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The Estimation of Serum Protein-Bound Iodine by Alkaline Incineration

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The term 'protein-bound iodine' (PBI) is used to describe that fraction of blood iodine which is non-dialysable and precipitates with serum or plasma proteins (Trevorrow, 1939). In the absence of structural damage to, or carcinoma of, the thyroid, about 75–100% of serum PBI consists of L-thyroxine, bound to protein, in a way as yet undefined, from which it can be released by denaturation. About 90% of the thyroxine is selectively combined with a 'thyroxine-binding-protein' fraction, intermediate in properties between α_1 - and α_2 -globulins, while the remainder is associated with albumin (Taurog & Chaikoff, 1948; Gordon, Gross, O'Connor & Pitt-Rivers, 1952; Petermann, Robbins & Hamilton, 1954; Robbins, Petermann & Rall, 1955). Of the other physiologically active compounds secreted by the thyroid, 3:5:3'-tri-iodothyronine is bound to protein in a similar way to thyroxine, though less strongly (Deiss, Albright & Larson, 1953), whereas no data are available for

3:3'-di-iodothyronine or for 3:3':5'-tri-iodothyronine (Roche, Michel, Wolf & Nunez, 1956).

Salter & McKay (1944) were the first workers to describe a method of estimating serum PBI, in which organic material was destroyed by alkaline incineration. Liberated iodide was then determined, after elution, by its catalytic effect on the reduction of ceric salts by arsenious acid (Sandell & Kolthoff, 1937). This technique was subsequently modified by Barker & co-workers (Barker & Humphrey, 1950; Barker, Humphrey & Soley, 1951). Brown, Reingold & Samson (1953) and Grossmann & Grossmann (1955) described further modifications, which were claimed to increase the accuracy of the method. Doubt was cast by some of these authors on certain aspects of the technique, different workers not being always in agreement about the efficiency of a particular step.

The present investigation was started with the dual object of resolving the contradictions between

the findings of previous workers and of developing from the latter a reasonably reliable method. A re-investigation of all the stages of the analysis was undertaken. The results obtained with the zinc hydroxide method of precipitating PBI were compared with those observed when trichloroacetic acid precipitation was used. The effect of increasing the ashing time from $2\frac{1}{2}$ to $3\frac{1}{2}$ hr. and that of increasing the ashing temperature range from 590 – 610° to 640 – 660° were investigated and the iodide-elution procedures described by the above authors were compared. The kinetics of the reaction between ceric and arsenite ions were re-investigated from the point of view of its analytical application, and interference with this reaction by zinc salts was studied. The percentage recovery of DL-thyroxine added to serum was determined. Preliminary reports have appeared (Acland, 1955, 1956).

EXPERIMENTAL

Determination of serum PBI

The complete method finally adopted for serum is described in this section. Variants tested are dealt with later. No elementary iodine is allowed to be introduced into the room in which analyses are carried out.

Glassware. Before being used, all glass apparatus was kept immersed, at least overnight, in an aqueous solution of Dymex (J. W. Towers and Co. Ltd.), an alkaline detergent, to remove traces of adsorbed iodide. Immediately before use, the glassware was first rinsed thoroughly with tap water, test tubes being cleaned with a brush, and then washed three times with water purified as described below. Pipettes were dried with acetone on a filter pump, other glassware in a hot-air oven at 90° .

Reagents. All chemicals were AnalaR grade except HCl, H_2SO_4 ('Microanalytical reagent,' Hopkin and Williams Ltd.) and ceric ammonium sulphate ('Laboratory reagent low in other rare earths', British Drug Houses Ltd.). All water was purified by distillation from a metal still and passage through two columns of Amberlite Monobed MB-1 ion-exchange resin. The second resin treatment was given immediately before use. Distillation from permanganate or from alkali was not tried as satisfactory results were obtained with water purified in this way.

The KI reference solution contained 130.8 mg. of the desiccator-dried salt/l. ($\equiv 100\mu\text{g. of I}^-/\text{ml.}$). The ceric ammonium sulphate solution contained 39 g. of salt in 1 l. of $3.5\text{N-H}_2\text{SO}_4$. Arsenious acid was prepared by dissolving 3.8 g. of As_2O_3 in 50 ml. of warm N-NaOH . To the cooled solution were added 50 ml. of water and $3.5\text{N-H}_2\text{SO}_4$ to 500 ml.

Precipitation of PBI. To 1 ml. of serum in a 16 mm. $\times$ 125 mm. Pyrex tube was added water (7 ml.), followed by 1 ml. of 10% (w/v) $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and 1 ml. of 0.5N-NaOH , the contents of the tube being thoroughly stirred after each addition by means of a glass rod. The protein precipitate was allowed to stand for 3 hr. and then separated by centrifuging in an angle centrifuge at 2000 rev./min. for 10 min. The supernatant was decanted and the precipitate washed three times by resuspending it in successive 10 ml.

portions of water, stirring with a glass rod, centrifuging and discarding the washings. A fine suspension of the precipitate in 1 ml. of $4\text{N-Na}_2\text{CO}_3$ was then prepared with the glass rod, from which traces of precipitate were washed into the tube by adding 0.5–1 ml. of water down the rod.

Incineration of protein precipitate. The precipitate was dried overnight at 95 – 98° in an electric oven. The tube containing the dried precipitate was then placed in a muffle furnace for $3\frac{1}{2}$ hr. at 600° , the furnace door being left slightly open (about $\frac{1}{4}$ in.) to create a draught. The Pyrex tubes were discarded after having been used three times, since they became badly etched and difficult to clean.

Estimation of released iodide. After the tube had cooled, 1 ml. of 2N-HCl was added carefully to the residue and thoroughly mixed with a glass rod. Water (6 ml.) was added and the contents of the tube were allowed to stand for $\frac{1}{2}$ hr., during which time they were stirred three times with a glass rod. The reaction mixture was then transferred to a conical centrifuge tube and the carbonized residue separated by centrifuging for 20 min. at 3000 rev./min., swing-out buckets being used.

Duplicate 3 ml. samples of the clear supernatant were pipetted into thin-walled 16 mm. $\times$ 150 mm. Pyrex test tubes. To each of these samples were added 1 ml. of water, 0.5 ml. of arsenious acid solution and 1 ml. of $3.5\text{N-H}_2\text{SO}_4$, in that order, and the contents of the tubes mixed by 'flicking' as described below. The tubes were then incubated for 10 min. at $37 \pm 0.1^\circ$ in a water bath. When this period had elapsed, 0.5 ml. of ceric ammonium sulphate solution at 37° was added and the contents of the tube were mixed quickly. The most satisfactory way of carrying out the latter operation was found to be the following: the tube is held just below its rim in the left hand and the bottom of the tube flicked with the four fingers of the right hand in quick succession, the movement being repeated about 15 times.

Exactly 12.5 min. after the time when the addition of the ceric ammonium sulphate was begun, the extinction at $415\text{ m}\mu$ of the solution contained in a 1 cm. cuvette was measured in a prism spectrophotometer (SP. 600, Unicam Instruments Ltd., Cambridge). Filling of the cuvette was begun 45 sec. before this time. It was found convenient to allow 1 min. between successive additions of ceric ammonium sulphate. This enabled a maximum of six serum samples to undergo iodide estimation in one batch, since duplicate iodide determinations were carried out on each sample.

The amount of 'added iodide' (i.e. iodide present in excess of that contaminating the reagents) in the reaction mixture was read from a calibration curve, connecting $E_{1\text{cm.}}$ at $415\text{ m}\mu$ with $\mu\text{g. of 'added iodide'}$, whose derivation is described below. Multiplication of the figure thus obtained by 700/3 will give the result in $\mu\text{g. of I}/100\text{ ml. of serum}$.

Reagent blanks. A reagent blank was run with each group of estimations, 1 ml. of water replacing the 1 ml. sample of serum, and the entire analytical procedure being carried out as with serum. The reagents have been found sometimes to take up small amounts of iodine on keeping.

Construction of the calibration curve. The calibration curve was constructed by adding known amounts of iodide to reagent blanks. This was most easily done during preparation of the final solutions for iodide estimation. In place of 1 ml. of water, duplicate 1 ml. samples of solutions containing respectively 0.02, 0.04, 0.06, 0.08 and $0.10\mu\text{g. of I}^-/\text{ml.}$ were added to tubes containing 3 ml. of the eluate from a reagent

blank; a reagent blank containing no added iodide provided the zero point. The reduction of the ceric ion was then initiated and measurements were carried out as described above for the serum samples. The mean value of $E_{1\text{cm}}$ was plotted on semi-logarithmic graph paper against $\mu\text{g.}$ of 'added iodide' and a straight-line curve drawn to express the relation between them (see Fig. 2c). A fresh curve was constructed whenever a fresh solution of any reagent was made up.

Variants in method of PBI determination

Precipitation of PBI with trichloroacetic acid. Trichloroacetic acid precipitation was carried out according to Tong, Taugo & Chaikoff (1954). A serum sample (1 ml.) was added to 1 ml. of 20% (w/v) trichloroacetic acid (TCA), followed by 3 ml. of 10% (w/v) TCA and the suspension was thoroughly stirred with a glass rod. The precipitate was centrifuged off, washed three times in the centrifuge with 10 ml. of 2.5% (w/v) TCA and dissolved with stirring in 1 ml. of 4N- Na_2CO_3 , the glass rod being washed down with 1 ml. of water. The subsequent analytical procedure was the same as for Zn(OH)_2 precipitation.

Calibration curves and reagent blanks for solutions containing no zinc. Solutions for constructing calibration curves appropriate to conditions in which Zn^{2+} and TCA are absent were prepared from reagent blanks, consisting of 1 ml. of 4N- Na_2CO_3 and 1 ml. of water, treated in the same way as has been described for Zn(OH)_2 precipitates. With TCA precipitation the reagent blanks consisted of 1 ml. of 4N- Na_2CO_3 , 0.5 ml. of 2.5% (w/v) TCA and 1 ml. of water, and were dried and ashed with the precipitated protein. Reagent blanks of this composition were used to prepare the iodide standards.

Elution of iodide after ashing of protein. (1) *Method according to Barker et al.* (1951). To the ash were added successively 2 ml. of 2N-HCl, 2 ml. of 7N- H_2SO_4 and 3 ml. of water, the suspension being stirred vigorously after each addition and the residue was centrifuged off when evolution of H_2S had ceased. Subsequent steps were the same as in the definitive method described above.

(2) *Method according to Brown et al.* (1953). The reagent volumes were modified in order to make the final dilution of eluate correspond with that used by Barker *et al.* (1951). To the ash was added 2.5 ml. of 2N-HCl followed by 4.5 ml. of water. The residue was centrifuged off when evolution of H_2S had ended and the remaining steps of the analysis were as described above.

In both these modifications, reagent blanks, and consequently the solutions used for constructing calibration curves, were treated in the same manner as the ashed serum.

Chromatography of plasma extracts

Extracts were made from a total of five samples of plasma (containing 0.1 mg. of heparin/ml.) from five different patients suffering from thyrotoxicosis. The samples were obtained 48–96 hr. after the administration by mouth of therapeutic doses of radio-iodine (4–20 mc.). Plasma proteins were precipitated with ZnSO_4 and NaOH, as described above, except that the protein suspension was shaken vigorously by hand in a stoppered container, and the washed precipitate was suspended in 5 ml. of water. The suspension was acidified to pH 4 with 2N-HCl, thereby redissolving the protein, and extracted three times with

10 ml. portions of water-saturated *n*-butanol. The *n*-butanol extracts were combined and evaporated to dryness under reduced pressure at 80° and the residue was taken up in 0.5 ml. of *n*-butanol. *n*-Butanol extracts were prepared in the same way from the combined supernatant and washings.

Duplicate samples (50 $\mu\text{l.}$) of the extracts were placed, 10 $\mu\text{l.}$ at a time, on Whatman no. 1 paper, and chromatographed by the descending method in *n*-butanol-dioxan-2N- NH_3 soln. (4:1:5, by vol.) as described by Gross, Leblond, Franklin & Quastel (1950). Carrier quantities (25 $\mu\text{g.}$) of non-radioactive thyroxine, di-iodotyrosine and tri-iodothyronine were added to the spots before developing the chromatogram. The amino acids were detected by spraying the paper with 0.2% ninhydrin in water-saturated *n*-butanol and heating it at 90° for 5 min.

The chromatogram was cut into strips 2 cm. wide, each strip corresponding to a different run, and the radioactivity present in each 1 cm. length was measured with a previously described apparatus (Acland, 1952). Each section of paper was counted for 5 min.

Recovery of PBI from serum

DL-Thyroxine solution was prepared by dissolving 157.3 mg. of the sodium salt in water, to which 2 ml. of N-NaOH had been added to increase the solubility of the amino acid, and diluting to 500 ml. with water. This solution was then diluted 1:200 with water. Both solutions were freshly made up when required. To 1 ml. of serum was added 0.1 ml. of the dilute thyroxine solution and the mixture incubated at 37° for 2 hr. The normal analytical procedure was then followed. The amount of thyroxine thus added to each serum sample increased its PBI concentration by 10 $\mu\text{g.}$ of I/100 ml.

RESULTS

Iodide estimation by the reaction $\text{Ce}^{4+} \rightarrow \text{Ce}^{3+}$

The use of the reaction between ceric salts and arsenious acid for iodide estimation (Sandell & Kolthoff, 1937) depends upon the fact that the reduction of the ceric ion is normally a pseudo-unimolecular reaction, the rate-limiting process being the reaction between ceric ions and iodide (Glasstone, 1948). Hence, at constant temperature, the kinetic equation for a reaction of the first order can be applied. The catalysis of this reaction by iodide is an example of homogeneous catalysis in solution. Hence we can write (Glasstone, 1948):

$$k = k' + k_1 C_1, \quad (1)$$

where k is the specific reaction rate for the reduction of ceric ion, k' is a constant, giving the specific reaction rate in the absence of 'added iodide' (added iodide is defined as iodide in excess of that contaminating the reagents), k_1 is the catalytic coefficient for 'added iodide' and C_1 is the concentration of 'added iodide'. Therefore

$$k' + k_1 C_1 = \frac{2.303}{t} \log_{10} \left(\frac{a}{a-x} \right), \quad (2)$$

where a is the initial concentration of ceric ion, and $(a-x)$ the concentration after time t . It follows

that, if t and α are constant, there is a linear relation between C_I and $\log_{10} (a-x)$. These conditions are fulfilled in the analytical procedure described above. The extinction at $415\text{ m}\mu$ can be substituted for the ceric ion concentration, provided that the two are linearly related. Hence a linear relation between extinction and iodide concentration should result if the graph is plotted on semi-logarithmic paper. A kinetic approach, somewhat similar to the above, has been used by Caraway (1952) to eliminate the use of graphs in the method of Barker *et al.* (1951).

Experiments were performed, as described below, to test the validity of the arguments put forward in this section.

Relationship between extinction at $415\text{ m}\mu$ and ceric ion concentration. The optical density of ceric salts in solution has been widely assumed, usually tacitly but sometimes explicitly (by Caraway, 1952), to follow Beer's law. That this is not necessarily the case is shown by the curve derived by Chaney (1940), which differs significantly from a straight line. The relationship between extinction and ceric ion concentration was re-investigated by making a series of dilutions of the ceric ammonium sulphate solution prepared for the iodide estimation at a constant concentration of sulphuric acid. The extinction of each solution at $415\text{ m}\mu$ was measured and Fig. 1 shows that Beer's law is strictly obeyed.

Effect of interfering substances on the kinetics of the reaction $\text{Ce}^{4+} \rightarrow \text{Ce}^{3+}$. Barker (1948) has shown that chloride, while not itself catalysing the reduction of ceric salts by arsenious acid, increases the catalytic effect of iodide. Klein (1954) has demonstrated that

Fig. 1. Relation between extinction at $415\text{ m}\mu$ and concentration of ceric ammonium sulphate in $0.29\text{ N-H}_2\text{SO}_4$.

sulphate decreases the catalytic effect of iodide. Experiments were performed to determine how, if at all, the kinetics of the reaction, $\text{Ce}^{4+} \rightarrow \text{Ce}^{3+}$, were affected by the presence of these ions, and also of zinc, whose influence on the reaction had not previously been studied.

Calibration curves were constructed relating iodide concentration to extinction at $415\text{ m}\mu$, both in the presence and in the absence of zinc. The solutions used for constructing these curves were prepared by adding known amounts of iodide to reagent blanks, as described in the Experimental section. The procedure was repeated with the reagents appropriate to each of the three methods of dissolving iodide from the ashed-protein precipitate which were tested, namely those of Barker *et al.* (1951), Brown *et al.* (1953) and Grossmann & Grossmann (1955), the last-named being described as part of the definitive method in the Experimental section. Fig. 2 shows that the expected linear relation between $\log_{10} E$ and iodide concentration is obtained in each case if all the reagents used in the analytical procedure, other than zinc, are present. In the presence of zinc, however, in the first two methods there is a decrease in the catalytic effect of iodide and the curve shows a departure from linearity (see Fig. 2a, b). It should be noted that in both the above cases, iodide is eluted from the ash in acid conditions, hence all the zinc passes into solution. Grossmann & Grossmann (1955) use an eluent which remains alkaline, and zinc is left behind in the residue. With this technique the addition of either zinc or trichloroacetic acid to the reagent blanks does not affect the calibration curve for the determination of iodide (see Fig. 2c). At the four lower iodide concentrations, the extinction values obtained for the three different solutions were too close together to be plotted separately.

The non-linearity of the calibration curve in the presence of zinc, with acid eluents, was further studied in the following experiment. Two series of iodide standards containing $0.1\text{ }\mu\text{g.}$ of added iodide were prepared from reagent blanks by the elution method of Barker *et al.* (1951). One series of tubes contained zinc and the other did not, and the kinetics of the reaction, $\text{Ce}^{4+} \rightarrow \text{Ce}^{3+}$, in the two conditions were investigated by plotting $E_{1\text{ cm.}}$ at $415\text{ m}\mu$ against reaction time on semi-logarithmic graph paper (see Fig. 3). Each point represents the average of duplicate determinations in different tubes. First-order reaction kinetics are followed both in the presence and in the absence of zinc for approximately the first 15 min. Hence the non-linearity of the calibration curves obtained in the presence of zinc after a reaction time of 12.5 min. (Fig. 2a, b) would appear to have arisen because the decrease in the catalytic coefficient of iodide caused by this ion is greater at higher iodide concentrations.

Extraction of iodide from ashed serum protein

Recent workers have chosen to acidify immediately the highly alkaline ash that remains after heating dried serum protein with sodium carbonate,

Fig. 2. Calibration of the reaction between ceric and arsenite ions for iodide determination. Relationship between $E_{1 \text{ cm.}}$ at 415 m μ after 12.5 min. reaction at 37° and weight of iodide added to the eluates from ashed reagent blanks containing protein precipitants. (a) Elution method of Barker *et al.* (1951); (b) method of Brown *et al.* (1953); (c) method of Grossmann & Grossmann (1955). Reagent blanks: x, 2N-Na₂CO₃ (2 ml.); o, 2N-Na₂CO₃ (2 ml.) and Zn(OH)₂ [$\equiv$ 1 ml. of 10% (w/v) ZnSO₄·7H₂O]; +, 2N-Na₂CO₃ (2 ml.) and 0.5 ml. of 2.5% (w/v) trichloroacetic acid.

in order to break up the residue and to make its soluble components easy to dissolve out (Barker *et al.* 1951; Brown *et al.* 1953). However, Grossmann & Grossmann (1955), although treating the residue with acid in order to break it up, do not add sufficient to neutralize all the alkali present. Acidification is delayed until the final stages of the iodide estimation. The Experimental section contains details of the above methods, which were modified slightly for the purposes of the present investigation, and the results of comparative tests undertaken to determine which was the best of the three procedures for dissolving iodide from the ash are given in Table 1. All determinations were carried out on samples from the same specimen of normal serum in a 2½ hr. ashing period. The highest estimate of the mean PBI concentration was observed when the extraction method of Grossmann & Grossmann (1955) was used. After extraction according to Barker *et al.* (1951) and Brown *et al.* (1953) some results were found to be below what has been stated to be the range in normal men: 4–8 $\mu\text{g.}$ of PBI/100 ml. of serum (Roche & Michel, 1955). Furthermore, a coefficient of variation of less than 5% was observed only when the elution method of Grossmann & Grossmann (1955) was employed. The last-named technique was consequently adopted when other stages of the analysis were tested. It is described in the Experimental section as part of the recommended procedure, whose overall efficiency was investigated in experiments to be described later.

Fig. 3. Kinetics of the reaction between ceric and arsenite ions in the presence of 0.1 $\mu\text{g.}$ of iodide added to the eluate from ashed reagent blanks, the elution method of Barker *et al.* (1951) being used. Symbols for reagent blanks are as in Fig. 2.

Table 1. *Estimated PBI concentration in the same serum specimen (1 ml. samples) with different methods being used to dissolve iodide from ashed protein*

Method	Eluent (ml.)			No. of determinations	PBI concn. ($\mu\text{g. of I/100 ml.}$)	
	2N-HCl	7N-H ₂ SO ₄	Water		Mean	Range
Barker <i>et al.</i> (1951)	2.0	2.0	3.0	62	2.9 $\pm$ 1.08 (s.d.)	0.63-4.67
Brown <i>et al.</i> (1953)	2.5	—	4.5	11	4.5 $\pm$ 0.88 (s.d.)	2.03-5.18
Grossmann & Grossmann (1955)	1.0	—	6.0	11	5.2 $\pm$ 0.215 (s.d.)	4.78-5.60

Fig. 4. Efficiency of PBI precipitation demonstrated by chromatograms prepared from the plasma of a thyrotoxic patient treated with ^{131}I . The distribution of radioactivity and the position of amino acids added as carriers are shown. (a) Protein precipitate from 1 ml. of plasma precipitated with 1 ml. of 10% (w/v) $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and 1 ml. of 0.5N-NaOH. (b) Supernatant and washings from (a). (c) Supernatant and washings from 1 ml. of plasma precipitated with 0.5 ml. of 10% (w/v) $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.5 ml. of 0.5N-NaOH. DIT, 3,5-di-iodotyrosine; THY, thyroxine; TRI, 3,5,3'-tri-iodothyronine. The peak of radioactivity in (b) and (c), with R_f approx. 0.3, is attributed to inorganic iodide.

Precipitation and ashing of PBI

The efficiency of the precipitation of PBI with zinc hydroxide was studied both chromatographically and by chemical methods. Zinc hydroxide and trichloroacetic acid precipitation were compared by means of repeated analyses on samples from the same serum specimen.

Thyroxine, in combination with serum or plasma protein, was thought to provide a suitable standard for testing the efficiency of the method since it is the largest component of serum PBI. Reproducibility formed a further criterion for the chemical

determinations. It was considered necessary that the coefficient of variation of the method should be less than 5% and that there should be no demonstrable difference between batches of analyses carried out on the same specimen at different times. No direct estimates of accuracy could be made since the serum specimens were not analysed by an independent method of known performance.

Efficiency of PBI precipitation. Chromatographic analysis of extracts, prepared as described above from the plasma of thyrotoxic patients who had been treated with radio-iodine, showed the following results. In the five samples studied, 2.5, 3, 3.5, 2 and

Table 2. Results of seventy-five determinations of the PBI content of a specimen of normal serum

PBI content is expressed as $\mu\text{g. of I/100 ml.}$ Ashing time was $2\frac{1}{2}$ hr.

Batch	...	1	2	3	4	5	6	7	8	9
No. of determinations		10	11	9	5	10	7	11	8	4
Mean		6.28	5.76	5.39	5.81	6.01	5.71	5.69	5.75	6.02
Range		6.00-6.42	5.51-6.18	5.13-5.67	5.67-6.13	5.70-6.16	5.25-6.23	5.48-5.83	5.53-5.99	5.90-6.23

1 % respectively of the total radioactivity associated with thyroxine was present in the combined supernatant and washings. The results obtained from the first serum are shown graphically in Fig. 4. Despite very vigorous shaking no difficulty was experienced in centrifuging down the precipitate, contrary to the findings of Barker *et al.* (1951). However, a further portion of the first serum was precipitated with half the usual concentration of zinc hydroxide. The washed precipitate was difficult to centrifuge down and Fig. 4c shows that 22 % of the thyroxine remained in the supernatant. In the protein precipitate, a small amount of radioactivity was associated chromatographically with tri-iodothyronine (about 5 % of that present in the thyroxine area). In no case did the combined supernatant and washings show radioactivity in the tri-iodothyronine area, the thyroxine spot alone among the amino acids containing radioactivity. The additional peak in Figs. 4b and 4c, with R_f approx. 0.3, is in the position where carrier-free radio-iodide was found in the same chromatographic system (Acland, 1952). This peak can therefore reasonably be identified with inorganic iodide, although further investigation with the ascending method and the same solvents gave an R_f for radio-iodide, both with and without carrier (50 $\mu\text{g. of KI}$), of 0.4-0.45. The R_f values in Fig. 4b are smaller than those in Fig. 4c, perhaps because of the higher salt concentration in the former extract.

In view of the errors inherent in the method of measuring the radioactivity in the chromatograms, the results quoted above cannot be more than semi-quantitative. It can, however, be concluded from them that large losses of endogenous PBI are unlikely to occur during protein precipitation with zinc hydroxide, provided that the method described in the Experimental section is adhered to.

Efficiency of the ashing procedure. A series of seventy-five determinations was carried out on a serum specimen obtained from a euthyroid subject (see Table 2). From these results a mean of 5.8 $\mu\text{g. of PBI/100 ml.}$ and a standard deviation of $\pm 0.31 \mu\text{g. of PBI/100 ml.}$ were calculated. In a further series of eight experiments, 0.1 $\mu\text{g. of iodine}$ was added as the sodium salt of DL-thyroxine to 1 ml. samples of the same serum. The mean recovery was 96.5 % with standard deviation of $\pm 2.1 \%$ (range 93.5-99.8 %). Superficially, these results,

Table 3. Comparison between zinc hydroxide and trichloroacetic acid as precipitants of PBI in samples from the same serum specimen

Serum PBI concn. is in $\mu\text{g. of I/100 ml.}$ Each result is the mean of five determinations.

Ashing time (hr.)	Zn(OH) ₂ precipitation		Trichloroacetic acid precipitation	
	Mean	Range	Mean	Range
$2\frac{1}{2}$	5.64	5.39-5.79	5.43	5.25-5.76
	4.94	4.78-5.02	5.47	4.78-5.95
	5.32	5.13-5.53	5.03	3.97-5.48
	4.84	4.78-4.90	5.46	5.06-5.67
	4.85	4.67-5.13	5.20	4.78-5.53
$3\frac{1}{2}$	5.10	4.83-5.44	5.15	4.78-5.53
	5.03	4.90-5.24	5.19	5.13-5.20
	4.79	4.60-5.02	5.11	4.85-5.44

which were quoted in a preliminary communication (Acland, 1956), seem satisfactory. However, on inspection of Table 2, the difference between batches of results which were obtained on different days appears larger than one would expect from the scatter of values within the same batch. This impression was strengthened by an analysis of variance which showed that the 'between-batch' variance was significantly greater than the 'within-batch' variance ($P \leq 0.001$).

As described below, this difference between batches was finally attributed to a failure in thermostatic control. Before this conclusion was reached, however, some experiments were carried out to determine whether trichloroacetic acid was a more satisfactory reagent for precipitating PBI than zinc hydroxide. These will be described next.

Batches of ten samples were taken from a serum specimen, five of which were precipitated by zinc hydroxide and five by trichloroacetic acid. The washed precipitates were ashed for $2\frac{1}{2}$ hr. The results of five such batches of determinations are given in Table 3.

With zinc hydroxide precipitation, the mean serum PBI concentration was found to be 5.1 $\mu\text{g./100 ml.}$ and the standard deviation $\pm 0.35 \mu\text{g./100 ml.}$ For trichloroacetic acid precipitates, corresponding figures were $5.3 \pm 0.41 \mu\text{g./100 ml.}$ Separate analyses of variance were carried out on the results of each precipitation method.

The difference between the means of batches precipitated by zinc hydroxide was again found to be statistically significant in an analysis of variance ($P \leq 0.001$). This 'between-batch' difference was not found when trichloroacetic acid was used as a precipitant. The latter method, however, shows greater variability within batches than the former. This difference in variability was found to be statistically significant ($P < 0.002$ in a two-tailed F test).

It was noticed that some of the finely divided, carbonized residue, derived from trichloroacetic acid precipitates, was not sedimenting on centrifuging and it was thought that this possible source of error might be eliminated by varying the ashing conditions. Three further batches of ten analyses were carried out with a $3\frac{1}{2}$ hr. ashing time on the same serum sample which had been ashed for $2\frac{1}{2}$ hr. Once again, in each batch five determinations were made on zinc hydroxide precipitates and five on trichloroacetic acid precipitates. The carbonized residue from the trichloroacetic acid precipitates centrifuged down completely. The results of this experiment are given in Table 3. The difference between batches of determinations on zinc hydroxide precipitates is again statistically significant ($0.01 < P < 0.05$). The 'within-batch' variance shown by trichloroacetic acid precipitates is decreased by increasing the ashing time. Comparison of the 'within-batch' variances for trichloroacetic acid precipitates calculated from the data used to construct Table 3 showed that this decrease was statistically significant ($0.02 < P < 0.05$ in a two-tailed F test).

At this stage in the investigation it was noticed that the thermostat controlling the furnace used for ashing was behaving erratically, sometimes allowing the furnace temperature to rise above 650° . An experiment was then carried out to examine the effect of varying the ashing temperature on the analytical results. A new serum specimen was obtained and PBI determinations were carried out on five batches of samples, each batch containing some zinc hydroxide precipitates and some tri-

chloroacetic acid precipitates. The zinc hydroxide precipitates were allowed to stand for 3 hr. before centrifuging, as recommended by Skanse & Hedenskog (1955), although no experiments were carried out to confirm the value of this procedure. For these determinations, the ashing temperature was not allowed to rise above 610° . A further two batches were ashed at 650° . An ashing time of $3\frac{1}{2}$ hr. was used, although the superiority of the longer ashing time was not demonstrated with zinc hydroxide precipitates. With trichloroacetic acid precipitates, ashed at the lower temperature, some of the finely divided residue did not sediment in the centrifuge. The results of this experiment are given in Table 4. Analysis of variance of the data for the samples ashed at 590 – 610° showed that the difference between batches of determinations on zinc hydroxide precipitates was no longer statistically significant. All the results can therefore legitimately be combined to give a grand mean of $4.3 \mu\text{g.}$ of PBI/100 ml. and a standard deviation of $\pm 0.17 \mu\text{g.}$ of PBI/100 ml. The two batches of determinations on zinc hydroxide precipitates ashed at 640 – 660° give a grand mean of $4 \mu\text{g.}$ of PBI/100 ml. The difference between these two means is statistically significant ($t = 2.96$; $0.001 < P < 0.01$). It seems likely, therefore, that inadequate thermostasis during ashing was responsible for the previously observed differences between batches of analyses on zinc hydroxide precipitates and for the higher value previously reported (Acland, 1956) for the variability of the method.

The results of determinations on trichloroacetic acid precipitates are erratic. In the series of analyses with the lower ashing temperature, there is a statistically significant difference between batches. On inspection of Table 4, the values are seen to decrease steadily from batch to batch. In addition, some of the determinations on precipitates ashed at the higher temperature show considerable losses.

It can be concluded that apparent losses of PBI from trichloroacetic acid precipitates are likely to occur if the ashing temperature rises above 650° . At lower temperatures, ashing for $3\frac{1}{2}$ hr. is inadequate

Table 4. *Effect of raised ashing temperature on the estimated PBI content of a serum specimen for both zinc hydroxide and trichloroacetic acid precipitation*

		Results are in $\mu\text{g.}$ of PBI/100 ml.									
		Ashing temperature 590 – 610°					Ashing temperature 640 – 660°				
Zn(OH) ₂ precipitation	No. of determinations	5	5	3	5	5	5	4			
	Mean	4.34	4.31	4.42	4.12	4.20	4.12	3.93			
	Range	4.27–4.50	4.27–4.36	4.36–4.48	3.97–4.25	4.04–4.67	3.99–4.32	3.64–4.11			
Trichloroacetic acid precipitation	No. of determinations	5	5	5	4	5	5	5			
	Mean	4.80	4.60	4.35	4.02	3.98	4.15	3.42			
	Range	4.62–5.04	4.43–4.71	4.08–4.54	3.87–4.32	3.70–4.48	3.81–4.43	3.30–3.66			

to oxidize all the finely divided, carbonized particles in the residue. Although a longer period of ashing might possibly give better results it would, in these circumstances, be inconvenient to carry out ashing and iodide determination on the same day. It was felt that since zinc hydroxide precipitation gave adequate results, it was not worth while to investigate further the feasibility of using trichloroacetic acid as a precipitant.

Recovery experiments

A series of five determinations was made with zinc hydroxide precipitation. Five samples were taken from the same serum specimen as was used to obtain the data in Table 4. To each 1 ml. sample was added 0.1 μ g. of iodine as DL-thyroxine. The percentage of iodine recovered in these experiments was respectively 89.1, 96.6, 94.7, 92.6 and 91.5, giving a mean recovery of 92.9%.

In a further series of five experiments, the percentage recovery of thyroxine, equivalent to 0.1 μ g. of iodine, added to the washed zinc hydroxide precipitates from reagent blanks, was respectively 75.8, 80.3, 77.0, 75.8 and 74.7, giving a mean recovery of 76.7%. Combination of organic iodine with protein would thus appear to prevent excessive losses of iodine in the ashing procedure.

DISCUSSION

Estimation of iodide by the reaction $Ce^{4+} \rightarrow Ce^{3+}$

The theoretical basis for using the reaction between ceric and arsenite ions to estimate iodide has been described above. It was shown that there are three prerequisites for the reaction to be applied in optimum conditions. First, ceric ammonium sulphate solutions must obey Beer's law. Secondly,

the reaction $Ce^{4+} \rightarrow Ce^{3+}$ must be rate-limiting. Thirdly, the catalytic coefficient for iodide must be constant over the whole useful range of iodide concentrations.

Some comment is necessary on the relation between extinction and ceric ion concentration. Chaney (1940) obtained a curve very far from a straight line for this relationship. However, he used a Corning no. 554 filter in conjunction with a photometer adapted for test tubes. A linear relation is more likely to be observed if, as in the present investigation, monochromatic light and cuvettes with optically flat surfaces are used (Kirk, 1950). Thompson, Klugerman & Truemper (1956) found that only the Beckmann DU spectrophotometer, of several tried, gave adequate results. Fig. 1 shows that the Unicam model SP. 600 has a comparable performance.

Some confusion appears to exist in the literature about the applicability of Beer's law to iodide

estimation by the reaction $Ce^{4+} \rightarrow Ce^{3+}$. The law can, of course, refer only to the relation between extinction and ceric ion concentration, not to that between extinction and iodide concentration as suggested by Klein (1954) and by Grossmann & Grossmann (1955). The latter relation is shown above to be a logarithmic one. It follows that Grossmann & Grossmann's (1955) linear-calibration curve, relating difference in extinction to iodide concentration, has no theoretical basis.

Fig. 3 demonstrates that first-order kinetics hold for a reaction time of 12.5 min., which is that used in the analysis. Hence the reduction of ceric ion is rate-limiting. Similar curves are shown by Thompson *et al.* (1956).

Figs. 2 and 3 show that zinc ion lowers the catalytic coefficient for iodide, this decrease being proportionately greater at higher concentrations. The reduction of the ceric ion follows first-order kinetics in the presence of this ion for as long a reaction time as in its absence. It should be noted that the original procedure employed by Barker *et al.* (1951) does not compensate for interference of this type. The addition of a known amount of iodide to an unknown will cause, in such a case, a smaller proportionate increase in the rate of reaction as the iodide concentration in the unknown becomes greater, while the relationship between $\log_{10} E$ and this time remains rectilinear. The errors thus caused with zinc are not of practical importance since their effect is to underestimate, by not more than about 10%, the PBI content of sera whose PBI concentration is greater than about 10 μ g./100 ml., well above the normal range. In any case, Barker (personal communication) now recommends '...that a calibration curve be employed rather than the cumbersome addition of a standard amount of iodide to each unknown'. He further states: 'The main reason this latter procedure was originally instituted was our concern over possible interference from substances in plasma or serum acting like mercury to alter the catalytic effect of iodide. Although mercurial diuretics will occasionally give such an effect, usually a one-day wait is adequate for elimination of the mercury from the bloodstream.' However, Grossmann & Grossmann's (1955) elution method is preferable because it eliminates interference by zinc and results in a theoretical straight-line calibration curve (see Fig. 2c).

Elution of iodide from ashed protein

The highly acid solutions used by Barker *et al.* (1951) and by Brown *et al.* (1953) to dissolve iodide from ashed protein were shown (Table 1) to result in losses of iodide. It follows that the normal limiting values for serum PBI concentration given by these workers are likely to be too low. A re-determination

of these limits would consequently appear to be necessary. Acidification after separation of the supernatant from the residue, as used in the method of Grossmann & Grossmann (1955), was shown to be satisfactory and was finally adopted with slight modifications. Acidification by itself does not therefore cause a loss of iodide. It is probable that acid conditions during elution favour the conversion of iodide into iodine and its retention by the carbonized residue derived from serum protein or on the walls of the Pyrex tube.

Precipitation and recovery of protein-bound iodine

From the results of the serial chemical determinations described above it is reasonable to conclude that, in the long run, with the same analytical method the percentage of added thyroxine recovered should be in the region of 90–95%. According to Tong *et al.* (1954) only 90% of [^{131}I]thyroxine added to serum is recovered in a zinc hydroxide precipitation. The chromatographic experiments reported here appear to indicate losses of not more than about 5% in this stage of the analysis. It may be that some of the radioactivity in the labelled thyroxine used by Tong *et al.* (1954) was present in the form of inorganic iodide.

The finding that apparent losses of iodine occur during the analysis when thyroxine is not associated with serum protein makes it unlikely that the method can be usefully applied to the determination of organic iodine in the absence of a large excess of protein. The protection afforded by protein may perhaps result from the adsorption of iodide on the carbonized residue, which might prevent occlusion in the walls of the combustion tubes. Losses of this type are perhaps more liable to occur at higher ashing temperatures, since sodium iodide melts at 651° and zinc iodide boils at 624°. It is possible that the bulkier zinc hydroxide precipitates are less liable than trichloroacetic acid precipitates to lose iodide into the walls of the combustion tubes between 590 and 610°. On the other hand, zinc hydroxide precipitates may lose iodide more rapidly than trichloroacetic acid precipitates between 625 and 650° as a result of the volatilization of zinc iodide. Above 650°, the melting of sodium iodide may increase the losses from trichloroacetic acid precipitates.

PBI estimations are not very specific chemically. There is probably no discrimination between the different iodothyronines. In addition, about 65% of di-iodotyrosine added to plasma co-precipitates with protein when zinc hydroxide, but not trichloroacetic acid, is used as precipitant (Tong *et al.* 1954). No work has been reported on the co-precipitation with plasma proteins of mono-iodotyrosine, reported to be present together with di-iodotyrosine in the blood of a goitrous patient by

Stanbury, Kassenaar, Meijer & Terpstra (1955). According to Taurog, Wheat & Chaikoff (1956) appreciable amounts of inorganic iodide (2–3%) may remain in a washed trichloroacetic acid precipitate, and therapy with potassium iodide is known to give rise to spuriously high analytical results when zinc hydroxide precipitation of PBI is used (Barker *et al.* 1951). Iodine in certain abnormal-serum components would also appear as PBI. Such compounds are thyroglobulin, found in blood after radio-iodine therapy (Robbins, 1954), and a serum-albumin component described by Robbins, Rall & Rawson (1955) in a patient with thyroid carcinoma. It is not yet certain how accurately the iodine content of these proteins would be estimated by ordinary methods of PBI determination. Finally, intravenous injection of radio-opaque substances containing iodine for special X-ray examinations causes the serum PBI to remain raised for a period of years (Barker *et al.* 1951).

Comparison of the present method with other methods of determining PBI

Methods of determining PBI based on the alkaline-ashing technique for converting organically bound iodine into iodide are preferable for routine use to those using acid digestion and distillation, since the former type requires less supervision and less stringent technical precautions (Barker & Humphrey, 1950). In addition, two well-known examples of distillation methods, those of Barker (1948) and Taurog & Chaikoff (1946), involve losses of iodine of the order of 10–15% during distillation.

Table 5 shows the reports of some other workers on the reliability of their methods. Results comparable to those reported here are claimed by Sobel & Sapsin (1952), using both their own and Chaney's (1940) acid-distillation methods, by Skanse & Hedenskog (1955), using a modification of the Barker *et al.* (1951) method, and by Thompson *et al.* (1956), using another modification of the same method. However, Chaney's (1940) method requires 3 ml. of serum and the distillation is time-consuming, whereas the results of Sobel & Sapsin's (1952) own method are stated by them to be affected if the door of the laboratory in which the estimations are being carried out is opened while the distillation process is proceeding. Thompson *et al.* (1956) have studied their own method with a quality-control technique, and find that it sometimes goes 'out of control'. These authors use the highly acid conditions which have been criticized above. Skanse & Hedenskog (1955) also use an acid solution to elute iodide from ashed protein, which probably accounts for their observing a 15% loss of iodine during the ashing process. They allow for this loss in their calibration of the reaction but,

Table 5. *Reliability of some published methods of determining serum PBI*

Author(s)	Method	s.d. or coeff. of variation for a single estimation	Mean difference between duplicates ($\mu\text{g. of I/100 ml.}$)
Gottschalk & Riggs (1952)	Man, Smirnow, Gildea & Peters (1942)	$\pm 0.46 \mu\text{g./100 ml.}$	—
Stanbury <i>et al.</i> (1954)	Barker (1948) modified	$\pm 0.39 \mu\text{g./100 ml.}$	0.42
Sobel & Sapsin (1952)	Chaney (1940)	$\pm 4.0\%$ *	0.15
	Sobel & Sapsin (1952)	$\pm 7.7\%$ *	0.34
Caraway (1952)	Barker <i>et al.</i> (1951)	—	0.5
Skanse & Hedenskog (1955)	Barker <i>et al.</i> (1951) modified	$\pm 0.22 \mu\text{g./100 ml.}$	—
Thompson <i>et al.</i> (1956)	Barker <i>et al.</i> (1951) modified	$\pm 5.7\%$ †	—
Acland (present work)	Barker <i>et al.</i> (1951) modified	$0.17 \mu\text{g./100 ml.}$	—

* Calculated from the 95% range for the mean of duplicate estimations as given by the authors (± 5.6 and $\pm 10.9\%$ respectively).

† Calculated from the coefficient of variation for the mean of triplicate determinations as given by the authors ($\pm 3.3\%$).

in view of the results with acid eluents which were obtained during the present investigation, it seems doubtful whether the loss would remain constant in the long run.

The possibility of stopping the reduction of the ceric ion at a fixed time, as recommended by Grossmann & Grossmann (1955) and by Meyer, Dickenson, White & Zak (1955), is attractive. It does, however, involve the running of simultaneous standards to counteract the effect of fading colour with time. It is simpler to read the iodide content directly off a calibration curve. No difficulty has been experienced in timing the extinction readings to within 1–2 sec. and the use of the same pipette for adding ceric ammonium sulphate has ensured constancy of delivery time.

The method of determining serum PBI described in the present communication seems sufficiently accurate and reliable to provide trustworthy results in a routine laboratory.

SUMMARY

1. A method of determining serum-protein-bound iodine (PBI) is described, which is suitable for routine use.

2. A series of twenty-three determinations on a serum specimen, with zinc hydroxide as a precipitant of protein-bound iodine, gave a mean of $4.3 \mu\text{g. of PBI/100 ml.}$ and a standard deviation of $\pm 0.17 \mu\text{g. of PBI/100 ml.}$ The mean recovery of thyroxine ($\equiv 10 \mu\text{g. of PBI/100 ml.}$) added to this serum specimen was 92.9%.

3. Analyses on trichloroacetic acid precipitates did not give consistent results owing to difficulties experienced in the ashing process.

4. Losses of iodine result if the ashing temperature rises above 650° .

5. Acidifying a suspension of ashed serum protein is shown to cause losses of iodine. Elution of iodide from the ash is satisfactory in alkaline conditions.

6. The mean recovery of thyroxine added to reagent blanks before ashing was found to be only 76.7%.

7. A linear calibration curve can be constructed for the reaction between ceric salts and arsenious acid, which relates the concentration of added iodide to the logarithm of the extinction measured at $415 \text{ m}\mu$ after the reaction has proceeded for 12.5 min.

8. Zinc is shown partly to inhibit the catalytic action of iodide on the reaction, $\text{Ce}^{4+} \rightarrow \text{Ce}^{3+}$, the effect being greater at higher iodide concentrations.

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Biochemistry of the Wood-rotting Fungi

9. VOLATILE METABOLIC PRODUCTS OF *STEREUM SUBPILEATUM* BERK. & CURT.*

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Our researches on the volatile metabolic products of some of the wood-rotting fungi led us to examine an organism which was isolated from the staves of a beer barrel constructed of Persian oak, *Quercus castaneaefolia* C. A. Mey. A batch of about eighty casks made of Persian oak, after being in use for 2-5 years, developed a curious scented odour which contaminated the beer so badly that the casks had to be destroyed.

Examination of staves from the defective casks showed small internal pockets of incipient white rot. Isolations from these areas yielded a vigorous golden-orange culture of a basidiomycete (now listed as Forest Products Research Laboratory no. 271A) which was found to be identical with a culture of *Stereum subpileatum* originally isolated in the U.S.A. by the late L. O. Overholts of Pennsylvania State College. Later, sporophores of the fungus developed on portions of staves kept in a damp chamber, and the structure of these agrees well with the description of *S. subpileatum* Berk. & Curt. This fungus has been reported by Long (1915) as a common cause of a 'honeycomb' white-pocket

rot in thirteen species of oak in the southern part of the U.S.A. He noted that the fungus can continue to grow in felled timber. It is highly probable that infection in the oak used for the manufacture of beer barrels was present at least in an incipient stage in the logs when they were felled.

The production of a sweet musty odour by *S. subpileatum* has been described as a characteristic feature of this fungus. The odour from a culture is both penetrating and persistent and resembles a scent used in cheap soaps. It has been suggested that it may be compared with 'a mixture of wintergreen and apricots plus a trace of thymol'. There is little doubt that the metabolic products of the fungus growing in the wood were responsible for the contamination of the beer, even though the casks were lined inside with a vegetable pitch.

In order to investigate the metabolic products of *S. subpileatum* responsible for the characteristic odour, the fungus was cultured in the laboratory and the volatile products were separated from the mycelium and culture fluid by steam-distillation as in previous cases. As preliminary tests had shown that a carbonyl compound and an acid were present in the ether extract of the steam-distillate, the

* Part 8: Birkinshaw & Chaplen (1955).