The Estimation of Iodine in Biological Materials: a Modification of the Method of Ellis & Duncan

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(Received 12 March 1958)

Most methods described in recent years for the estimation of iodine in biological materials employ a preliminary step in which organic matter is ashed and iodine is converted into iodide. This may be achieved by an acid wet-ashing technique during which the iodine is oxidized to iodate by chromium trioxide or potassium permanganate; the iodate is treated with phosphorous acid or oxalic acid and the liberated iodine distilled into alkali, forming iodide (Chaney, 1940; Talbot, Butler, Saltzman & Rodriguez, 1944; Taurog & Chaikoff, 1946; Barker, 1948; Connor *et al.* 1949; Kydd, Man & Peters, 1950; Moran, 1952; Ellis & Duncan, 1953; Butler & Johnson, 1957). Alternatively, iodide is liberated from organic material by fusion with alkali and eluted with acid (Barker, Humphrey & Soley, 1951; Brown, Reingold & Samson, 1953; Grossmann & Grossmann, 1955; Acland, 1957). The amount of iodine isolated by these procedures is usually determined by measuring its catalytic effect on the ceric sulphate-arsenious acid reaction, which can be followed spectrophotometrically.

We required a method which could be applied to the analysis of iodine in water, milk and urine, and of protein-bound iodine in serum. The alkaline-incineration technique was discarded because of the large sample volumes. A method employing the Riggs modification of the Chaney still was found to be unsatisfactory in our hands, as the recovery of iodine from iodide in aqueous solution was 80% with a standard deviation of 23. The procedure used was based on the methods of Talbot *et al.* (1944) and Taurog & Chaikoff (1946), and is used in the Veterinary Laboratory of the Ministry of Agriculture and Fisheries, Weybridge, Surrey (private communication). Butler & Johnson (1957) have stressed the importance of the dimensions of the still, and our stills may have been faulty.

The technique described by Ellis & Duncan (1953) also gave very variable results when applied to the analysis of iodide in aqueous solution. After the digestion and distillation procedures, as well as the spectrophotometric estimation of iodide had been modified, a method was developed which permits a more reliable estimation of micromilli-

gram quantities of iodine in water, milk, urine, serum and other biological materials.

EXPERIMENTAL

General procedure for all samples

Cleaning of apparatus. All glassware is cleaned overnight in chromic acid-sulphuric acid. It is thoroughly washed with tap water and then five times with a special water which has been purified by a Permutit Deioniser (portable Deminrolit plant) and subsequent distillation from an all-glass still. Glassware is dried in a hot-air cabinet at 50°.

Reagents. [Mainly according to Ellis & Duncan (1953) and Lein & Schwartz (1951).] Chromium trioxide solution: British Drug Houses Ltd. laboratory reagent (recrystallized), 50% (w/w) solution in water. Sulphuric acid: A.R., concentrated. Phosphorous acid: 1000 g. of H_3PO_3 (British Drug Houses Ltd. laboratory reagent, crystalline) was boiled with 1.5 l. of water for 2 hr., cooled and diluted to 2 l. with water. Hydrogen peroxide: A.R. 30% (w/v). Arsenious acid solution, 0.1 M: 9.9 g. of As_2O_3 (A.R.) was dissolved in 60 ml. of hot N-NaOH and 200 ml. water was added; the solution was neutralized (pH 7) with sulphuric acid and diluted to 1 l. with water. Sodium chloride reagent: 50 g. of NaCl (A.R.) dissolved in 7 N- H_2SO_4 to give 1 l. of solution. Ceric ammonium sulphate solution, 0.01 M: 6.33 g. of $Ce(SO_4)_2 \cdot 2(NH_4)_2SO_4 \cdot 2H_2O$ (low in other rare earths, Harrington Brothers Ltd.) was dissolved in 3.6 N- H_2SO_4 to give 1 l. of solution. Sodium arsenite solution: $NaAsO_2$ (British Drug Houses Ltd. laboratory reagent) was dissolved in water to give 1% (w/v) solution. Standard potassium iodide solution: 1 ml. of solution contained 100 μ g. of iodine. When the iodine content of the combined reagents exceeds 100 μ g., this is usually due mainly to the sulphuric acid or the chromium trioxide. The impure reagents are replaced by sufficiently pure ones.

Apparatus. Digestion is carried out in a 125 ml. Kjeldahl flask, and from this flask the iodine vapour is subsequently distilled. The distillation apparatus (Fig. 1) is based on the Ellis & Duncan (1953) still, with the following modifications:

- Introduction of the three-way tap (T).
- Introduction of potassium hydroxide and calcium chloride traps.
- Alterations in the sizes of jets A, B and C to 1.2, 1.6 and 1.2 mm. respectively.
- Connexion of the Kjeldahl flask and the traps with rubber tubing to allow flexibility.

The flow meters were supplied by Rotameters Ltd., and were calibrated to measure from 100 to 5000 ml. of air/min.

Digestion of sample. Duplicate samples, between 0.5 and 50 ml., are measured into 125 ml. digestion flasks. After addition of 5 ml. of chromium trioxide solution and six glass beads (diam. 4.5 mm.) the mixture is shaken and cooled in ice-water while 30 ml. of concentrated sulphuric acid is added carefully, with constant mixing. A 0-250° thermometer is placed in the flask, which is then heated on a sand bath; the sand is 0.5 cm. deep and heat is supplied by Bunsen burners fitted with flame spreaders operating at maximum intensity with the top of the flame touching the sand tray. When the temperature of the mixture reaches 180° the flask is removed, the contents allowed to cool to 100° and 15 ml. of water is then added. Into the flask is introduced a piece of right-angled glass tubing of internal diameter 0.5 cm. and with the vertical arm drawn out into a jet of internal diameter, 0.2 cm., which extends to the bottom of the flask. The mixture is heated to 200° and then removed from the sand bath. A stream of air from a compressed-air cylinder is blown through the glass tubing into the flask at a pressure of 1-2 lb./in.² for 3 min. to remove volatile interfering substances, mainly SO₂. When the digest has cooled to 100°, the thermometer and glass tubing are washed with water and removed from the flask; water is added until the volume of liquid is about 50 ml.

Distillation. The flask is placed in the water bath at 90° for 10 min. and the sections of the distillation apparatus are connected by means of rubber tubing of 6 mm. internal diam. which has been washed with purified water. Volumes (2 and 1 ml.) of 1% sodium arsenite solution are placed in traps 1 and 2 (see Fig. 1) and air is drawn through the apparatus at a rate of 1600 ml./min., the three-way tap being turned so that all the air first passes through the potassium hydroxide solution. Phosphorous acid (3 ml.) is measured into a 10 ml. beaker and drawn from there into the apparatus by holding the beaker at point X (Fig. 1) and turning the tap momentarily in the appropriate direction. The beaker is washed twice with small volumes of water and the washings are drawn into the digest. Hydrogen peroxide (0.2 ml.) is added in the same way. After 5 min., another 3 ml. portion of phosphorous acid is mixed with the liquid in the flask, and this is repeated 5 min. later. After a further 5 min. the air flow is stopped, the two traps are detached from the still and their contents quantitatively transferred to a 25 ml. graduated cylinder, the volume being made up to 20 ml. with purified water.

Spectrophotometric estimation of iodine. Sodium chloride reagent (2 ml.) and 0.1 M-arsenious acid (2 ml.) are measured

into 15 cm. × 1.5 cm. Pyrex test tubes. Duplicate 5 ml. portions of the distillates are added and the total volume in each tube is made up to 10 ml. with water. All the tubes are covered with a piece of Parafilm (Gallenkamp and Co. Ltd., Sun Street, London, E.C. 2), and shaken well and placed in a water bath at room temperature, which usually lies between 15° and 26°. The bath is placed in a draught-free position so that the temperature of the water does not vary by more than $\pm 0.2^\circ$ over a period of an hour. After 15 min. has been allowed for equilibration, 1 ml. of 0.01 M-ceric ammonium sulphate solution is added to the first tube and, at 45 sec. intervals, to the remaining tubes, each one being shaken thoroughly before being replaced in the bath. After a suitable time interval, which varies from 20 min. at 26° to 40 min. at 15°, some of the solution from the first tube is transferred to a 1 cm. glass cuvette, and the extinction ($E_{1\text{ cm.}}$) measured at 363 m μ in a Unicam spectrophotometer (SP 600). The amount of iodine present in the distillate portion is calculated from a calibration curve prepared as described below, which relates $\log E_{1\text{ cm.}}$ to iodide concentration. The total amount of iodine in both the sample and the reagents together is found by multiplying this value by four.

Reagent blank. Purified water equal in volume to the sample is analysed in duplicate as described above, and the amount of iodine present in the reagents is calculated. This amount is subtracted from the value for sample and reagents to give the iodine content of the sample.

Calibration curve. Volumes (0.25, 0.50, 0.75 and 1.00 ml. respectively) of the standard potassium iodide solution are added to tubes containing sodium chloride reagent (2 ml.) and arsenious acid (2 ml.); 0.75 ml. of sodium arsenite solution is measured into each tube to equal the amount of this reagent in 5 ml. of distillate. A fifth tube containing no added iodide is prepared in a similar manner. The total volume of all five tubes is made up to 10 ml. with purified water and the spectrophotometric analysis carried out as described above. The plot of the logarithm of the extinction against the amount of iodine added as iodide to each tube is linear.

A calibration curve is prepared for each set of estimations, and the five tubes containing the standard iodide solution are mixed randomly with those prepared from the samples.

Iodine estimation in various materials

Natural waters. A volume (50 ml.) of the water is measured in a digestion flask, and the digestion reagents are added. The analysis is carried out as described above.

Milk. Approx. 2 g. of milk is added to a 10 ml. beaker, and the exact weight is determined. The milk is then transferred to a digestion flask together with 20 ml. of water. The iodine content is estimated by the general procedure.

Urine. The human urine is collected over a 24 hr. period, in a 2.5 l. bottle containing 20 ml. of toluene. The volume of urine analysed by the general procedure varies from 2 to 8 ml., depending on the intake of iodine.

Protein-bound fraction in serum. A volume (3 ml.) of serum is mixed with 20 ml. of water in 70 ml. polythene bottles fitted with screw caps, and 3 ml. of 10% (w/v) sodium tungstate and 3 ml. of 0.67 N-sulphuric acid are added (Moran, 1952). The bottle is completely filled with water before centrifuging to prevent collapse at the neck,

Fig. 1. Diagram of the distillation apparatus. For explanation see text. (Scale: 3.2 mm.: 1 in.)

and is then spun at 5500 g in a BaraGyro centrifuge for 20 min. The supernatant is decanted and the residue suspended in water by shaking vigorously. A volume (3 ml.) of 0.67 N-sulphuric acid is added, and then 3 ml. of 10% sodium tungstate solution, and the contents of the bottle are well mixed. The bottle is filled with water and centrifuged as before. The procedure is repeated once more, and the washed precipitate transferred with 20–25 ml. of water to a digestion flask.

Recovery experiments

Recovery of ^{131}I . In all radioactivity measurements, 10 ml. of solution was counted in a Twentieth Century Electronics liquid counter, type M6H. Counts were corrected for background count, and were accurate to $\pm 2\%$ unless otherwise stated. Radioactive iodide was obtained in the form of tracer ^{131}I in dilute sodium thiosulphate solution at pH 8–10 (Radiochemical Centre, Amersham, Bucks). The tracer iodine was diluted with potassium iodide solution until the total concentration of iodine was approx. 100 $\mu\text{mg./ml.}$, and the radioactive count was approx. 2000 counts/min./ml. A volume (2 ml.) of this solution was used to determine the recovery of iodine from small volumes of an aqueous solution of potassium iodide, and from volumes of urine (sample A) varying from 2 to 10 ml.

Recovery of iodine from potassium iodide. (1) *In aqueous solution.* Volumes of potassium iodide solution varying from 0.5 to 5 ml. were added to digestion flasks, and the sides of the flasks were washed down with small volumes of water. The iodine contents of the samples were determined, and the percentage recovery of iodine was calculated.

To determine the recovery of iodine under the conditions used in the analysis of natural waters, 2 ml. of the standard iodide solution was diluted to 50 ml. before digestion.

(2) *In milk.* The iodine content of milk (sample A) was determined in the way described above; 2 ml. of standard iodide solution was added to ten samples of milk A and the mixtures were analysed. The iodine content of milk (sample B) was estimated and 0.5 ml. of standard iodide solution was added to each of five samples of this milk.

(3) *In urine.* Samples (3 ml.) of a urine (sample B) were analysed as described above. A volume (2 ml.) of the standard iodide solution was used to determine the percentage recovery of iodine from urine in the presence of toluene.

Recovery of protein-bound ^{131}I . ^{131}I -radio-iodinated human-serum albumin (0.24 ml.), containing 4.8 mg. of albumin (Radiochemical Centre, Amersham, Bucks.) was diluted with bullock serum until 3 ml. of the diluted material gave a count of approx. 2000 counts/min. The radio-iodinated albumin contained not more than 2% of free iodine, and a correction was applied for this amount. The serum was treated as described in this paper, and the recovery of the bound iodine was calculated. The effect on the recovery when tungstic acid was omitted in the washing procedure was determined.

Two solutions, A and B, containing known amounts of ^{131}I were prepared by diluting tracer ^{131}I in sodium thiosulphate solution with potassium iodide solution. In solution A 0.2 ml. contained about 30 $\mu\text{mg.}$ of iodine, and 0.2 ml. of solution B contained about 330 $\mu\text{mg.}$ of iodine. Four polythene bottles, each containing 0.2 ml. of solution A and 3 ml. of bullock serum, were incubated for 2 hr. at 37°. The protein-bound iodine was precipitated and washed in the usual way, and the supernatant, washings and

washed precipitate were all collected, and their radioactivity was determined as a percentage of the amount added. This was repeated by using 0.2 ml. of solution B, and incubating for 16 hr. with 3 ml. of bullock serum before precipitating the protein.

RESULTS AND DISCUSSION

Comparison of the present method with that of Ellis & Duncan

Digestion. The digestion stage of chromic acid-ashing methods for estimating iodine was regarded by Moran (1952) as being the most critical part of the analysis. He suggested that excessive heating results in loss of iodine, probably due to partial reduction of chromic compounds and of iodate, and that insufficient heating does not expel chlorides, which interfere with the catalytic action of iodine on the ceric sulphate–arsenious acid reaction. Ellis & Duncan (1953) reported that if only one portion of water is boiled off in the digestion, the passage of air through the digest for 10 sec. periods at 1–2 min. intervals is necessary in order to remove substances which would cause inaccuracies in the spectrophotometric estimation of iodine. In this Laboratory, passing a current of air through the liquid for 3 min. after digestion has been completed has been found to be equally satisfactory in removing these interfering substances.

When the end points of the two stages of the digestion were determined according to the method of Ellis & Duncan (1953) the mean recovery of radioactive iodine from radioactive Na^{131}I was 86%, S.D. 12.6% (five estimations). With the method described in this paper, the recovery of ^{131}I was 94%, S.D. 2.2% (four estimations). Calculation of the variance ratio showed that there was a significant decrease in accuracy when the procedure of Ellis & Duncan (1953) was adopted ($P = 0.01$ – 0.001).

If the temperature at the end of the first heating in the digestion was raised from 180° to 200°, the recovery of radioactive iodine was unaffected, and this was also true if the digest was heated to 220° in the second stage of the oxidation process.

Distillation. The estimation of iodine by the use of a still designed by Chaney (1940) and a chromic acid-ashing method was investigated by Moran (1952), who found that the ratio of the weight of phosphorous acid added in the distillation to the weight of chromium trioxide used in the digestion is an important factor in obtaining the maximum recovery of iodine, the optimum ratio being 3 g. of phosphorous acid to 2 g. of chromium trioxide.

In the method of Ellis & Duncan (1953), the ratio of the weights of phosphorous acid to chromium trioxide is 1:3.2. We found it convenient to use 4 g. of chromium trioxide rather than 3.2 g.

because in milk analyses 2 g. of a high-fat milk reduces more than 3 g. of chromium trioxide. The addition of 1.25 g. of phosphorous acid in the distillation gives approximately the same ratios of the reagents to each other as those quoted by Ellis & Duncan (1953); this amount of phosphorous acid is contained in 2.5 ml. of the 50 % phosphorous acid (Table 1, group *a*). Chaney (1940) found that with some bottles of phosphorous acid the recovery of iodine is inexplicably low, but that the addition of 2 drops of 30 % (w/v) hydrogen peroxide improves the recovery (cf. Moran, 1952 and Table 1, group *b*). Taurog & Chaikoff (1946) did not use hydrogen peroxide in their method of estimating iodine as they found that it frequently caused a green colour to appear in the distillate.

Moran (1952) reported that the recovery of iodine increases with the length of the distillation time, and concluded that the reduction of iodate does not take place immediately on the addition of the reducing agent, possibly due to the presence of a complex between iodate and chromic acid. For this reason phosphorous acid was added in several portions (Table 1, groups *e*, *f*, *g*).

In order to test the efficiency of the distillation under different conditions, known amounts of Na^{131}I were ashed and the percentage of ^{131}I recovered in the distillate was calculated (see Table 1). It is shown by Student's *t* test after

using an angular transformation that there was no significant difference in the mean percentage recoveries of groups *b* to *g* inclusive, but that the addition of hydrogen peroxide in the distillation causes a significant increase in the percentage recovery of ^{131}I ($P < 0.001$). There was a significant decrease in variance when 6 ml. of phosphorous acid was added in two fractions at intervals of 5 min. rather than as one portion at the beginning of the distillation ($P = 0.05-0.02$). Omission of the arsenious acid affected neither the mean percentage recovery nor the variance. Arsenious acid has therefore been omitted in the final method.

With the treatment described in group *g* (Table 1) and sodium arsenite as the trap solution the mean recovery of 100–400 $\mu\text{mg.}$ of iodine added as potassium iodide was 90.3 % (29 estimations); with the 200 $\mu\text{mg.}$ amount, the s.e. of a single observation was 5.0 %. At the point in the estimation at which the distillation was usually terminated, a further 3 ml. of phosphorous acid was added to the flask and distillation continued for a further 5 min. Eleven determinations were thus carried out in which the samples for analysis were 200 $\mu\text{mg.}$ quantities of iodine as potassium iodide, and 12 determinations were made of 50 $\mu\text{mg.}$ amounts of iodine. The mean recovery of 200 $\mu\text{mg.}$ of iodine was 98 %, s.e.m. 1.9, and that of 50 $\mu\text{mg.}$ of iodine was 102 %, s.e.m. 5.8. The increases in the mean percentage recoveries of 200 and 50 $\mu\text{mg.}$ of iodine were significant ($P = 0.02-0.01$ and $P = 0.01-0.001$ respectively) but there was no significant increase in reproducibility.

The method of distillation shown in Table 1, group *g*, was adopted as the usual procedure, after considering the accuracy of the technique, the mean percentage recovery of added iodide and the time taken to carry out the estimation.

Trapping of distilled iodine. The composition of the solution which traps the distilled iodine in methods using chromic acid and phosphorous acid has varied considerably. Sodium hydroxide was found to be satisfactory by Taurog & Chaikoff (1946) and Moran (1952). In his early experiments, Barker (1948) used sodium hydroxide to trap the iodine and obtained very low recoveries when the iodine in the distillate was estimated spectrophotometrically, but on replacing the sodium hydroxide by sodium sulphite the recovery was increased to 90 %. In this procedure the sulphite had to be removed before the colorimetric estimation, as otherwise it caused immediate fading of the ceric ammonium sulphate. Ellis & Duncan (1953) trapped the iodine with a mixture of sodium hydroxide and sodium arsenite; the arsenite did not interfere with the spectrophotometric estimation.

By the method described in this paper, except that the trap solutions were replaced by those of

Table 1. *Effects of varying the types and quantities of reagents added during the distillation*

Four analyses were carried out for each group of results. Portions (2 ml.) of a solution of Na^{131}I containing approx. 200 $\mu\text{mg.}$ of iodine were treated as described in the text and distilled under the conditions specified in the table. The amount of ^{131}I recovered in the distillate was determined as described in the text. The arsenious acid solution contained 0.013 g. of arsenic trioxide/ml. in 2.5 N-sulphuric acid and was added in three 1 ml. portions at the beginning of the distillation. Hydrogen peroxide was added at the beginning of the distillation. When more than one portion of phosphorous acid was added, the first addition was made at the commencement of the distillation, and subsequent additions were made at 5 min. intervals. Distillation was continued for 15 min. after the addition of the first batch of reagents. The solution used to trap the iodine was a mixture of sodium hydroxide and sodium arsenite (Ellis & Duncan, 1953).

Group	Volume of reagents added during distillation (ml.)			Mean recovery (%)	S.D.
	H_3PO_3	As_2O_3	H_2O_2		
<i>a</i>	2.5	3	—	52	16.8
<i>b</i>	2.5	3	0.2	87	6.3
<i>c</i>	2.5	3	0.5	87	8.7
<i>d</i>	6.0	3	0.2	89	8.3
<i>e</i>	2 × 3	3	0.2	95	2.6
<i>f</i>	3 × 3	3	0.2	96	1.3
<i>g</i>	3 × 3	—	0.2	93	2.3

Ellis & Duncan (1953), the mean percentage recovery of radioactive iodine from Na^{131}I was 92 %, s.d. 5 % (14 estimations). Under the same conditions the apparent recovery of iodine from

potassium iodide, determined with the spectrophotometric procedure as the final stage of the analysis, was very variable, frequently being of the order of only 50 %. The iodine was therefore being carried into the trap, but either (i) the iodine was present in a non-catalytic form, such as iodate, or (ii) some substance was interfering with the catalytic action of the iodine. Iodine distilled over into sodium hydroxide in the form of iodine or hypoiodous acid gives rise to iodate.

Ellis & Duncan (1953) added 1 ml. of 1 % (w/v) sodium hydroxide and 3 drops of 0.1N-sodium arsenite to trap 1 and 0.5 ml. of 1 % sodium hydroxide to trap 2. These were changed to 1.5 ml. of 1 % (w/v) sodium arsenite in trap 1 and 0.5 ml. of 1 % sodium arsenite in trap 2. Recoveries of iodine estimated spectrophotometrically averaged 87 % over the linear portion of the curve but fell when the sample iodine exceeded 280 $\mu\text{mg.}$ (Fig. 2a). Increasing the volume of arsenite to 2 ml. in trap 1 and 1 ml. in trap 2 increased the recovery of iodine to 89 % in the higher range (Fig. 2b).

The importance of the rate of air flow through the still was stressed by Ellis & Duncan (1953), who reported that the use of an air flow lower than 1500 ml./min. results in incomplete transfer of iodine into the traps, whereas when this rate is exceeded, the distillate is coloured green owing to the transference of chromium ions. With the jet sizes quoted in their paper, experiments showed that even at an air flow of 1500 ml./min. some of the distillates were pale green in colour. In the Ellis & Duncan (1953) still, the tips of the jets increased in size in the direction in which the air passed through the still. A slight vacuum was therefore formed in the Kjeldahl flasks during the distillation and this appeared to facilitate the transfer of chromium ions as well as of iodine into the traps. It was found that if the jets were made approximately equal in diameter, thus avoiding the vacuum, the flow could be raised to over 2000 ml./min. without a green colour appearing in the distillate or the percentage recovery of iodine from iodide altering significantly.

Spectrophotometric determination of iodide

In most methods in which the reduction of Ce^{4+} ions by arsenious acid has been followed in the visible part of the spectrum, the wavelength at which measurements have been made has been 415 or 420 $\text{m}\mu$. The absorption curve of ceric ammonium sulphate solution is shown in Fig. 3. Because the absorption of this solution at 363 $\text{m}\mu$ is about nine times greater than at 420 $\text{m}\mu$ the former wavelength was used. Although there is a maximum in the absorption curve at 315 $\text{m}\mu$ (Weybrew, Matrone & Banley, 1948; Freedman &

Fig. 2. Effect of the volume of 1 % sodium arsenite solution in the still traps on the relation between the amount of iodine added to the reagents before analysis and the extinction at 363 $\text{m}\mu$ at the end of the spectrophotometric determination of iodide. (a) Volumes of potassium iodate solution containing 88–346 $\mu\text{mg.}$ of iodine were analysed as described in the text, except that the still traps contained 1.5 and 0.5 ml. of 1 % sodium arsenite solution respectively. (b) Volumes of potassium iodide solution containing 100–400 $\mu\text{mg.}$ of iodine were analysed as described in the text, the volume of 1 % sodium arsenite solution in the traps being 2 and 1 ml. respectively.

Hume, 1950), measurements could not be carried out at wavelengths below $363\text{ m}\mu$ owing to limits imposed by the spectrophotometer.

The effect of the concentration of I^- ions on the rate of the reduction of Ce^{4+} ions by arsenious acid is shown in Fig. 4. The reaction can be followed satisfactorily at room temperature and the time required for the extinction to change by a suitable amount varied from 40 min. at 15° to 20 min. at 26° .

In the spectrophotometric analysis of solutions containing known amounts of iodine, the standard error of a single result was $1.4\text{ }\mu\text{mg.}$, for amounts of iodine between 25 and $100\text{ }\mu\text{mg.}$ (48 results).

*Estimation of iodine in the presence
of other compounds*

It is more convenient to prepare a calibration curve by adding iodide to water and sodium arsenite, rather than to the distillate from a reagent blank. This practice assumes that no compound which distills from the reagents into the traps has any effect on the catalytic action of iodine on the ceric-arsenite reaction.

To determine if this assumption is correct, twenty-five tubes containing known amounts of standard iodide solutions were analysed spectrophotometrically, and the log extinction values at the end of the reaction time were calculated. At the same time nineteen portions of the distillates from

reagent blank analyses were mixed with known amounts of iodide solution, and treated in a similar manner. The regression coefficients of the log extinction, on the amount of iodide added, were calculated.

The experiment was completed by the analysis of duplicate samples of 500 mg. of sodium chloride and of 500 mg. of sodium bromide. The distillates from these analyses were divided into portions and to each portion a known amount of iodide was added. After spectrophotometric analysis of these solutions, the regression coefficients of $\log E$, on the amount of iodide added, were calculated (Table 2).

The values of the regression coefficients showed that there was no interference with the catalytic action of iodine due to any compounds distilled from the reagents, and that even the presence of very large quantities of chloride and bromide in the sample analysed did not affect the rate at which the ceric-arsenite reaction was catalysed by the iodine.

The analyses did not show any difference between the iodine content of the reagents, and of

Fig. 3. Absorption curve of ceric ammonium sulphate. The absorption of a 0.3 mm -solution of ceric ammonium sulphate was measured in a Unicam spectrophotometer model SP. 600, in 1 cm. glass cuvettes.

Fig. 4. A calibration curve for the spectrophotometric estimation of iodide. Volumes (0.25 , 0.50 , 0.75 and 1.00 ml.) of standard potassium iodide solution were added to 2 ml. of sodium chloride reagent (100 mg. of NaCl in $7\text{N-H}_2\text{SO}_4$), 2 ml. of 0.1M -arsenious acid and 0.75 ml. of 1% sodium arsenite solution. Water was added to give a total volume of 10 ml. , and the tubes were placed in a water bath at room temperature (15.1°) for 15 min. A volume (1 ml.) of 0.01M -ceric ammonium sulphate solution was added to each solution, 45 sec. elapsing between successive additions. After a reaction time of 37 min. the extinction $E_{1\text{ cm.}}$ was measured at $363\text{ m}\mu$ in a Unicam spectrophotometer model SP. 600.

Table 2. *Effect of compounds other than iodides on the catalytic action of iodine on the ceric ammonium sulphate-arsenious acid reaction*

The reagents, and the reagents with added chloride or bromide, were digested, and the iodine was distilled as described in the text. Volumes of standard iodide solution from 0.25 to 1.0 ml. were added to portions of the distillates and the solutions analysed spectrophotometrically.

Solution to which KI was added	$10^3 \times$ regression coefficient (log $E/\mu\text{mg.}$ of I added)	No. of points from which regression coefficient was calculated
Water (calibration curve)	5.589	25
Distillate from reagent blank	5.597	19
Distillate from analysis of 500 mg. of NaBr	5.639	8
Distillate from analysis of 500 mg. of NaCl	5.671	8

the reagents with added chloride or bromide. In order to ensure that the presence of chloride did not cause the loss of any iodine from the sample being analysed, the recovery of iodine from potassium iodide containing 200 $\mu\text{mg.}$ of iodine in the presence of 500 mg. of sodium chloride was determined and found to be 94 %.

If the spectrophotometric determination is carried out with the omission of the sodium chloride reagent, the presence of large amounts of chloride in the sample analysed results in interference with the catalytic action of the distilled iodine on the reduction of Ce^{4+} ions by arsenious acid. If the sample contains a large amount of bromide, this effect is not observed. The addition of Cl^- ions in the spectrophotometric analysis of iodide has been recommended by several workers (Barker, 1948; Connor *et al.* 1949; Carr, Graham, Ober & Riggs, 1950; Lein & Schwartz, 1951). Lein & Schwartz (1951) have suggested that this addition of a large excess of Cl^- ions prevents the

combination of I^- ions with undefined contaminants. These contaminants would otherwise have interfered with the catalytic action of the iodide. It is possible that the reactions between chloride, sulphuric acid and chromium trioxide lead to the subsequent distillation of similar contaminants; when bromide is substituted for chloride these contaminants do not seem to be formed.

Fluoride ions at concentrations less than 50 $\mu\text{g.}/10 \text{ ml.}$ do not interfere with the spectrophotometric determination of iodide. At larger concentrations of fluoride the catalytic action of iodide is partially inhibited.

Recovery experiments

Aqueous potassium iodide solution. The recovery of quantities of iodine between 50 and 400 $\mu\text{mg.}$ added in the form of potassium iodide in from 0.5 to 4 ml. of solution was determined by the present method (Table 3).

There was no significant difference between the variance values for the different amounts of iodine, but there was a significant difference in the recovery of 50 $\mu\text{mg.}$ of iodine compared with that of 100 $\mu\text{mg.}$ of iodine ($P=0.02-0.01$). The standard error of a single estimation was 10.1 $\mu\text{mg.}$ of iodine, and the mean percentage recovery of quantities between 100 and 400 $\mu\text{mg.}$ of iodine inclusive was 90.3 %.

Table 3. *Recovery of iodine from small volumes (0.5-4 ml.) of an aqueous solution of potassium iodide*

Volumes (0.5, 1.0, 2.0, 3.0 and 4.0 ml.) of standard potassium iodide solution were analysed by the present method.

Iodine added ($\mu\text{mg.}$)	No. of estimations	Mean recovery (%)	S.D.
50	10	72	14
100	14	90	11
200	6	90	4
300	5	93	5
400	4	88	2

Table 4. *Recovery of iodine from milk samples*

The iodine content of weighed portions of milk (1.74-3.329 g.) was determined as described in the text. Standard potassium iodide solution (2 or 0.5 ml.) was added to further weighed portions of the same milk and the recovery of the added iodine was calculated.

Milk sample	No. of determinations	Mean wt. of milk analysed (x g.)	Iodine added to x g. of milk ($\mu\text{mg.}$)	Mean total iodine in x g. of milk ($\mu\text{mg.}$)	Variance	Mean recovery (%)	Mean total iodine in milk sample $\pm$ S.E.M. ($\mu\text{g./kg.}$)
A	10	1.82	—	53	22.9	—	28.9 ± 0.8
A	10	1.82	200	244	87.3	95.3	—
B	5	2.62	—	24	92.0	—	9.3 ± 1.4
B	5	2.37	50	70	23.3	96.8	—

The low concentration of iodine in natural waters necessitated the use of 50 ml. samples in order to obtain an amount of iodine within the range of the method. Experiments were carried out to determine if the use of these large volumes interfered with the recovery of iodine. The mean recovery of 200 μ mg. of iodine was found to be 90 %, s.d. 6.2 % (eight estimations). It was concluded that the large volume of water taken did not affect the recovery of iodine from iodide in aqueous solution.

Milk. Samples of two milks, A and B, were analysed to determine their iodine content, and then known amounts of iodine were added as potassium iodide, and the determinations were repeated (Table 4).

There was only a small difference between the recovery of 50 and 200 μ mg. quantities of iodine, and no significant difference between the variance values for amounts of iodine between 24 and 244 μ mg. The lower limit for the estimation of iodine in milk is of the order of 50 μ mg. of iodine, compared with 100 μ mg. in waters.

Urine. Stanbury *et al.* (1954), using the modification of the method of Barker (1948) described by Carr *et al.* (1950), found that the recovery of radioactive iodine from urine decreases as the volume of urine taken for analysis increases. In order to determine whether this is true for the

method described in this paper, the following experiment was carried out. Volumes of urine measuring between 2 and 10 ml. (urine A) were pipetted into digestion flasks and the sides of each flask washed down with water. Radioactive iodine was added to each sample and the results of the analyses are shown in Table 5.

The mean percentage recovery increased when the volume of urine used was increased from 8 to 10 ml., and this increase was significant ($t=3.1$, $P=0.02-0.01$). For volumes between 2 and 8 ml. there was no change in the percentage recovery of iodine.

The effect of toluene in the urine on the analysis of iodine was tested in samples of urine B before and after the addition of known amounts of iodine as iodide. The iodine content of 3 ml. of urine was found to be 106 μ mg., s.e.m. 2.9 (nine estimations). The 95 % confidence limits for the true mean recovery of 200 μ mg. of added iodine lay between 93 and 102 %. Thus the recovery of iodine from urine is not affected by the presence of toluene in the sample. The coefficient of variation for the determination of iodine in urine, calculated from the data for urine B, was 8.2 %.

The mean recovery of iodine from volumes of urine between 2 and 8 ml. was 89.8 % (Table 5).

Serum-protein-bound iodine. Volumes (3 ml.) of ^{131}I radio-iodinated serum were analysed by the procedure described for estimation of protein-bound iodine. The mean percentage recovery of protein-bound ^{131}I was 85 %, s.d. 2.6 (eleven estimations). In a series of analyses in which the addition of sodium tungstate and sulphuric acid was omitted during the washing of the precipitate the mean recovery was 66 %, s.d. 3.3 % (six estimations).

In order to test whether non-protein-bound iodide can become occluded with the precipitate, especially when the level of iodide is abnormally high, a series of experiments were carried out in which radioactive iodide was incubated with serum before precipitation. The division of the iodide between the supernatant, washings and washed

Table 5. Recovery of ^{131}I from different volumes of urine

Portions (2 ml.) of Na^{131}I solution containing approx. 200 μ mg. of iodine were added to samples of urine (2-10 ml.). Recovery of ^{131}I was determined as described in the text.

Volume of urine (ml.)	Percentage recoveries		Mean percentage recovery
2	88.3	93.2	90.7
4	88.5	88.7	88.6
6	88.5	91.5	90.0
8	91.1	88.5	89.8
10	92.7	96.7	94.7

Table 6. Recovery of ^{131}I added as inorganic iodide to serum before precipitation of protein-bound iodine

A volume (0.2 ml.) of a solution of Na^{131}I containing approx. 33 μ mg. of iodine was added to 3 ml. of bullock serum and the mixture incubated for 2 hr. at 37°. The protein-bound iodine was precipitated and washed and the amount of ^{131}I in the supernatant and washings was determined as described in the text. The washed precipitate was dissolved in sodium tungstate solution and the radioactivity of the solution determined. This was repeated with 0.2 ml. of Na^{131}I solution containing approx. 330 μ mg. of iodine. Radioactivity in the supernatant was determined to an accuracy of 2 %, that in the first washing to 7 % and that in subsequent washings and the residue to 10 %.

Approx. amount of I added (μ g.)	No. of estimations	Mean recovery of ^{131}I (% of total)				
		Supernatant	Wash 1	Wash 2	Wash 3	Residue
33	4	91	6	0	0	0
330	8	92	9	2	1	1

precipitate was determined (Table 6). It was concluded that only two washings were necessary even if the level of inorganic iodine in the serum was abnormally high.

Some other aspects of the method. Alkaline-incineration techniques appear to be satisfactory only when protein is present in the sample being analysed (Acland, 1957). A similar effect of protein has also been observed in some acid-ashing methods (Taurog & Chaikoff, 1946; Barker, 1948). In the present method the recovery of iodine is satisfactory in the presence or absence of protein, and no protein need be added to the sample. Acland (1957) has pointed out that one disadvantage of some acid-ashing processes is the relatively low recovery of the iodine present in the sample. In our method the recovery of iodine from aqueous potassium iodide solutions can be increased from 90 to 98 % if desired by extending the distillation time and adding more phosphorous acid. Wet acid ashing does, however, require more constant attention than alkaline-incineration methods, but it can be applied to a wider range of materials. One further disadvantage is the relatively high iodine content of the blank, mainly due to iodine in the sulphuric acid and chromium trioxide. Matthews, Curtis & Brode (1938) have recommended boiling the acid with concentrated hydrochloric acid to remove the iodine, but we have not found this to be satisfactory.

SUMMARY

1. The method of Ellis & Duncan (1953) for the estimation of protein-bound iodine in serum has been extensively modified and applied to the estimation of iodine in natural waters, milk and urine.

2. Factors which were found to be relevant were the temperatures during digestion, the quantities and types of the reagents added during distillation and the nature of the reagents used for trapping the distilled iodine.

3. The iodide obtained on distillation was determined by a modification of the method of Lein & Schwarz (1951), which rests on the catalytic action of iodide on the reduction of Ce^{4+} ions by arsenious acid. The reduction was measured spectrophotometrically at 363 m μ and the reaction was carried out at room temperature.

4. Chloride and bromide were without effect on the estimation of iodine, even when the ratio of chlorine or bromine to iodine exceeds 1.5×10^6 to 1. Concentrations less than 50 $\mu g.$ of fluoride/10 ml. did not interfere with the spectrophotometric estimation of iodide.

5. The recovery of iodine, added as iodide, from an aqueous solution of potassium iodide (200 $\mu g.$

of iodide in 50 ml. of solution) was 90 %, s.d. 6.2 (eight estimations). Five determinations on a sample of cow's milk gave a mean of 9.3 $\mu g.$ of iodine/kg. of milk, s.d. ± 3.7 ; ten determinations on another sample of milk gave a mean of 28.9 $\mu g.$ of iodine/kg. of milk, s.d. 2.6. The mean recovery of iodine from milk samples was 96 % (sixteen estimations). The mean iodine content in a 3 ml. sample of human urine was 106 $\mu mg.$, s.e.m. 2.9 (nine estimations), and the mean percentage recovery of iodine from urine was 89.8 % (eight estimations). The mean recovery of ^{131}I -radioiodinated protein-bound iodine from serum was 85 % (eleven estimations).

We wish to thank Professor Sir Hans Krebs, F.R.S., Dr D. E. Hughes and Dr Dagmar C. Wilson for their interest in the work, Dr A. Rodgers for much helpful advice and Mr J. F. Scott of the Department of Biometry for assistance with the statistical analyses. The work was aided by a grant from the Chilean Iodine Educational Bureau.

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