2\text{SO}_4$ and 0.1 ml. of 1% (w/v) ceric sulphate in $\text{N-H}_2\text{SO}_4$ to 3 or 4 drops of the eluate; 2 μg . of di-iodotyrosine or 1 μg . of iodide was shown to cause a rapid loss of colour from the solution. A drop of each fraction containing iodotyrosine was applied to a strip of Whatman no. 1 chromatography paper for development in butan-1-ol-2N-acetic acid (1:1, v/v). The developed chromatogram was stained with a solution of equal parts of 5% sodium arsenite in $\text{N-H}_2\text{SO}_4$ and 1% ceric sulphate in $\text{N-H}_2\text{SO}_4$ and sprayed with 1% *o*-phenylenediamine solution in acetone (Fletcher & Stanley, 1955).

Only 60–75% of [^{131}I]di-iodotyrosine could be recovered from the column even when elution was continued long enough to strip the stationary phase from the column. Mono- and di-iodotyrosine could not be separated from each other either by elution with butan-1-ol- CHCl_3 (4:1, v/v) as used by Gross & Pitt-Rivers (1952, 1953) for purification of 3:5:3'-tri-iodothyronine or with butan-1-ol-propanol (4:1, v/v) equilibrated with 2N-NaOH (Flock *et al.* 1956, 1957). Separation and recovery were not improved by variation in the pH of the solvent used in packing.

Packing the column in the bottom phase of butan-1-ol- CHCl_3 -water (4:1:5, by vol.) and eluting with the top phase, followed by the top phase of butan-1-ol-propanol-water (4:1:5, by vol.) gave a better recovery of the applied iodine compounds. Iodide was rapidly eluted, followed by thyroxine and more slowly by iodotyrosines. Chromatographic analysis showed that there was some overlap of components, some fractions containing a mixture of thyroxine and iodotyrosine, but the method was found to be useful in fractionation of the mixture of ^{131}I -labelled compounds concentrated from urine after ^{131}I therapy because a better recovery of applied radioiodine was achieved.

Urine collections were made over 24 hr. periods in bottles containing 5 g. of Na_2SO_3 to reduce the likelihood of artifact formation by oxidation, 1 g. of NaI to lower the specific activity of any possible artifacts and 5 g. of Na_2HPO_4 to maintain the pH near neutrality. This last

precaution was necessary because it had been found, in control experiments with normal urine to which ^{131}I -labelled compounds were added, that at alkaline pH values some deiodination of di-iodotyrosine occurs and that, at acid pH values, di-iodotyrosine forms artifacts which have a high R_F on paper chromatograms of the urine run in butan-1-ol-dioxan-aq. 2N- NH_3 soln. (4:1:5, by vol.).

The radioactivity of a sample (10 ml.) of the total collection was measured in the liquid-counting apparatus described in the preceding paper. The proportion of organic radioiodine in the sample was determined by measuring the radioactivity remaining after removal of iodide on a small column of AgCl (1 cm. $\times$ 7 cm.) according to the method developed by Fletcher (1957*a*). Depending upon the proportion of ^{131}I in organic combination, a volume of 100–300 ml. of urine was taken such that 10–20 μC of organic radioiodine could be recovered after removal of radioiodide on a large column of AgCl (3 cm. $\times$ 40 cm.). The organic fraction was concentrated on Zeo-Karb 215 (200–400 mesh, Na form) and eluted in a minimum volume (100–200 ml.) of ethanol-aq. 2N- NH_3 soln. (1:1, v/v) (Fletcher, 1957*a*). The solution was concentrated under reduced pressure at 50° to a volume at which crystallization of the salts still associated with the organic iodine compounds first began to occur (20–50 ml.). The concentrated solution was taken to pH 1–2 with 9% (w/v) HCl and extracted twice with an equal volume, and once with a half volume, of butan-1-ol. The extract was made alkaline with ethanol-aq. 2N- NH_3 soln. (1:1, v/v) and concentrated to 2–5 ml. for column chromatography.

The column was prepared by mixing 75 g. of acid-washed kieselguhr with the bottom phase of butan-1-ol- CHCl_3 -water (4:1:5, by vol.) to form a thin slurry, which was poured into a column (2 cm. $\times$ 50 cm.), care being taken to avoid entrapping air. During the next 4 hr., as more of the

Fig. 1. Elution of the radioiodine compounds of urine from a kieselguhr column. Solvents used were: (1) butan-1-ol- CHCl_3 (4:1) equilibrated with water, followed by (2) butan-1-ol-propanol equilibrated with water. The change of solvent is indicated by the arrow.

bottom phase was introduced, the column slowly packed by sedimentation. The top of the column was allowed to approach dryness in order that the aqueous solution of the radioiodine-containing material could be applied, followed by 1 ml. of water used to wash the flask. In some instances a few drops of concentrated NH_3 soln. were added to the aqueous solution to improve the solubility of the material to be fractionated and thus keep the volume to a minimum (2-5 ml.). When the top of the column again became almost dry, a head of the eluting solvent (the top phase of butan-1-ol- CHCl_3 -water) was introduced. Elution proceeded at the rate of 0.25 ml./min. The eluate was collected in 10 ml. fractions, the radioactivity of each of which was measured in the liquid-counting apparatus. After elution of one large and one small peak, the eluting solvent was changed to the top phase of butanol-propanol-water (4:1:5, by vol.). A typical elution curve is shown in Fig. 1.

The fractions which comprised each peak were selected for further chromatography. For a single peak, the fractions which together contained the major part of the radioiodine compounds were mixed and concentrated to a small volume under reduced pressure at 60°. The residual solutions were applied in narrow bands to sheets of Whatman no. 3MM chromatography paper (6 in. $\times$ 18 in.) for development in butanol-dioxan-aq. 2N- NH_3 soln. (4:1:5, by vol.). Radioautographs were made by leaving the developed chromatograms in contact with Ilford Industrial X-ray film for 16-36 hr. The sections of the chromatograms which contained radioactive components, located by comparison with blackened areas of the film, were cut out. The organic radioiodine component of each section of paper was eluted into ethanol-aq. 2N- NH_3 soln. and the radioactivity of the solution was measured in the liquid-counting apparatus. Samples of the eluates were taken for chromatography in butan-1-ol-dioxan-aq. 2N- NH_3 soln. The ^{131}I -labelled compounds were located by use of a radioautograph or of a scanning device employing a mica end-window Geiger tube. By this means the components of R_F greater than that of iodide in butanol-dioxan-aq. 2N- NH_3 soln. were shown to have been well separated, as no fraction of eluate contained more than one ^{131}I -labelled compound. Since most of the compounds were of unknown structure it was not possible to do control experiments, but the recovery of [3':5'- $^{131}\text{I}_2$]thyroxine was shown to be about 80%.

RESULTS

A selection of analyses is presented in Table 1, in which the recovery of organic radioiodine at each stage of concentration and fractionation is indicated. The fractions listed as *B*, *C* and *D* were all contained in the first peak eluted from the column (Fig. 1, Table 1). Their ^{131}I -labelled compounds were revealed on the radioautograph of the chromatogram of the eluate as sharply defined bands with R_F values greater than that of iodide in butanol-dioxan-aq. ammonia. Fraction *A* was eluted in the second and third peaks (Fig. 1) and consisted of radioiodine compounds of R_F less than that of iodide in butanol-dioxan-aq. ammonia. The major components of this fraction were identified by Fletcher (1957*b*) as 3:5-di-iodotyrosine and 3:5-di-iodo-4-hydroxyphenyl-lactic acid.

Table 1. *Fractionation of urinary iodine after ^{131}I therapy*

No.	Diagnosis	Dose (mc)	Time after dose (hr.)	Amount ($\mu\text{C}/\text{ml.}$ of urine)	Percentage of total activity in organic form	Percentage of organic iodine recovered from resin	Percentage solubility of organic iodine in butanol	Percentage of organic radio-iodine components
1	Thyroid carcinoma	80	24-48	4.2	1.0	58	45	<i>A</i> , 15; <i>B</i> , 25
2	Thyroid carcinoma	150	72-96	4.0	3.3	60	31	<i>A</i> , 15; <i>B</i> , 10; <i>C</i> , 5; <i>D</i> , 5; iodide 5
3	Thyroid carcinoma	150	96-120	1.8	3.8	86	86	<i>A</i> , 30; <i>B</i> , 25; <i>D</i> , 10
4	Thyrototoxicosis	15	24-48	0.1	13.5	84	43	<i>A</i> , 12; 3:5:3'-tri-iodothyronine, 5; iodide, 15

A, Components of R_F less than that of iodide in butan-1-ol-dioxan-aq. 2N- NH_3 soln. (4:1:5, by vol.); *B*, components of R_F greater than that of iodide but less than that of thyroxine in butan-1-ol-dioxan-aq. 2N- NH_3 soln. (4:1:5, by vol.); *C*, components of the same R_F as thyroxine in butan-1-ol-dioxan-aq. 2N- NH_3 soln. (4:1:5, by vol.); *D*, components of R_F greater than that of 3:5:3'-tri-iodothyronine in butan-1-ol-dioxan-aq. 2N- NH_3 soln. (4:1:5, by vol.).

Elution was not always taken to completion, as in Fig. 1, because only the first peak contained the compounds of particular interest for subsequent identification studies. In experiments where complete elution was performed the recovery was at least 75%, that of the example shown in Fig. 1 being 90%. When elution was not completed, the amount of fraction *A* of ^{131}I -labelled compounds (Table 1) was estimated by the difference between the amounts of the applied radioiodine and of the radioiodine eluted in the first peak, applying a correction, if necessary, for any of fraction *A* which was found in the first peak. That such an estimate was justified could be shown by extrusion of the packing material from the column after elution of the first peak, and fractionation of the remaining ^{131}I -labelled compounds by extraction. Of the radioiodine of samples of this material in butan-1-ol solution 80–90% could be removed by one extraction with an equal volume of 2*N*-sodium hydroxide. As a similar distribution of $^{131}\text{I}_2$ diiodotyrosine between 2*N*-sodium hydroxide and butan-1-ol can be demonstrated, it is probably justifiable to regard the ^{131}I -labelled compounds remaining on the column after elution of the first peak as $^{131}\text{I}_2$ diiodotyrosine and $[3:5\text{-}^{131}\text{I}_2]$ diiodo-4-hydroxyphenyl-lactic acid. The amounts of fractions *B*, *C* and *D* were the radioactivities of the ^{131}I -labelled compounds eluted from the chromatograms of the first peak.

Fraction *A* formed the major part of the ^{131}I in organic combination which was recovered after column chromatography of the organic radioiodine of any 24 hr. urine collected from 48 to 142 hr. after ^{131}I therapy for either thyroid carcinoma (19 experiments) or for thyrotoxicosis (two experiments). In some experiments fraction *A* was also the major component during the first 48 hr. after thyroid-carcinoma therapy.

Compounds running between iodide and thyroxine in butanol-dioxan-aq. 2*N*-ammonia (fraction *B*) usually comprised 5–10% of the organic radioiodine in urines collected after thyroid-carcinoma therapy. In two cases, however, 25% of the organic radioiodine was found in this component in urine collected between 24 and 48 hr. after thyroid-carcinoma therapy. None of this component was observed in the urine of two cases examined after ^{131}I therapy for thyrotoxicosis.

The radioactivity of the component of R_f similar to that of thyroxine in butan-1-ol-dioxan-aq. 2*N*-ammonia (fraction *C*) was usually about 5% of the total organic radioiodine in urines collected more than 48 hr. after thyroid-carcinoma therapy, but was frequently absent between 0 and 48 hr. This component was not detected in the urine after thyrotoxicosis therapy and was shown

by work described in the next paper to be separable by paper chromatography from thyroxine and from 3:5:3'-tri-iodothyronine.

Thyroxine and 3:5:3'-tri-iodothyronine were never detected in the urine after thyroid-carcinoma therapy, but 3:5:3'-tri-iodothyronine formed a substantial part of the organic radioiodine fraction after thyrotoxicosis therapy (see Table 1).

The zone near the solvent front in the butanol-dioxan-aq. 2*N*-ammonia system (fraction *D*), containing 5–25% of the organic radioiodine, probably consisted of a physical mixture of ^{131}I iodide and pigments, or of chemically unstable artifacts. When the fraction was eluted and subjected to a second paper-chromatographic fractionation, only ^{131}I iodide was detected on radioautographs or on scans. As the compounds formed by di-iodotyrosine in urine at acid pH have R_f values in the butanol-dioxan-aq. 2*N*-ammonia system similar to that of this zone, but are chemically stable, it is unlikely that the zone consists of any such artifact.

DISCUSSION

The failure of the methods of kieselguhr-column chromatography developed by Gross & Pitt-Rivers (1952, 1953) and by Flock *et al.* (1956, 1957) to give a satisfactory recovery of urinary organic radioiodine compounds is probably caused by the presence of a preponderance of organic material other than iodine compounds in the mixture of material which is concentrated from urine and applied to the column. Under these conditions the chromatographic properties of the iodine compounds will have little or no effect on the order of elution of the components of the mixture of organic compounds. The method developed here is probably more like an extraction process than a true chromatographic fractionation, but considerable advantages are gained by its introduction into the procedure of Fletcher (1957*a*) for concentration and fractionation of the iodine compounds of urine after ^{131}I therapy. First, the iodine fraction may be more rapidly and completely purified, and secondly the components of the iodine fraction are more easily fractionated in subsequent paper chromatography. It was found that if the method developed by Fletcher were used, minor components had to be submitted to several paper-chromatographic fractionations before a degree of purification was achieved which was sufficient to permit further study to be undertaken. Each stage of paper chromatography was, of course, accompanied by losses due to radioactive decay and to deiodination. In the method described here these fractions may be eluted from the first paper chromatograms in a state of purity enabling further studies to be begun at once.

The method cannot be used to measure the amount of each ^{131}I -labelled compound quantitatively because of the losses of organic radioiodine (see Table 1) at each step. Quantitative analyses of the organic radioiodine fraction is probably best performed by removal of iodide on a column of silver chloride followed by butanol extraction and paper chromatography of the remaining organic iodine compounds. The method described here is primarily useful for concentrating the organic iodine compounds for qualitative study. Its use is limited to urines of relatively high radioactivity, at least $0.003\ \mu\text{C}$ of organic radioiodine/ml., and it is therefore not applicable to study of organic radioiodine compounds of urine after tracer doses of ^{131}I . As the method is not quantitative, the relative amount of each component does not give any insight into the turnover of iodine *in vivo*, and may in fact reflect merely the efficiency of the method, some compounds being more completely recovered than others.

SUMMARY

1. A method for fractionation of urinary radioiodine activity after ^{131}I therapy has been modified to give improved resolution and recovery of organic radioiodine compounds.

2. The organic radioiodine compounds of the

urine after ^{131}I therapy for thyroid carcinoma were divided into four fractions, one of which (fraction A) contained di-iodotyrosine and 3:5-di-iodo-4-hydroxyphenyl-lactic acid, previously identified by Fletcher (1957*b*).

3. After ^{131}I therapy for thyrotoxicosis, fraction A was isolated from the urine but the other fractions were not detected.

4. The method is not suitable for analysis of urines excreted after tracer doses of ^{131}I , nor for quantitative measurement of the amounts of the organic radioiodine compounds of urine after ^{131}I therapy.

REFERENCES

- Fletcher, K. (1956). Ph.D. Thesis: University of London.
Fletcher, K. (1957*a*). *Biochem. J.* **67**, 136.
Fletcher, K. (1957*b*). *Biochem. J.* **67**, 140.
Fletcher, K. & Stanley, P. G. (1955). *Nature, Lond.*, **175**, 730.
Flock, E. V., Bollman, J. L. & Grindlay, J. H. (1957). *Amer. J. Physiol.* **189**, 420.
Flock, E. V., Bollman, J. L., Grindlay, J. H. & Orvis, A. L. (1956). *Amer. J. Physiol.* **187**, 407.
Gross, J. & Pitt-Rivers, R. (1952). *Lancet*, **1**, 439.
Gross, J. & Pitt-Rivers, R. (1953). *Biochem. J.* **53**, 645.
Horst, W. & Heuwieser, H. (1957). *Strahlentherapie*, **102**, no. 3.
Rall, J. E. (1950). *J. clin. Endocrin.* **10**, 996.
Tong, W., Taurog, A. & Chaikoff, I. L. (1954). *J. biol. Chem.* **207**, 59.

Radioiodine Compounds of Human Urine after ^{131}I Therapy

2. CHROMATOGRAPHIC COMPARISON WITH IODINE COMPOUNDS OF KNOWN STRUCTURE

By CHRISTINA CAMERON

Medical Research Council, Department of Clinical Research, University College Hospital Medical School, London, W.C. 1

(Received 4 May 1959)

Thyroxine is the major iodine-containing compound in the circulation (Taurog & Chaikoff, 1948) but in certain circumstances, for example after administration of ^{131}I in therapeutic amounts for the treatment of thyroid carcinoma or thyrotoxicosis, labelled thyroxine (Robbins & Rall, 1952; Gordon, Gross, O'Connor & Pitt-Rivers, 1952), 3:5:3'-tri-iodothyronine (Gross & Pitt-Rivers, 1952), 3:5-di-iodotyrosine and 3-mono-iodotyrosine (Benua & Dobyns, 1955), thyroglobulin (Robbins & Rall, 1952) and an iodoprotein resembling serum albumin (Robbins, Rall & Rawson, 1955) may also be found. The labelled iodine compounds of urine have not been as

thoroughly investigated as those of plasma. 3:5-Di-iodotyrosine and thyroxine were reported to be present by Rall (1950), thyroxine and 3:5:3'-tri-iodothyronine by Horst & Heuwieser (1957). Fletcher (1957*a, b*) detected a total of eight labelled iodine compounds in the urine 6-7 days after ^{131}I therapy for thyroid carcinoma. Two were identified as 3:5-di-iodotyrosine and 3:5-di-iodo-4-hydroxyphenyl-lactic acid, but neither thyroxine nor 3:5:3'-tri-iodothyronine was detected. With the method developed by Fletcher (1957*a*), modified as described in the preceding paper, some of the unidentified urinary iodine compounds were isolated for further study.