QUANTITATIVE STUDIES OF IODINE METABOLISM

BY

M. H. RICHMOND

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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S U M M A R Y

This thesis describes a series of investigations of the quantitativ aspects of iodine metabolism in relation to thyroid disease. Few studies in this field have combined accurate chemical measurement of the quantitie of iodine involved at each stage with radioiodine measurements of the dynamic aspects of iodine metabolism. The work described here is primar concerned with the development of reliable microchemical techniques for the determination of iodine in body fluids and tissues. The application of these techniques and of radioiodine measurements to the study of iodine metabolism in healthy subjects and in patients with thyroid disease is described.

A simplified chemical method for the microdetermination of iodine in biological samples was developed which proved accurate and reliable in routine laboratory use. An alternative method of iodine microdetermination by neutron activation analysis was also investigated but consist results were not obtained. The successful chemical procedure involved chloric acid digestion of organic material followed by colorimetric determination of iodine utilising the iodide catalysed ceric sulphate - arsenious acid redox reaction. In the application of this technique particular emphasis was placed on the determination of the protein-bound fraction of blood iodine (P.B.I.), which is the most direct index at present /

present available of the level of circulating thyroid hormone. The relationship between serum P.B.I. and thyroid function was investigated in a large series of subjects with normal and disordered iodine metabol

The dietary iodine supply in the West of Scotland was studied by chemical determination of the iodine content of food and water. Fish was shown to be by far the richest source of dietary iodine available. The figures obtained for milk and eggs were high in comparison with earlier published values. The low iodine content of Glasgow tap water indicated that the dietary supply from this source was of little significance. Diet histories of hospital patients revealed that subjects with simple goitre had a significantly smaller iodine intake than normal individuals.

A method for urinary iodine analysis was developed and its accuracy assessed. The urinary excretion of iodine was measured in a series of patients with thyroid disease and in a normal control group. Faecal excretion of iodine was similarly studied in a small number of subjects.

The level of the plasma inorganic iodine, which is of great importance in the regulation of thyroid function, was determined by a specific activity technique and the validity of the procedure was demonstrated.

QUANTITATIVE STUDIES OF IODINE METABOLISM

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by

M.H. RICHMOND

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PREFACE

The work described in this thesis was carried out between 1958 and 1961 in the University Department of Medicine, the Gardiner Institute, Glasgow, under the supervision of Professor E. J. Wayne. Financial support was received from the Secretary of State for Scotland on the recommendation of the Advisory Committee on Medical Research. Facilities and equipment for radiochemical studies were generously provided by the Western Regional Physics Department and by the United Kingdom Atomic Energy Research Establishment, Harwell.

The author wishes to express his indebtedness to Professor Wayne for guidance and encouragement given during the course of this work. Useful discussions with Mr. L. P. Farrell and Dr. H. Smith on the chemical aspects of the work and with Drs. D. A. Koutras, W. D. Alexander, W. W. Buchanan and J. Crooks, on the medical aspects are gratefully acknowledged.

Published papers listed under the following references include in part the results of work described in this thesis: Koutras et al. (1960), Richmond et al. (1960), Alexander et al. (1961), Buchanan et al (1961), Farrell and Richmond (1961).

CONTENTS

GENERAL INTRODUCTION

Iodine occupies a unique position in human physiology in that the sole known function of this element is its participation in the synthesis of the thyroid hormones thyroxine and triiodothyronine. Iodine metabolism and thyroid physiology are therefore inextricably linked.

The work described in the present thesis is concerned with the investigation of quantitative aspects of iodine metabolism. Much research has been carried out in this field in the past, but few investigators have employed the combination of chemical and radioiodine tracer procedures necessary to obtain a clear picture of iodine metabolism. Radioiodine studies, though ideal for measuring the proportion of iodine in the body which follows a particular metabolic pathway and for studying the rate of turnover of iodine, do not indicate the actual amounts of iodine which are metabolised. This information can only be obtained by accurate chemical determination of the quantities of iodine present in the various compartments of the body. However, due to the minute amounts of iodine present in biological material, chemical analysis provides many technical difficulties and requires extremely sensitive techniques. The purpose of the research undertaken for this thesis was to develop reliable micro-chemical techniques for the determination of iodine in biological material, and to apply these techniques together with radioiodine tracer studies to the investigation of iodine metabolism in health and disease.

A /

A simplified chemical method for the micro estimation of iodine in biological material is presented together with evidence of its accuracy and consistency. An investigation of the application of neutron activation analysis to the measurement of the trace quantities of iodine is also described; the inconsistent results obtained with this technique, however, indicate that further research will be necessary in order to develop a reliable activation analysis procedure. The chemical procedure involves chloric acid digestion of organic material followed by determination of iodine utilising the iodide catalysed ceric sulphate arsenious acid redox system. This technique is used as the basis of analytical methods developed for the measurement of iodine in a variety of biological samples. In its application, however, particular emphasis has been placed on determination of the protein-bound fraction of blood iodine (P.B.I.) which is the most direct index at present available of the level of circulating thyroid hormone. The results of a survey of the relationship between P.B.I. and thyroid function are given for a large number of patients with thyroid disease and for a normal control series.

Estimation of the iodine excreted in the urine has long been used for the detection of iodine deficiency, but many of the analytical techniques used for this purpose have been unreliable. A method of urinary iodine estimation is described here, together with experimental verification of its accuracy and reliability. In addition a simple technique /

technique for separating inorganic iodine from organic iodinated compounds in urine is suggested.

Chemical analysis of faecal iodine has received much less attention from research workers than has urinary iodine, althoughthe loss of iodine in faeces has been implicated as a possible cause of goitre. A method for chemical determination of faecal iodine is presented together with the results of an investigation into the significance of iodine losses by this route in a small series of normal subjects and patients with thyroid disease.

The plasma inorganic iodine (P.I.I.) is of paramount importance as a regulator of thyroid function. However, its importance, although always recognised and frequently referred to, has had little actual experimental verification. This is due to the fact that in man the P.I.I. is present in such extremely small amounts that direct chemical estimation under normal conditions is not possible with the techniques available at present. Using an indirect method which is dependent upon specific activity measurements of urinary iodine, the level of P.I.I. has been determined in a series of hospital patients with thyroid disease and in normal subjects. The accuracy and reproducibility of results obtained by this method have been fully assessed.

Iodine intake in humans is entirely dependent upon the amount ingested in food and water. Although it has long been recognised that diets deficient in iodine are an important cause of goitre, little recent information /

information is available on the iodine content of common articles of diet. In the present thesis an investigation of the iodine content of food and water supplies in the West of Scotland is described. The incidence of thyroid disease in relation to dietary insufficiency of iodine in this area has also been studied and evidence is presented in favour of introducing compulsory salt iodisation in Great Britain.

PART I.

BACKGROUND TO INVESTIGATION

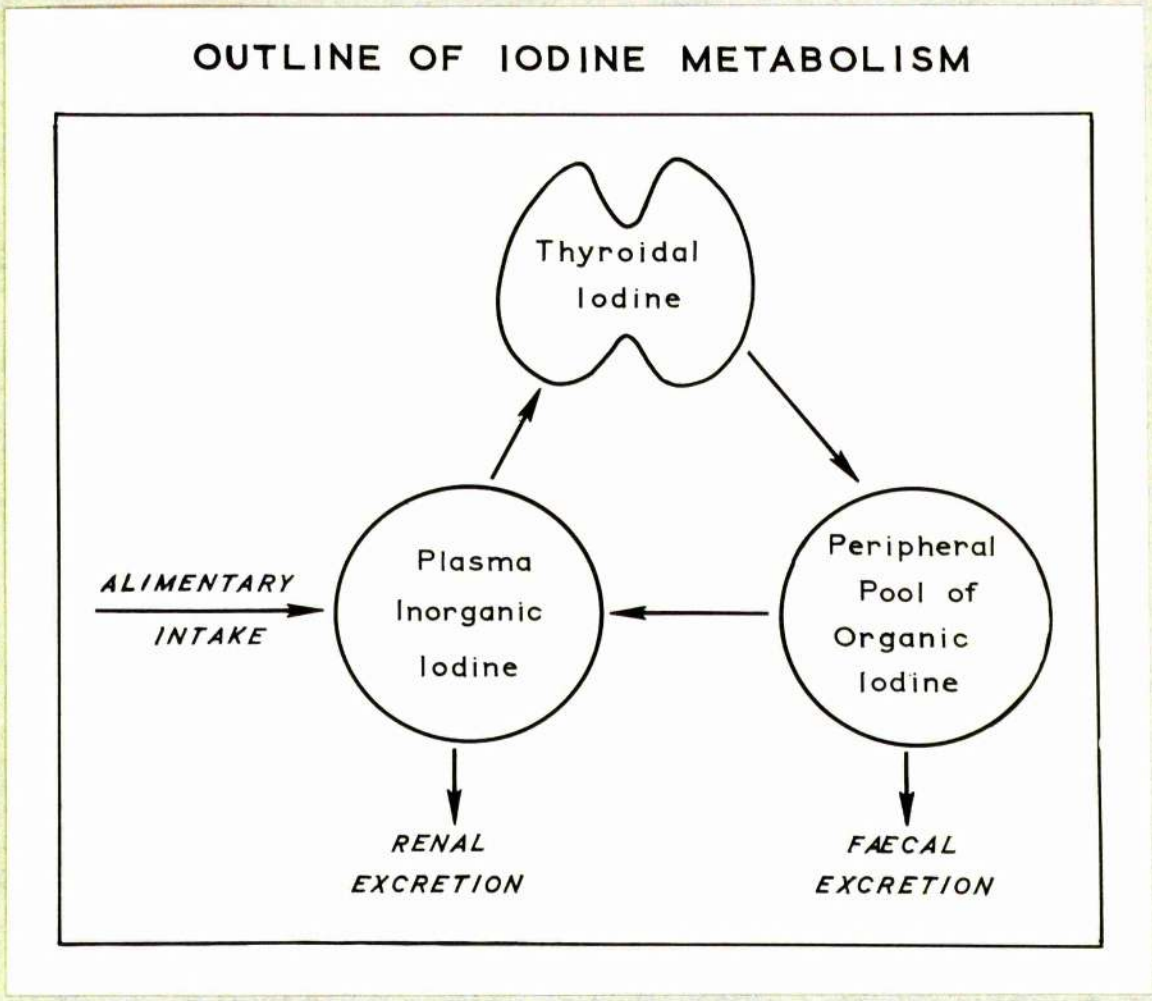
Chapter 1

IODINE METABOLISM

Early studies on the distribution and metabolism of iodine were considerably hampered by the lack of a reliable micro-technique for the determination of iodine; only by administering doses of iodine far in excess of a physiological range could its metabolism be studied. In recent years three factors have largely contributed to the advances that have been made in our knowledge of the subject. Firstly, refinements in chemical technique have enabled fractions of a microgramme of iodine to be measured with accuracy. Secondly the use of radioactive isotopes of iodine, principally I^{131}, has enabled workers to study the fate of iodine administered in a physiological range of doses, while chromatographic analysis of I^{131} labelled compounds has revealed iodinated substances in thyroidal and extrathyroidal tissue in extremely small amounts. Thirdly, two types of antithyroid drugs have been discovered - those of the thiocyanate and perchlorate class, which inhibit the thyroidal iodide concentrating mechanism, and those related to thiourea which permit iodide concentration by the gland but prevent its incorporation into organic compounds. These drugs have allowed separate investigation of the thyroidal iodide concentration mechanism and the iodine binding mechanism.

The general scheme of iodine metabolism is illustrated in Fig I.

Fig I.



Absorption and circulation of iodine.

Iodine ingested with food and water is rapidly absorbed from the gastrointestinal tract (Hamilton 1938). If administered as molecular iodine or iodate, reduction to iodide must occur before absorption can take place (Cohn 1932). Absorbed iodide appears in the circulation as the plasma inorganic iodide (PII). Apart from intake, the PII is constantly replenished by iodide derived from metabolic deiodination of the thyroid hormones.

The level of PII influences thyroid function in several ways. In order to obtain an adequate amount of iodide the thyroid gland must clear a specific volume of plasma per unit of time. When, however, the PII level falls the gland is required to clear a larger volume of plasma to obtain the same amount of iodide. The increased plasma iodide clearance in iodine deficiency goitre is therefore compensatory to the low PII level (Koutras et al 1960).

Although a modest increase in thyroid clearance may be obtained without goitre formation, (Roche 1959) when the PII decrease is marked and sustained the compensating rise in thyroid clearance must also be equally marked, and goitre formation irrevocably proceeds (Riggs, 1952).

The PII level is determined both by the dietary intake and the loss of iodine from the body. The renal clearance of iodide appears to be an important factor as it does not adjust to the PII level to keep it constant (Stanbury et al, 1952; Fraser, 1960) and differs from individual to individual (Alexander et al, 1961). Cassa no et al, (1959) emphasised the importance of an increased renal clearance of iodide as a cause of sporadic iodine deficiency goitre. Theoretically there is no doubt that persons with a high renal clearance of iodide are more predisposed to iodine deficiency goitre than those with a low one, however, in 21 cases studied in Glasgow the renal iodide clearance was only slightly raised, (Koutras et al 1960). Presumably, in practice, the role of renal

clearance is overshadowed by the more important role of the dietary intake of iodine. On the other hand increased PII levels have been found in patients with renal failure and diminished renal iodide clearance (Perry and Hughes, 1952).

Thyroid hormone biosynthesis.

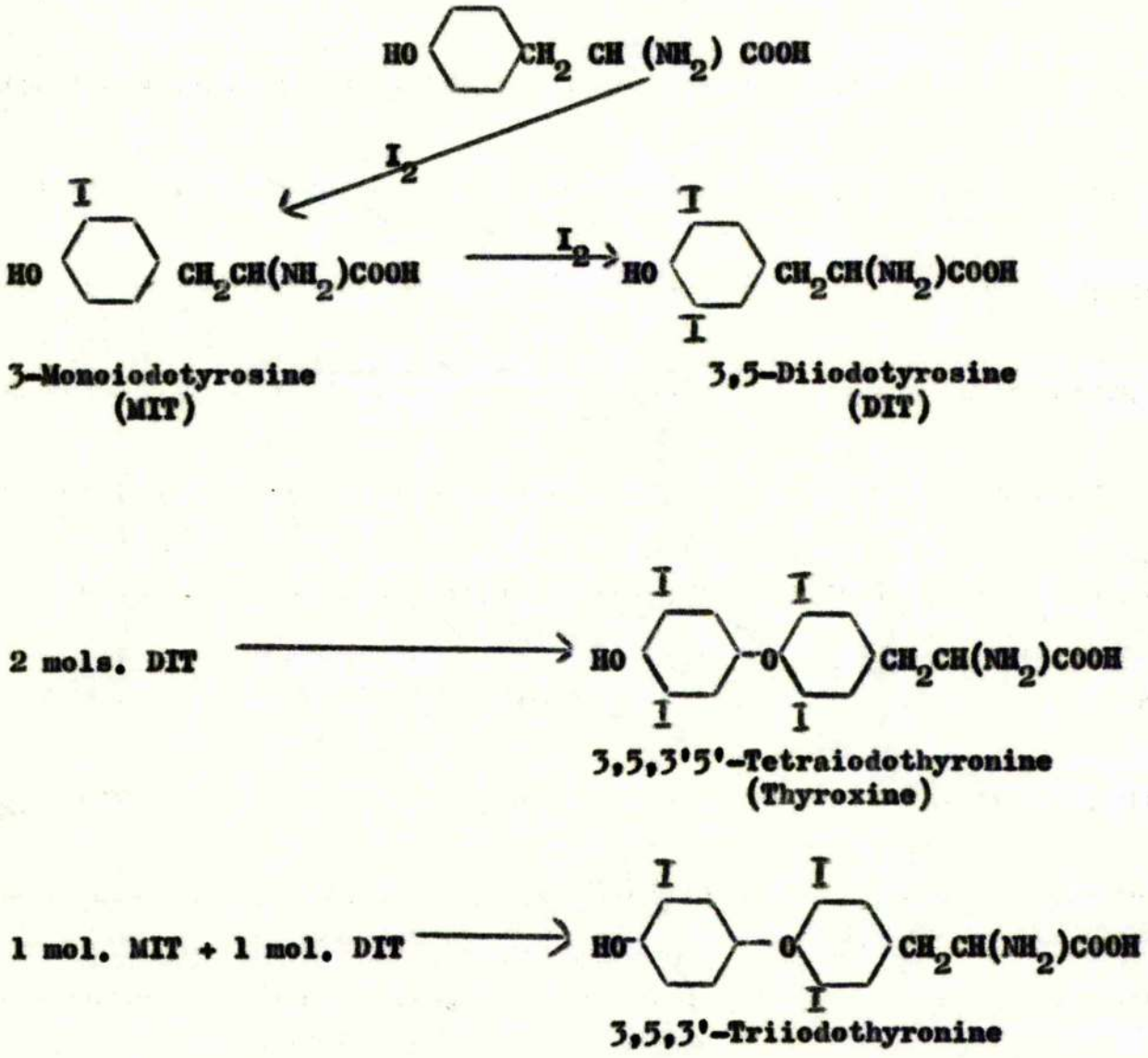
In vertibrates the thyroid gland alone possesses the mechanisms necessary to elaborate the thyroid hormones thyroxine and 3,5,3' - triiodothyronine. It is generally believed that biosynthesis of these compounds occurs in the following stages (Fig II):-

1. The thyroid actively concentrates iodide from the blood.
2. Iodide is oxidised to elemental iodine.
3. Tyrosine molecules in thyroglobulin are iodinated first to 3-monoiodotyrosine and then to 3,5-diiodotyrosine.
4. Two molecules of diiodotyrosine are coupled to form the diphenylether thyroxine (3,5,3',5'-tetraiodothyronine), with the subsequent splitting off of one aminopropionic side chain. 3,5,3'-Triiodothyronine is similarily formed by the coupling of one molecule each of mono- and di-iodotyrosine.

Thyroxine and 3,5,3 ' triiodothyronine are the only biologically active compounds synthesised by the thyroid and secreted into the blood. 3,3' Diiodothyronine and 3,3',5'- triiodothyronine have also been detected in the thyroid (Roche et al 1956) but neither of these compounds shows any significant thyromimetic

Fig. II

Thyroid Hormone Biosynthesis



activity (Gemmill, 1956 ; Stasilli et al, 1959).

Very little is yet known about the enzymes responsible for thyroid hormone biosynthesis. Dempsey (1944) and Alexander (1959) have demonstrated that the conversion of iodide to elemental iodine is carried out by a thyroid peroxidase. The small amounts of diiodotyrosine obtained by the latter author indicate that a different enzyme system is responsible for stage 3 of the biosynthetic route. There is no definite evidence that an enzymatic mechanism is responsible for the coupling reactions in stage 4. Tubiana and Vallée (1959) postulate that this condensation may be due to the favourable spatial arrangement of the iodotyrosine residues in the thyroglobulin framework.

Thyroxine is stored within the follicles of the thyroid gland in peptide linkage with thyroglobulin. Thyroglobulin is broken down by the action of a proteolytic enzyme (De Robertis, 1949) and thyroxine and triiodothyronine are liberated together with mono- and di-iodotyrosine. The iodotyrosines, however, unlike thyroxine and triiodothyronine, are not released into the circulation but are deiodinated within the gland through the medium of the enzyme deiodinase (Roche et al, 1952). The iodine thus released is retained by the thyroid and reutilised for the biosynthesis of new thyroid hormone.

Evidence exists which suggests that not all of the iodine liberated from mono- and di-iodotyrosine is retained within the

thyroid. Nagataki et al (1959) showed in dogs that after administration of thyrotropic hormone (TSH) the thyroid liberates both inorganic I^{131} and PBI^{131} into the thyroid vein. This has apparently been confirmed in humans by Rosenberg et al (1961) who observed increased urinary excretion of I^{131} after TSH. Whether such a leak of iodide from the thyroid occurs only after increased TSH stimulation, or takes place continuously, is a matter of speculation. Tubiana and Vallée (1959) suggest that this thyroidal iodine leak may be more pronounced in pathological states.

The circulating Thyroid hormones.

Until recently thyroxine and triiodothyronine were generally thought to be the only iodinated products of thyroid elaboration present in significant amounts in the blood of normal subjects, (Taurog and Chaikoff, 1948; Laidlaw, 1949; Gross and Leblond, 1951; Roche and Michel, 1955). Thyroxine, the principal thyroid hormone, was shown by the above authors to comprise more than 90% of blood iodine. However, two separate studies of the iodinated constituents of blood indicate that as much as 50% of the blood iodine in normal individuals represents mono- and di-iodotyrosine (Block et al 1960; Beale and Whitebread, 1960). More recent work by Wynn (1961) and Pitt Rivers and Rall (1961) has not confirmed the presence of appreciable quantities of the iodotyrosines and is consistent with the findings of earlier authors.

Trevorrow (1939) first observed that thyroxine in the blood was not dialysable and could be coprecipitated with plasma proteins; it was later shown that the thyroxine thus precipitated was extractable in n-butanol (Taurog and Chaikoff, 1948). It is apparent from these facts that thyroxine is firmly bound to plasma protein by a strong physical link. This property of thyroxine is utilised in the determination of 'protein-bound iodine' (PBI) in serum or plasma, since precipitation of serum proteins affords a convenient means of separating thyroxine iodine from any inorganic iodine present.

No protein has yet been isolated which can account for the binding of thyroxine in blood. Electrophoretic studies employing radiothyroxine have shown that thyroxine is bound to a protein fraction whose mobility is intermediate between that of α_1 and α_2 globulins (Gordon et al, 1952; Robbins and Rall, 1952 and 1957); the term thyroxine binding protein (TBP) is applied to this fraction. In vitro studies have revealed that TBP also binds triiodothyronine; this binding is weak and TBP is more easily saturated with respect to triiodothyronine than thyroxine (Deiss et al, 1953; Dingledine et al, 1955). This relatively weaker binding of triiodothyronine is also demonstrated by the ready displacement of bound triiodothyronine by added thyroxine and by the difficulty of displacing thyroxine by triiodothyronine.

Thyroid hormone metabolism.

Deiodination is the first and most important reaction in the metabolism of the thyroid hormones. Little is known about the succeeding stages of metabolic breakdown since labelled hormones of high specific activity have only so far been obtained with I^{131} as the label. For this reason the deiodination process has been intensively investigated.

All organs and peripheral tissues possess some ability to deiodinate thyroxine and triiodothyronine. The liver plays an important part in this process (Flock et al, 1956 ; Dragunova and Langer, 1956) and the iodide so released mixes readily with the PII pool. Part of this iodide is recirculated to the thyroid and utilised for the synthesis of new hormone, and another part is eliminated by the kidney. However, not all of the iodine contained in the circulating thyroid hormone returns to the PII pool. A significant proportion of thyroxine is excreted as the glucuronide by the liver into the gastrointestinal tract in the bile and of this, some is reabsorbed by the plasma and some lost with the faeces. Myant (1956) studied the enterohepatic circulation of radiothyroxine in humans. The biliary clearance rate varied from 200 to 600 ml of the plasma per day, but as part was reabsorbed, only thyroxine corresponding to 170 to 350 ml of plasma daily was finally lost in the faeces. This would correspond to 8.5 - 17.5 µg of iodine per day at a PBI level of 5.0 µg / 100 ml.

Recently, Van Middlesworth (1960) has determined the faecal iodine loss in a few euthyroid individuals and postulates that increased faecal losses are possibly an important factor in iodine deficiency goitre.

Chapter 2

MICRODETERMINATION OF IODINE IN BIOLOGICAL MATERIAL

Introduction

Accurate measurement of the exceedingly minute quantities of iodine in biological material is of paramount importance in the investigation of iodine metabolism. H ighly sensitive techniques are required for this purpose. In this section the principal chemical methods of iodine analysis are reviewed and their application to the microdetermination of iodine in biologica l material is discussed. The use of a radioactivation analysis technique for trace determination of iodine is described separately in Part IV.

Historical Background

Early scientific interest in the chemistry of iodine centred in France following its discovery there by Courtois in 1811. In 1850 the French botanist Gaspard Adolphe Chatin carried out the first quantitative study of the occurrence of iodine in Nature and spent 25 years testing the iodine content of air, water, soils, and foods from different localities. As a result of his analyses Chatin formulated the hypothesis that simple goitre was related to deficiency of iodine. A commission appointed by the French Academy of Science was unable to repeat his chemical findings and repudiated his hypothesis. Chatin remained discredited until Baumann (1896) discovered the presence of iodine in the thyroid gland.

Following Baumann's discovery great interest was taken in the

iodine content of biological material and numerous attempts were made to develop methods of analysis. Hunter (1910) reviewed the early methods and exposed many errors both of principle and technique. He described at the same time a new procedure for iodine estimation which is used in principle at the present time. This was as follows: Iodine was liberated from organic combination (as a mixture of iodate and iodide) by total alkaline incineration with a mixture of sodium and potassium carbonates and potassium nitrate; the iodide was oxidised by chlorine in dilute acid solution to iodate and, in the presence of excess iodide and acid, the iodate converted to iodine according to the equation;

$$IO_3^- + 5I^- + 6H^+ \rightleftharpoons 3I_2 + 3H_2O$$

The iodine released was then determined by titration with standard sodium thiosulphate using starch as indicator.

In recent years, many refinements in chemical technique stemming principally from the work of Sandell and Kolthoff (1937) have led to the production of more reliable results. During work carried out in 1936 , these workers found that the rate of reduction of ceric ion by arsenite ion in acid medium was proportional to the iodide concentration present. They applied this relationship successfully in the determination of small amounts of iodide. In 1940 Chaney adapted this procedure to the estimation of iodine in blood, using a preliminary chromic acid, sulphuric acid digestion

of organic matter. Since then, most published methods for micro-determination of iodine have utilised the extremely sensitive ceric - arsenite reaction.

Methods of Iodine Analysis

The determination of iodine in biological material is a two stage process involving the liberation of iodine in an inorganic form by destruction of organic matter, followed by determination of the inorganic iodine. The methods of analysis described since 1910 depend in the main upon two principles; (1) conversion of the inorganic iodine liberated by combustion or digestion to iodate, which is determined by the H unter (1910) volumetric method; (2) conversion of the inorganic iodine to iodide, which is determined by means of the iodide catalysed ceric sulphate - arsenious acid reaction. Numerous modifications based on these principles have been introduced and many different techniques for the destruction of organic material and the release of an inorganic form of iodine have been described. Much conflicting evidence is apparent in the description of these modified procedures, and it is now clear that some of the modifications have been ill-founded. The combined contributions however, clarify the fundamental chemical problems, but leave some details which require further attention.

It is convenient to group the methods of analysis according to the type of digestion procedure employed. Table I summarises the principal methods which have been developed since 1940 in this way.

Table I PRINCIPAL METHODS FOR MICRODETERMINATION OF IODINE.

Chromic Acid digestion and distillation	Acid permanganate digestion and distillation	Chloric acid digestion and direct estimation	Alkaline ashing and direct estimation
Chaney, 1940, 1950.	Riggs and Man, 1940, 1942.	Zak et al, 1952.	Salter and McKay, 1944.
Taurog and Chaikoff, 1946, 1948.	Bassett et al, 1941.	O'Neal and Simms, 1953.	Salter and Johnson, 1948.
Barker, 1948.	Talbot et al, 1944.	Leffler, 1954.	Decker and Hayden, 1951.
Shakrokh and Chesbro, 1949.	Kydd et al, 1950.	Fischl, 1956.	Barker and H umphrey, 1951.
Conner et al, 1949.	Rall et al, 1950.	Astwood, 1957.	Chesky et al, 1952.
Lachiver and Leloup, 1949.		Bodansky et al, 1958.	Brown et al, 1953.
Nesh and Peacock, 1950.			Starr, 1954.
Danowski et al, 1950.			Grossman and Grossman, 1955.
Thomas et al, 1950.			Murray, 1955.
Carr et al, 1950			Sunshuk and Aconsky, 1955.
Zachere and Stocke, 1951.			Skanse and Hedenskog, 1955.
Moran, 1952.			Meyer et al, 1955.
Klein, 1952.			Thomson et al, 1956.
Sobel and Sapsin, 1952.			Sanz et al, 1956.
Carr and Riggs, 1953.			Acland, 1957.
Ellis and Duncan, 1953 .			Marlow, 1958.
Kirkpatrick, 1953.			
Friis, 1955.			
Spitz and Lieb, 1956.			
Rodgers and Poole, 1958.			

SEPARATION OF IODINE FROM ORGANIC MATERIAL

Methods devised for achieving the separation of iodine from organic combination can be divided into two groups:

(1) Destruction of organic matter by heat and air in the presence of sufficient alkali to prevent loss of iodine.

(2) Acid digestion with an active oxidising agent.

Alkaline Ashing Methods.

Alkaline ashing methods were used by the early investigators of iodine biochemistry. Destruction of organic material is brought about by the action of heat and air. Temperatures in the region 520 - 620°C are normally employed. The presence of alkali is essential to prevent loss of iodine by volatilisation. Sodium or potassium hydroxide, carbonates and mixtures of carbonates and nitrates have been used for the purpose.

In modern methods (Salter and McKay, 1944; Barker and Humphrey, 1951; Acland, 1957) the ash remaining after incineration is dissolved in acid for the final quantitative measurement of iodine, and no attempt is made to isolate the iodine from its salts. Sulphuric acid, hydrochloric acid and a mixture of both have been used.

Acid Digestion Methods.

(a) Chromic Acid and Permanganate Methods.

In acid digestion techniques, an active oxidising agent is used in excess and destroys organic material with the formation of CO_2, N2, H_2O or other simple compounds. All of the iodine

present is oxidised to iodic acid. Both chromic acid and permanganate in the presence of sulphuric acid are used for this purpose. The quantities of acid and oxidising agent required are principally determined by the quantity of organic material to be destroyed. An excess of oxidising agent must be present however, to prevent loss of iodine during the digestion.

The final acid digest contains both the excess oxidising agent and iodic acid. Iodine is isolated by reducing both the remaining oxidising agent and the iodic acid and distilling the iodine into a suitable absorbing medium, (dilute alkali is normally used for this purpose.)

It is generally agreed that the distillation stage is the one in which the problem of recovery is greatest. Losses of iodine are liable to occur in several ways, such as incomplete distillation, inefficient condensation of the distillate or adsorption on glass surfaces. Because of the inherent blank in the reagents it is sometimes difficult to decide the magnitude of these losses. An account of the sources of error that may be encountered in distillation methods is given by Moran (1952).

(b) Chloric Acid Method.

Chloric acid has a high oxidation potential and was first applied to iodine micro-analysis by Zak et al. (1952). This reagent readily releases iodine from organic combination and oxidises it to iodate. Excess chloric acid is volatilised at temperatures in the region of 160° C, and iodate determined directly

in the residue. The presence of a small amount of chromate is considered necessary to prevent iodine loss during digestion. Loss of iodine is indicated by reduction of the red Cr^{6+} ion to green Cr^{3+} ion.

The principal advantage of the chloric acid technique over the other acid digestion methods is that iodine can be determined directly in the digestion residue. No distillation step is necessary as in the chromic and permanganate methods.

QUANTITATIVE DETERMINATION OF IODINE.

(a) Volumetric Methods.

A detailed study of the volumetric estimation of micro-quantities of iodine was reported by Harvey (1935). The technique used by Hunter (1910) is the basis of all volumetric methods. Oxidation of iodine to iodate by chlorine or bromine water is followed by the addition of iodide and titration of the liberated iodine with thiosulphate using starch as indicator.

The volumetric method is rarely used for micro-analysis and is more suitable for measuring large quantities of iodine. There a re several sources of error. The figure obtained by titration measures the oxidising power of the solution. Hence, in the presence of oxidising agents other than iodate (chlorine, bromine, bromate, iron, vanadium etc.) the final value is not a true measure of the iodine originally present. Furthermore, inthe presence of

bromide, nitrite or organic matter, the iodate may be partially reduced.

(b) Direct Colorimetric Methods.

Iodine in solutions of carbon tetrachloride, carbon disulphide or chloroform can be measured by colorimetric comparison with standards containing known amounts of iodine. McClendon (1928), Hartley (1929), Andrew (1930) and Ovenstone et al. (1951) have applied this method to the determination of small quantities of iodine.

Direct estimation of iodine can be achieved also by colorimetric measurement of the blue starch-iodine complex and comparison with standards of known iodine content. Talbot et al. (1944), Koenig and Gustavson (1945) and H ouston (1950) have used this technique.

The accuracy of the above techniques is limited and they can normally be applied only to the analysis of samples containing more than 1µg of iodine. They are therefore not suitable for biological samples unless either the iodine content is high or large samples are taken.

(c) Isotopic Exchange Method.

Raben (1950) has described a method for the determination of iodine which utilises an isotopic exchange reaction between stable iodate and iodide - I^{131}. The unknown in the form of iodate is trea ted with an excess of labelled iodide. The iodine

formed is extracted with carbon tetrachloride and the radioactivity in the latter determined by γ-ray counting.

The reaction can be represented as:-

$$I^{127}O_3^- + 5I^{-\,127.131} + 6H^+ \rightarrow 3I_2^{\,127.131} + 3H_2O$$

At the instant of formation the specific activity of the $I_2^{\,127.131}$ is $^5/_6$ of that of the I^- 127.131. The exchange reaction between iodine and iodide occurs rapidly and the specific activity of the iodine approximates that of the iodide by exchange with the excess of iodide present.

The sources of error in this method are similar to those involved in volumetric estimation of iodine by starch- thiosulphate titration. The presence of any agent capable of reducing iodine will invalidate the results. In addition the method cannot be applied to the determination of iodine in biological samples from patients who have been treated with radioiodine.

(d) Polarographic Methods.

Polarographic determination of small quantities of iodine as iodate has been reported (Rylich, 1935; Cizek, 1944; Godfrey et al. 1951; Pliska, 1959). From the characteristics of the current voltage curves obtained on electroreduction of iodate, using a dropping mercury electrode, the iodine concentration can be determined. The method is sensitive because 6 electrons are involved in the reduction of IO_3^-.

Godfrey et al. (1951) isolated iodine from organic material by chromic acid digestion and distillation. Following oxidation of iodine to iodate with ozone, polarographic analysis was carried

out. The limit of sensitivity quoted is 0.5 μg I with a maximum error of ± 10 %.

(e) Ceric-Arsenite Catalytic Method.

The quantitative adaptation of Sandell and Kolthoff's Catalytic detection method for iodine has become the preferred procedure because of its extreme sensitivity.

The reduction of ceric ion in acid solution by trivalent arsenic is an extremely slow reaction in the absence of catalysts, since it involves the simultaneous transfer of two electrons from the arsenic to two different ceric ions. In the presence of a catalyst such as iodine, which exhibits multiple states of oxidation, the reaction rate is greatly increased. Experimentally the reaction rate ma y be made directly proportional to iodine concentration if a suitable choice of conditions is made. The reduction of the yellow ceric to colourless cerous ion is usually followed photometrically, and a series of empirical standardisation curves relating photometer readings to known quantities of iodine at predetermined reaction times is employed.

$$2Ce^{4+} + AsO_3^{3-} \xrightarrow{I^-} 2Ce^{3+} + AsO_3^{-}$$

yellow colourless

The sensitivity of the reaction is such that quantities as small as 0.005 μg of iodine can be measured accurately.

Conditions Influencing the Catalytic Reaction: Numerous investigations of the catalytic reaction have been carried out (Chaney, 1940 and 1950;

Lein and Schwartz, 1951; Kirkpatrick, 1953; Dubravcic, 1955; Escobar and Ríos, 1958). The following factors are of principal importance: (a) the temperature and reaction time, (b) the effect of acidity and of other cations, (c) substances affecting the catalysis, (d) the oxidation state of iodine, (e) the relative concentrations of arsenious acid and ceric sulphate.

The reaction rate is temperature dependent and careful control is necessary. A rise in temperature of 10°C approximately doubles the rate of reaction. The temperatures employed in different methods range from 10 - 50° C. Reaction times of 5 - 40 mins. are used.

Acidity is controlled by using either sulphuric or hydrochloric aced. Chaney (1950) showed that the reaction rate increases with acidity up to 3N and then decreases at higher concentrations. The catalytic effect of some interfering substances (chromium ion) is also dependent upon acidity (Carr et al, 1950).

Sandell and Kolthoff (1937) found that osmium is the only substance which causes catalysis comparable in rate to iodine. Catalysis by chloride and platinum is very weak, and by bromide, weak unless a manganous salt is present. The catalytic effect of chromate is of importance since traces may be present in distillates, or may be intentionally added, as in chloric acid digestion methods.

The reaction rate is reduced in the presence of appreciable

quantities of Na^+, K^+ and NH_4^+ (Chaney, 1950; Thomson et al. 1956). The effect of Na^+ is of importance in alkaline ashing procedures since the ash solution contains relatively high concentrations of the element.

Mercury and silver react with iodide at low concentrations and inhibit the reaction completely if present in concentrations equivalent to the iodine. Dubravcic (1955) showed that this effect is decreased in the prese ce of chloride. Other ions which poison the catalytic mechanism include fluoride, cyanide and copper (Patterson and Man, 1959).

Inhibitors have been employed in several methods to stop the catalysis completely at a specified time and enable colorimetric measurement of Ce^{4+} to be carried out with greater ease and a ccuracy. Mercuric salts (Strickland and Maloney, 1950; Meyer et al. 1955), silver salts (Kontaxis and Pickering, 1958), morphine (Sandell, 1950) and brucine (Shemyakin et al. 1938; Grossman and Grossman, 1955; Fischl, 1956) have been utilised for this purpose.

The oxidation state of iodine is of importance. Iodine is generally in the form of iodide in distillates and ash solutions. When iodine is oxidised to iodate, it has little catalytic effect except under conditions in which reduction occurs during measurement. Iodate is apparently readily reduced and becomes catalytically

active when chlorides are present in high concentration. When iodine is organically combined as in iodinated tyrosines and thyronines catalytic activity is retained, but its reaction rate is different and varies with the structure of the compound (Escobar and Rios, 1958).

Considerable variation is apparent in the relative concentrations of arsenious acid and ceric sulphate employed in different methods. Lein and Schwartz (1951) found that the optimum ratio of arsenious - ceric concentrations for the study of iodide catalysis consists of a large excess of arsenious acid. Oneal and Simms (1953) propose that the (arsenious: ceric) equivalent ratio should be not less than 10 : 1. In the methods of Connor et al. (1949), Chaney (1940), Taurog and Chaikoff (1946) and Zak et al. (1952) the recommended (arsenious : ceric) ratios are outwith this range.

Summary and Conclusions.

1. The quantities of iodine present in biological material are extremely minute and very sensitive methods are required for accurate analysis.

2. Most methods entail the destruction of organic material with acid or alkali followed by determination of iodine in the digestion residue.

3. From the extensive literature it is apparent that the iodide catalysed ceric sulphate - arsenious acid reaction is the most sensitive method at present available for iodine microanalysis.

4. Alkaline ashing techniques are time consuming and prone to iodine loss by volatilisation at the high temperatures employed.

5. Acid digestion methods involving distillation of iodine require special apparatus, and the number of analyses performed is limited owing to the close attention that must be paid to each individual sample during distillation.

6. Chloric acid digestion provides a simple and efficient means of releasing iodine from organic combination. It has the great advantage over other techniques that all steps in the preparation of the sample up to colorimetric determination may be carried out in the same container, thereby eliminating iodine loss through transfer or distillation.

PART II.

SERUM PROTEIN-BOUND IODINE

Chapter 1.

DETERMINATION OF SERUM PROTEIN-BOUND IODINE BY A CHLORIC ACID DIGESTION METHOD.

Introduction.

A major part of the work carried out for the present thesis was concerned with the development of a simple and reliable procedure for the microdetermination of iodine in biological material. A method was required which could be applied to the analysis of serum, urine, faeces, water and foodstuffs. First consideration was given to measurement of the protein bound fraction of serum iodine (PBI) because of the outstanding importance of this parameter in the assessment of thyroid function. This section deals with the chemical and technical aspects of serum PBI analysis. A full account of the significance and clinical application of PBI is given in Chapter 2.

Choice of Method.

It was decided, after careful consideration of the existing methods for microdetermination of iodine, that a chloric acid digestion procedure with catalytic determination of iodine was most suitable for the purposes of the present study. The digestion procedures described by Zak et al. (1952) and Oneal and Simms (1953) were used as the basis of the method. In order to

simplify the technique and provide a method which gives consistent and reliable results in routine use several modifications were introduced.

The original methods involve digestion of 2 - 3 ml serum samples with 25 ml aliquots of 28 % chloric acid. Digestion is carried out in 250 ml beakers on a hot plate. It was considered in the present case that a reduced sample size of 0.5 ml would cause less inconvenience to patients and enable digestion to be accomplished in less time with a reduced volume of chloric acid. Trials showed that complete digestion of 0.5 ml samples could be achieved in 1½ - 2 hrs. with 5 ml of chloric acid. The samples were digested in 50 ml centrifuge tubes heated either on a sand bath or a specially constructed aluminium block heater.

Separation of inorganic iodine from serum PBI was initially carried out by precipitation of serum proteins with trichloroacetic acid. Subsequently and alternative method was devised whereby inorganic iodine was removed by ~~treat~~ment in a column of anion exchange resin. The resin procedure proved to be both simpler and more effective in removing inorganic iodine.

The reagent concentrations used by Oneal and Simms (1953) were adjusted to suit the reduced sample size. Sodium chloride, added to arsenious acid during preparation helped to increase the sensitivity of the catalytic determination. A sulphuric acid

concentration of approximately 0.2 N was used for the final spectrophotometric reaction.

Technical difficulties involved in chronological observation of catalysis end points were overcome by using a brucine inhibitor to arrest the Ce^{IV} - As^{III} reaction at the required time.

EXPERIMENTAL

Precautions.

Because of the extreme sensitivity of the catalytic procedure employed and the minute amounts of iodine being determined (0.5 ml of serum normally contains less than 0.03 µg of iodine) rigorous precautions were taken to prevent contamination from outside sources of iodine, oxidising-reducing agents and catalytic inhibitors. The following points were given primary consideration : (1) Isolation of the analytical apparatus and reagents, (2) The consistent use of absolutely clean glassware, and of water and chemical reagents of the highest purity, (3) Thorough destruction of the organic matter present in the sample being analysed, (4) Extreme care in manipulation.

The work described in this thesis was carried out in a small laboratory, set aside solely for iodine analysis, which was kept scrupulously clean and free of all sources of contamination. Water was purified by distillation from a glass still followed by treatment with ion exchange resin. Chemicals used were of 'Analar' grade in every case with the exception of ceric sulphate (low in rare earths, Hopkins and Williams, Ltd.). All new stocks of reagents were carefully tested before use on unknown specimens. Glassware was steeped overnight in chromic

acid, rinsed thoroughly with tap water, and washed at least three times with distilled deionised water.

Apparatus.

Water was deionised, after distillation, by passage through twin columns each 20" X 2½", containing Amberlite MB 3 ion exchange resin. With this apparatus 20 - 30 litres of pure water were obtained in about 6 hours.

The sand bath initially used for the digestion stage was constructed from a 'Heatrae' 1,500 watt 12" X 8" hot plate with 'Simmerstat' temperature control. A cast iron frame fitted to the hot plate contained a ¾" layer of fine iron filings above which was a copper plate supporting a 1½" deep layer of fine acid washed sand. At a working temperature of 160° C the temperature fluctuation across the bath was between 5 and 10°. Up to 24 tubes were treated in the bath at the one time.

An improved digestion apparatus was later developed based on a design by Albert-Recht and Fraser (1961). This consisted of an electrically heated, thermostatically controlled, solid aluminium block containing holes drilled to fit digestion tubes. The apparatus had a capacity of 30 tubes and maintained a constant temperature of 160 ± 1°C.

Digestion was carried out in 50 ml 4" X 1" hard glass centrifuge tubes supplied by M.S.E. Ltd.

Glass columns 3 x 120 mm. drawn out to a fine point at one end and plugged at the tip with a wisp of cotton wool.

A thermostatically controlled water bath accurate to $\pm 0.5^{\circ}C$ was used for the catalytic reaction.

Colorimeter readings were taken on an Rel photoelectric colorimeter using a 420 mμ violet filter.

An EKCO N550 well type scintillation counter was used for measuring I^{131} in test tube samples.

Reagents

Trichloroacetic Acid : 50 gm. dissolved in 1,000 ml H_2O

Amberlite IRA 400 (Cl) Anion Exchange resin (B.D.H. Ltd.) : 200 g. of resin was washed thoroughly with 10 - 15 litres H_2O under suction in a 21 cm. Buchner funnel. It was necessary to dry the resin sufficiently to prevent hydration or dehydration of serum. Suction was left on for 1 hr. for partial drying. The resin in 50 g. aliquots was then spread out on Whatman 3MM filter paper, dried at room temperature for 25 - 30 mins., and stored in airtight polythene bottles. Each batch of resin prepared was tested before use on unknown samples by processing control serum specimens of known iodine content.

Potassium Iodate Standards : Stock solution contained 269.6 mg of dessicator-dried KIO_3 per litre. Dilution of 1 in 2,000 gave the reference solution containing the equivalent of 8 μg I per 100 ml. Standards of 4, 8, 12 and 16 μg I per 100 ml were run with each batch of s era.

Chloric Acid with Chromate : 500 g. $KClO_3$ and 200 mg Na_2CrO_4

were dissolved in 1,000 ml H_2O. To the hot solution was added slowly with stirring 370 ml of 72 % perchloric acid. After cooling and standing overnight the supernatant acid was filtered through Whatman 541 paper and stored below 4°C.

Arsenious Acid with Sodium Chloride : Stock solution was prepared by dissolving 12g. As_2O_3 and 8g. NaOH in 400 ml H_2O. The solution was neutralised to phenolphthalein with 10 % H_2SO_4, 100 ml 50 % H_2SO_4 and 30 g. NaCl were added with stirring. The solution was cooled, diluted to 1,000 ml with H_2O and stored below 4°C. Working solution was prepared fresh for each analysis by 1 in 10 dilution of stock with H_2O. The final acidity was approximately 0.18 N with respect to H_2SO_4.

Ceric Sulphate : To 100 g. $Ce(SO_4)_2$ H_2O in a 1 litre beaker was added 100 ml of conc. H_2SO_4 followed by the careful addition of 70 ml H_2O to the stirred mixture. The solution was heated under a low flame for 20 min., cooled, and a further 600 ml H_2O added. The volume was made up to 1,000 ml and the solution filtered. The working solution was prepared by 1 : 1 dilution with 10 % H_2SO_4. The final acidity with respect to H_2SO_4 was approximately 3.6 N

Brucine Sulphate (0.5 %) : 5g. brucine sulphate dissolved in 1,000 ml 5 % H_2SO_4.

Procedure.

Separation of PBI from Inorganic Iodine:

(a) Serum Protein Precipitation: Serum proteins were precipitated with 10 ml of 5 % trichloroacetic acid, centrifuged at 2,500 r.p.m. for 10 min. and the supernatant acid carefully decanted.

(b) Resin Column Treatment of Serum: The glass columns were packed with resin to a depth of 10 cm. and 2.5 ml of serum added from a pasteur pipette without delay. The rate of flow of serum through the column was about 1 ml per min. One treatment was sufficient for each serum aliquot, the resin being discarded after use.

Chloric Acid Digestion: Serum samples were analysed in duplicate. To 0.5 ml of treated serum in a centrifuge tube was added 5 ml of chloric acid. The tube was then placed in the sand bath and the contents digested for $1\frac{1}{2}$ - 2 hrs. at 160°C. Standards and a 0.5 ml water blank were treated in the same way as serum samples. At the completion of digestion approximately 0.5 ml of an amber-coloured solution remained in the tubes. On cooling, the liquid became colourless and deposited red crystals of chromium trioxide.

Care was taken to avoid over-digestion of samples and the resultant iodine loss. Over-digestion was indicated by excessive fuming and the presence of green Cr^{3+} ion.

The Ceric-Arsenite Reaction: 15 ml of freshly prepared arsenious acid wa s added to the cooled tubes and thoroughly mixed with the digestion residue by shaking. The tubes were placed in the water bath at 37° C and the contents allowed to stabilize for 10 - 15 mins. 1 ml of ceric sulphate was added at 30 sec. intervals. Each tube was carefully shaken to mix the contents. After a set time (20 - 40 mins), 1 ml of brucine sulphate was added to terminate the catalysis. The tubes were then removed from the water bath a nd allowed to attain room temperature.

Calculation of Results: Colorimeter readings of the cerate-brucine complex were taken 10 - 15 mins after brucine addition. A curve of iodine content vs. extinction was plotted for the standards and blank. A fresh curve was constructed for each batch of sera analysed. The results of unknown specimens were read off from the calibration curve and expressed as µg per 100 ml of serum.

RESULTS AND DISCUSSION

Separation of Serum P.B.I.

(a) Serum Protein Precipitation.

The natural thyroid hormone is present as a constituent of thyroxine binding protein and is quantitatively precipitated with total serum proteins by a number of general protein precipitants. On the other hand, inorganic iodine is not precipitated but remains in the aqueous filtrate. Accordingly protein precipitation affords a means of separation of the hormonal iodine or PBI from any inorganic iodine present.

The trichloroacetic acid precipitation procedure first used in the present method provided an effective separation of PBI in the absence of high concentrations of inorganic iodide. 'Analar' grade trichloroacetic acid (B.D.H. Ltd.) was found to have a negligeable iodine content and was completely oxidised during the digestion procedure, leaving no non volatile products to interfere in the cataly tic reaction. However, in cases where the inorganic iodide concentration is very large compared to that of the hormone fraction it is impracticable to free the protein precipitate from the inorganic fraction. Repeated washing of the protein precipitate assists the separation, but a proportion of hormonal iodine is lost in the process. In the present method the mean loss of hormonal iodine during precipitation was 3.6 %.

This was shown by adding thyroxine - I^{131} to serum and measuring the change in I^{131} concentration after protein precipitation.

(b) Anion Exchange Resin Treatment of Serum.

The removal of inorganic iodine from serum by treatment with ion exchange resin was investigated in an attempt to devise a more efficient separation technique.

Anion exchange resins have been used by several workers (Zieve et al, 1956 ; Scott and Reilly, 1954; Fields et al., 1956; Dowben, 1960; Penna-Franca, 1960) for the separation of iodide from organic iodine in the determination of serum or plasma protein-bound radio-iodine (PBI^{131}). Only Slade (1956) has attempted to apply resin separation to chemical PBI determination. He found, however, that a precipitation step was necessary following treatment with 'Dowex 1 or 2' because of iodide contamination of samples from the resin.

The anion-exchange resin technique described by Zieve et al. (1956) for PBI^{131} analysis was applied in modified form to the present PBI procedure. The 21 cm. column used by these authors was replaced by one of 10cm. and 2.5 ml serum aloquots were treated. Before the technique was applied to unknown samples it was necessary to ensure that iodide wa s removed

efficiently and without loss of hormonal iodine. Accordingly experiments were carried out on serum specimens to assess the validity of the method.

The capacity of the resin column to separate inorganic iodide was shown in two ways. Using serum containing added NaI^{131} it was possible to remove no less than 98 % of iodide as I^{131} (Table I). Additional evidence of the high efficiency of separation was obtained by determining the PBI concentration, after resin treatment, of samples containing a high concentration of added iodide (800 µg / 100 ml). It was shown that only 0.22% of iodide was retained by the serum following treatment.

Table I. Separation of NaI^{131} from Serum by Resin Treatment

Serum No.	Na I^{131} in Serum c/100 sec.		NaI^{131} removed
	Untreated	Resin-treated	%
1	41,532	384	99.08
2	70,124	970	98.62
3	63,210	892	98.59

The effect of resin treatment on PBI was measured by adding thyroxine - I^{131} to serum and counting 2 ml aliquots before and after passage through the column. The results of this study are given in Table II The mean loss of thyroxine was only 0.53 % (Ta ble II).

Table II. Recovery of Thyroxine - I^{131} after Resin Treatment.

Serum No.	Thyroxine - I^{131} c/100 sec.		Recovery %
	Untreated	Resin Treated	
1	33,308	33,119	99.43
2	31,025	30,903	99.61
3	30,397	30,227	99.44
4	30,142	29,973	99.43

In a further investigation the PBI content of a serum sample containing an additional 6.4 µg I /100 ml, added as l - thyroxine, was determined before and after resin treatment. Six determinations were carried out in each case and the recovery of thyroxine iodine calculated. There was a close agreement between the recovery figures, (Table III).

Table III Recovery of Added Thyroxine Iodine (6.4 µg I/100 ml) from Serum.

SAMPLE NO.	Thyroxine Iodine Recovered. UNTREATED µg / 100ml	%	RESIN TREATED µg / 100ml	%
1	6.8	106.3	6.7	104.7
2	6.2	96.9	6.4	100.0
3	6.1	95.3	6.5	101.3
4	6.4	100.0	6.1	95.3
5	6.7	104.7	6.4	100.0
6	6.3	98.4	6.5	101.6
MEAN		100.3		100.5

The results of the above experiments indicate that the change in hormonal iodine concentration occuring during the resin separation stage is sufficiently small to have no appreciable effect on the final PBI value.

Particular care was taken to ensure that the resin was dried sufficiently to prevent hydration or dehydration of serum samples. Each batch of resin prepared was tested for proper moistness by mea suring the PBI content of serum before and after treatment in two consecutive columns of resin. Since the first column removes inorganic iodide the PBI values beforeand after treatment in the second column should be identical. The results in Table IV illustrate the application of this test technique. The serum used in the example given contained a high concentration of iodide as was indicated by the drop in iodine concentration after passage through column No. 1.

Table IV. Effect of Resin Treatment on Serum PBI Concentration.

		Duplicate values (μg I / 100 ml)	
Untreated serum ;	total iodine;	12.0	11.8
Treated in column 1;	PBI	8.0	8.2
" " " 2;	PBI	8.2	8.2
" " " 3;	PBI	8.2	8.4
" " " 4;	PBI	8.1	8.5

In addition to providing a check on the moisture content of the resin the above technique may also be used as a test for iodide contamination of serum from the resin. No significant contamination from this source was experienced in the present

investigation. As an additional test of the resin in this respect, the following experiment was carried out. Resin was soaked in 1 % sodium iodide solution containing added NaI^{131}. The I^{131} - labelled resin was then filtered, washed and dried by the standard procedure, and packed into a column. Serum was passed through and the activity of 0.5 ml aliquots of the eluates counted. The results showed that no more than 0.15 % of I^{131} was taken up by serum from the resin.

The resin and trichloroacetic acid separation techniques were compared by determining the PBI content of serum samples from euthyroid subjects by both methods. In 19 samples tested in this way the values obtained by the resin method averaged 0.2 µg / 100 ml less than those obtained by the precipitation procedure. In 7 of the samples identical results were obtained and of the remaining 12 only 3 gave results differing by more than 0.3 µg / 100 ml.

The foregoing experiments demonstrate that treatment of serum with amberlite IRA 400 (cl) is an efficient method of separating PBI from inorganic iodine. Provided care is taken in preparing the resin for use, both iodide contamination from the column and hormonal iodine losses are insignificant. A single treatment with resin provides a quicker and more effective separation of serum PBI in the presence of large amounts of inorganic iodine than does protein precipitation.

Chloric Acid Digestion

The prevention of iodine loss during digestion depends upon the maintenance of iodine in the form of iodate. Chloric acid maintains iodine in the oxidised form but the acid is lost during digestion, partly by decompsition to volatile products and partly by conversion to perchloric acid. Zak et al. (1952) found that loss of iodine occurs in fuming mixtures of iodic and perchloric acid in the absence of chromium. Hot concentrated perchloric acid oxidises Cr^{3+} to Cr^{6+} and the presence of Cr^{6+} maintains iodine in the form of iodate throughout digestion. Both Zak and Oneal and Simms (1953) found that 5 mg. amounts of Na_2CrO_4 were sufficient to keep iodine in the oxidised form. Goodwin et al. (1957), on the other hand, studied the loss of I^{131} during digestion and concluded that the recovery of iodine was unaffected by the presence or absence of chromate providing digestion was stopped before complete dryness was reached.

In the present method the amount of chromate in 5 ml of chloric acid is approximately 1 mg. which is equivalent to the 5 mg. quantities used by Zak and Oneal and Simms in 25 ml of acid. The effect of chromate was investigated by adding Na I^{131} to serum and comparing the recovery of iodine obtained after digestion in the presence of chromate with that obtained in the absence of chromate. Each sample contained 0.05 µg I. The results (Table V)

indicate that chromate has no effect on iodine recovery. In every case digestion was stopped when approximately 0.5 ml of amber-coloured residue remained. Excessive iodine loss occurred however, when samples were digested beyond this stage as was indicated by the presence of green Cr^{3+} ion.

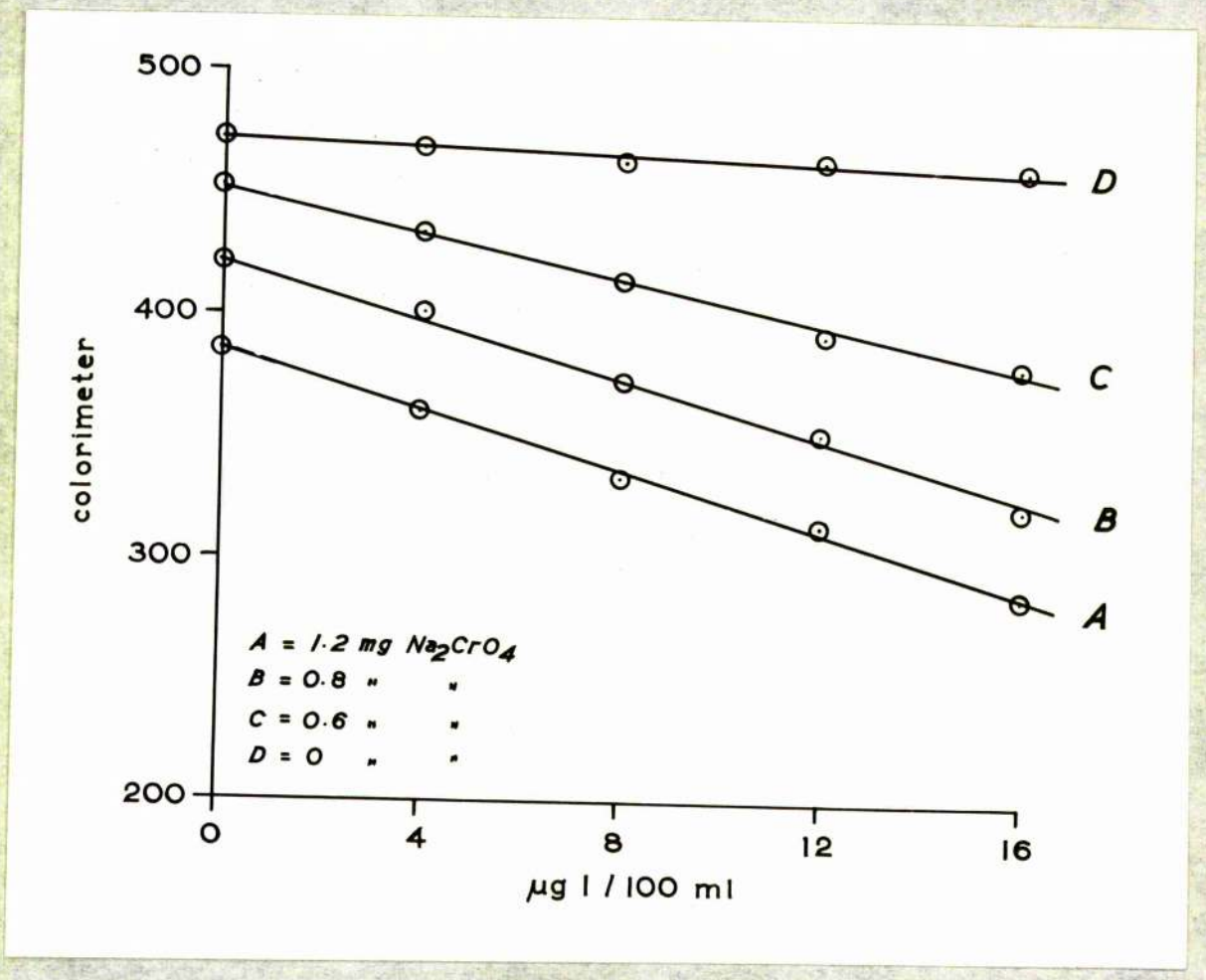
Table V. The Effect of Chromate on Iodine Recovery.

Sample No.	Na_2CrO_4 concn. mg.	I^{131} before digestion C/100 sec	I^{131} after digestion C/100 sec	Recovery of I^{131} %
1	1.0	5,591	5,465	97.74
2	1.0	5,537	5,401	97.54
3	1.0	5,468	5,305	97.02
4	1.0	5,534	5,493	99.26
5	0	5,587	5,509	98.60
6	0	5,412	5,280	97.56
7	0	5,540	5,367	96.88
8	0	5,526	5,499	99.51

Carr et al. (1950) using the chromic acid distillation technique of Barker (1948) demonstrated that chromate ion exerted

a strong catalytic effect on the reduction of ceric sulphate by arsenious acid. In the present method the catalytic effect of chromate was studied by adding Na_2CrO_4 to the standard arsenious acid - ceric sulphate system at 37°C. Amounts of 0.4 - 1.2 mg. were added to tubes containing iodide standards. A series of iodide standards without added chromate was included for comparison. The results shown in Fig I demonstrate the strong catalytic effect exerted by chromate.

Fig I. Catalytic Effect of Chromate.



Colorimetric Determination of Iodine.

Various techniques have been used to measure the extent of reduction of ceric sulphate in iodine micro-estimation. Sandell and Kolthoff (1937) determined the amount of iodide present in test solutions by measuring the time required for complete disappearance of the yellow ceric colour. In Chaney's (1940) method, and many modern methods, measurements of the ceric colour intensity are taken after a specific time interval while the reaction is still in progress.

Chaney's method, if carefully applied gives good results; but taking accurate photometric readings while reduction is continuing is technically very difficult and can give rise to considerable experimental error. Accuracy may be improved and the analytical procedure simplified by completely arresting the $Ce^{IV} - As^{III}$ redox. reaction before measuring the colour of the test solution. The catalytic inhibitors already described (Part I) have been employed for this purpose.

Grossman and Grossman (1955) found that brucine arrested the catalytic reaction by forming a stable complex with cerate ions in which the cerate colour was greatly intensified. These authors added brucine sulphate solution to a cooled reaction medium in which the ceric ion was dormant with respect to its activity in the catalysis. In the present case, after trial of

the original method, it was discovered that a simplified procedure involving direct addition of brucine sulphate to the reaction system at 37°C gave excellent results. The brucine solution instantaneously arrested the Ce^{IV} - As^{III} reaction with the initial formation of a deep red colour which faded in a few seconds to orange - brown.

The stability of the cerate - brucine complex was studied by measuring the colour intensity of a series of standard test solutions over a period of 4 days. It was found that a slight, almost imperceptable colour increase occurred initially, reaching a maximum after 20 hours. This increase, however, amounted to less than 5 % of the initial value and was not significant. Colour intensity readings taken 4 days after brucine addition showed no change from the 20 hour value.

The conditions employed in the present method were such that a linear relationship was obtained on plotting the iodine content of standards against the brucine - cerate colour intensity (see Fig I), as measured on an Eel photoelectric colorimeter using a 420 mμ violet filter. The reaction time allowed depended on the iodine content of the reagents used and normally varied from 20 to 40 mins at 37°C. Consistent results were obtained when the optical density of the test solutions ranged from 0.15 for the standard containing 16 μg I % to 0.45 for the reagent blank.

The direct-reading Eel colorimeter used in this work enabled measurement of the brucine-cerate complex to be accomplished in a much shorter time than with a photoelectric spectrophotometer, the time factor becomes increasingly important when a large number of samples are processed at the one time. The results obtained on serum samples using both the Eel and a Hilger spectrophotometer were compared and showed no significant difference..

Sources of Contamination Encountered.

Although every precaution was taken throughout this investigation considerable time was lost due to the use of contaminated reagents and apparatus.

Great difficulty was experienced in obtaining supplies of potassium chlorate which did not contain appreciable quantities of iodine. It was finally necessary to test samples from several batches provided by B.D.H.Ltd., and Hopkins and Williams, Ltd., and obtain a large quantity of the batch which proved most satisfactory.

The most troublesome source of contamination encountered was that caused by mercury. For a long period of time results could not be obtained due to complete inhibition of the $Ce^{IV} - As^{III}$ catalytic reaction. Finally, a trace of mercury was detected in

the drying cupboard used for glassware; when this was removed satisfactory colorimetric determination was again achieved.

Laboratory Studies of the Reliability of Serum PBI Estimation.

Recovery Experiments: The accuracy of the analytical procedure was determined by adding known quantities of KI, KIO_3, diiodotyrosine and thyroxine to serum samples and measuring the recovery of iodine. The results obtained in a series of recovery experiments are given in Table VI. Each value quoted is the average of 4 estimations. The mean recovery of added iodine is 98.1 %.

Table VI. Recovery of Iodine added to Serum.

Substance	Amount I added (µg%)	Amount I Recovered %
KI	3.0	96.6
	4.0	97.0
	6.0	100.0
	8.0	101.0
	16.0	92.1
KIO_3	3.0	101.7
	4.0	95.0
	6.0	98.5
	9.0	99.0
	12.0	93.6
Thyroxine	6.4	101.3
	9.6	102.0
	12.8	98.2
Diiodo-tyrosine	5.4	97.2

Determinations of Known Internal Control Sera: One of the guides to reliability used in the present method was the inclusion of a serum of established value in each batch of unknown samples analysed. Control sera for this purpose were obtained from Dade Reagents Inc., Miami, Florida, U.S.A. Over a period of 3 months 50 duplicate determinations were carried out on a control sample of established value 6.3 - 6.9 μg I/ 100 ml. The results showed a range of 6.0 - 7.0 μg I/ 100 ml (S.D. $\pm$ 0.2). The maximum difference between duplicate values was 0.4 μg I/ 100 ml and the mean difference less than 0.2 μg / 100 ml.

Chapter 2.

DETERMINATION OF SERUM P.B.I. IN THE ASSESSMENT OF THYROID FUNCTION.

Introduction.

Measurement of the serum or plasma protein-bound iodine (PBI) provides in most instances an accurate estimate of the level of circulating thyroid hormone. The PBI value represents the balance between the amount of thyroid hormone secreted by the thyroid and the amount peripherally metabolised or excreted during the same time. At the present time it is the best available measure of the activity of the thyroid and the most accurate clinical criterion for the diagnosis and treatment of thyroid disorders. This chapter will be devoted to consideration of the clinical applications of serum PBI estimation.

Several comprehensive reviews on blood iodine analysis are available (Rapport and Curtis, 1950; Blackburn and Power, 1955; Chaney, 1957). Early attempts to measure blood iodine as a diagnostic procedure were disappointing because of the inadequacy of the analytical procedures employed. Early determinations on whole blood disclosed that it contained iodine in both organic and inorganic form. Subsequently it was found that the organic fraction was confined to serum. The demonstration that the major part of the iodine in serum is non-dialysable and is

co-precipitated with the proteins (Trevorrow, 1939; Man et al. 1942) led to the development of methods for determining the iodine precipitated with protein. The terms "serum precipitable iodine" (SPI), "hormonal iodine " and "protein-bound iodine"(PBI) have been applied to this protein-bound fraction.

Materials and Methods.

Estimations of PBI were carried out in duplicate on 0.5 ml serum samples by the method described in Chapter 1. The variability of the method was tested by frequent analysis of control serum samples of known iodine content.

The records of more than 1,500 patients in whom serum PBI was measured between December 1958 and September 1961 were reviewed. The cases were arranged into groups on the basis of all the clinical and laboratory evidence available exclusive of PBI.

Patients in whom a definite diagnosis had not been established were not included in this study. Also excluded were patients recently treated with antithyroid drugs, thyroxine, triiodothyronine, inorganic iodine, iodised radiopaque dyes and therapeutic doses of radioiodine.

The diagnosis of thyrotoxicosis was based on typical clinical features (all patients scored > 20 on the diagnostic index of Crooks et al., 1958) and characteristic radioiodine tests (Wayne, 1960). Hypothyroid patients were chosen who showed the classical clinical picture (all scored > 15 on the hypothyroid index of Wayne, 1960) together with a typical pattern of radioiodine excretion. Patients with Hashimoto's thyroiditis had a firm goitre, either diffuse or nodular, and the diagnosis was confirmed in every

case by positive results with the precipitin test (Rosenberg, 1960) and / or histologic examination of the gland (Buchanan et al. 1961). Further evidence of Hashimoto's disease was provided by: (1) the thyroid complement fixation test (Anderson et al. 1959); (2) electrophoresis of serum proteins; and (3) the standard serum flocculation test. The diagnosis of simple non-toxic goitre was made after full clinical and laboratory investigation to exclude, as far as possible known causes of goitre such as thyrotoxicosis, neoplasm, thyroiditis and dyshormonogenesis. These patients were as a group iodine deficient as was indicated by a low PII level (mean 0.08 µg / 100 ml; range 0.02 - 0.35 µg / 100 ml) and low urinary excretion of iodine.

RESULTS AND DISCUSSION.

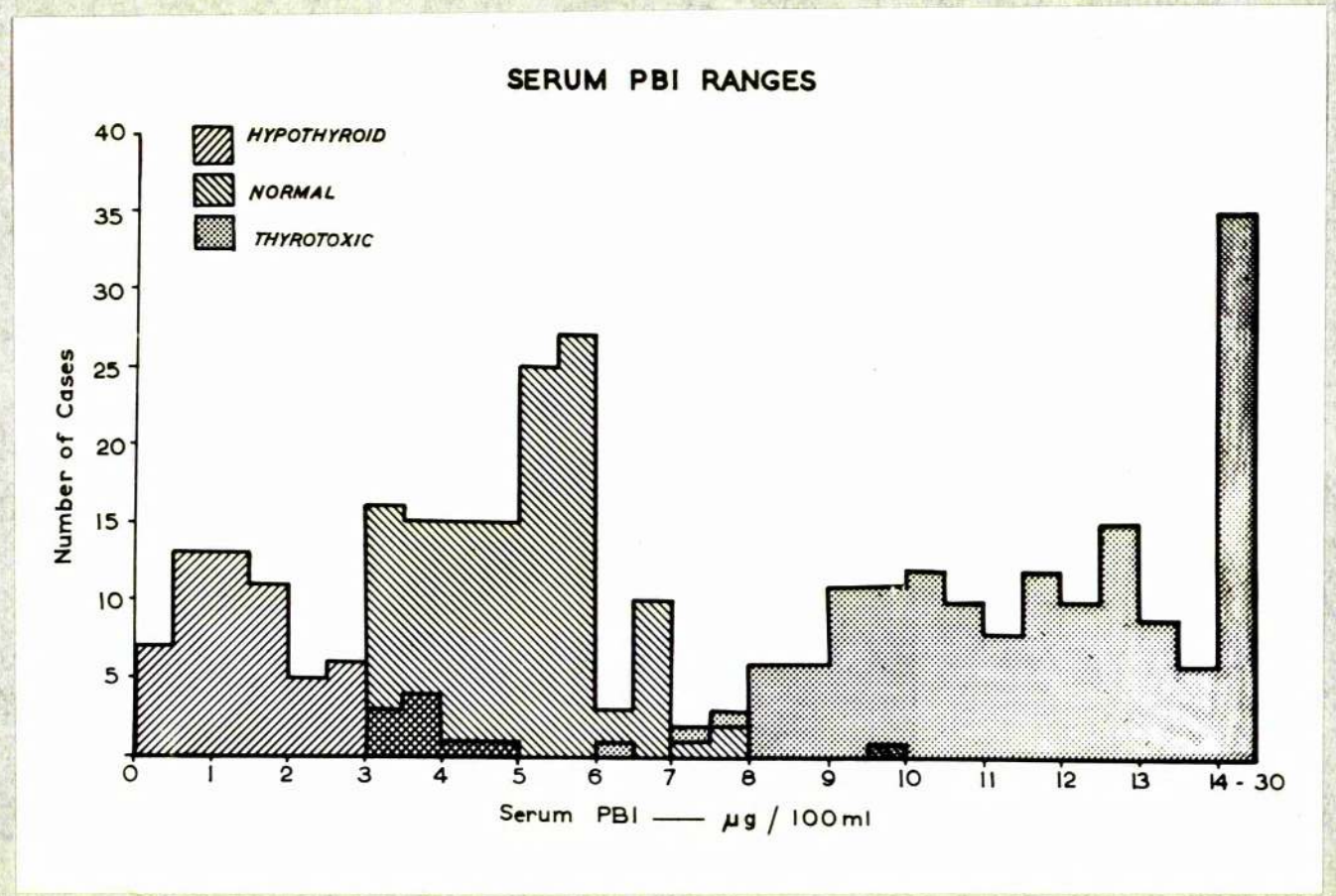
Relationship of PBI to Hypo and Hyper-thyroidism and Normal Range.

The most important use of PBI determination is in the diagnosis of hypo- and hyperthyroidism. High levels of PBI are associated with hyperthyroidism and low levels are found in hypothyroidism. It is essential to determine the limits of the normal range before using PBI estimations for diagnostic purposes.

In order to establish a normal range PBI estimations were carried out on serum samples fron 130 euthyroid subjects. These were clinically euthyroid members of the staff and students or were hospital patients in whom other tests had confirmed the absence of thyroid disease. The results were in good agreement with those of other authors (Table I). Values ranged from 3.0 - 9.3 µg / 100 ml with a mean of 4.9 µg / 100 ml (S.D. 1.16). The distribution of values was not a normal one (Fig I), and since only two values (7.8 and 9.3) lay outside the range 3.0 - 7.5 this was taken as the normal range, since it provides the best separation between normal subjects and patients with hyper- and hypo-thyroidism.

In 163 cases of thyrotoxicosis the PBI range observed was 4.8 - 30.0 µg / 100 ml (mean 12.5; S.D. 3.9). The distribution

Fig I.


SERUM PBI RANGES
HYPOTHYROID
NORMAL
THYROTOXIC
Number of Cases
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14 - 30
Serum PBI —— µg / 100ml

of values is shown in Fig I where it can be seen that a slight overlap of the upper normal range occurred. However, since only 4 values (4.8, 6.2, 7.4, and 7.4) were responsible for this overlap, an extremely good separation of thyrotoxic cases was obtained.

Serum PBI values in 64 cases of hypothyroidism ranged from 0.3 - 4.5 µg / 100 ml (mean 1.6, S.D.1.1). The separation of this group from the normal series was not as clear as that obtained for the thyrotoxic cases. A significant overlap of higher hypothyroid values occurred at the lower limit of the normal range (Fig I), for which 9 cases were responsible.

The foregoing results indicate that the PBI estimation used in this study provides a reliable separation of hypo- and hyperthyroid subjects from the normal series. From Table II it can be seen that the PBI ranges obtained are in general agreement with those of other authors.

Our results do not support the statement by Starr (1955) that values for PBI less than 4 or more than 8 µg / 100 ml by definition indicate the existence of disordered thyroid function. Such a rigid biochemical definition of abnormality ignores the existence of a small range of values for PBI common to both euthyroid persons and those who have disordered thyroid function.

Table I.

Values for PBI in Euthyroid Subjects.

Author	PBI (µg / 100ml serum)			
	No. of Cases	Mean	Range	S.D.
Turner et al. 1940	40	6.3	3.5 - 10.4	-
Salter et al. 1941	21	5.7	4.0 - 8.0	1.0
Riggs 1947	55	5.1	3.5 - 7.5	-
Perry and Cosgrove 1949	34	5.9	4.0 - 9.3	1.3
Kydd et al. 1950	83	5.3	3.8 - 8.6	1.0
Starr et al. 1950	100	5.5	4.0 - 8.5	0.9
Barker et al. 1951	68	5.1	3.4 - 8.0	1.0
Tucker and Keys 1951	402	5.8	2.6 - 11.1	1.3
Hallman et al. 1951	37	5.4	3.2 - 7.6	1.08
Sunderman and Sunderman 1953	65	5.0	2.9 - 7.9	1.0
Zak et al. 1952	120	7.3	3.5 - 11.3	-
Oneal and Simms 1953	10	5.2	-	-
Winikoff 1954	106	5.1	2.7 - 8.0	-
Zieve et al. 1954	50	6.7	4.6 - 9.3	1.0
Blackburn and Power 1955	530	5.2	2.5 - 8.3	1.2
Sanz et al. 1956	12	5.2	3.8 - 6.0	-
Astwood 1957	117	-	4.0 - 8.0	-
Levy 1959	49	-	3.5 - 7.4	-
Vannotti and Beraud 1959	30	5.1	4.2 - 6.1	-
Tanaka and Starr 1959	103	-	5.0 - 7.0	-
Present Study	130	4.9	3.0 - 7.5	1.16.

Table II. Values for PBI in Hypothyroidism and Hyperthyroidism.

Author	HYPERTHYROIDISM			HYPOTHYROIDISM		
	No. of Cases	PBI (μg/100 ml) MEAN	RANGE	No. of Cases	PBI (μg/100 ml) MEAN	RANGE
Turner et al. 1940	20	15.9	6.7 - 38.5	-	-	-
Winkler et al. 1945, 1946	87	13.8	4.9 - 55.1	25	1.3	0.2 - 2.5
Kydd et al. 1950	206	-	7.1 - 48.2	93	-	0.0 - 3.0
Starr et al. 1950	208	12.3	3.5 - 25 +	39	2.1	-
Sunderman and Sunderman 1953	56	-	6.5 - 29.0	15	-	0.4 - 3.4
Blackburn and Power 1955	354	12.1	4.4 - 30.0	113	1.3	0.0 - 5.2
Present Study	163	12.5	4.8 - 30.0	64	1.6	0.3 - 4.5

Serum PBI in Hashimoto's Thyroiditis.

Thirty nine patients with Hashimoto's thyroiditis were studied. PBI values were low or low-normal and ranged from 0.5 - 5.3 µg / 100 ml (mean 2.5; S.E. 0.2). In 22 clinically euthyroid members of this group the mean PBI level of 3.1 µg / 100 ml was significantly lower ($p < 0.001$) than the mean normal value (4.9 µg / 100 ml).

Low or normal values of PBI in Hashimoto's disease have been reported by most authors, (Statland et al, 1951; Skillern et al. 1956; Skillern and Evans 1957; Owen and McConahey 1956) with the exception of Gribetz et al. (1954) who in 6 children found high values in 3 (8.8, 9.6 and 9.3 µg / 100 ml). Our study, which included a larger number of cases than any previously published, indicates that the low PBI level in this disease reflects decreased thyroid hormone production. The alternative explanation - increased peripheral degradation of thyroxine - is most unlikely, since the patients tended to be hypothyroid and thyroxine degradation is decreased in hypothyroidism (Ingbar, 1960).

Serum PBI in Simple Goitre.

In 52 patients the PBI level ranged from 2.5 - 7.5 µg/100 ml. The mean value of 5.1 µg/100 ml (S.E. 0.17) in this group did not differ significantly from the mean obtained in the normal

control series (4.9 µg/100 ml), and only 3 values (2.5, 2.7 and 2.7) lay outside the limits of the normal range. The normal PBI level found in this series indicates that these patients have fully compensated iodine deficiency and are, therefore, euthyroid.

Serum PBI in Hepatic Cirrhosis.

PBI estimations were carried out in 16 patients with hepatic cirrhosis in whom no obvious signs of thyroid dysfunction were apparent. Values ranged from 3.0 - 8.0 µg/100 ml (mean 5.0, S.D. 1.4), and only one value (8.0 µg/100 ml) lay outside the limits of the normal range. A normal PBI level in cirrhosis has previously been reported by Kydd and Man (1951) and Tanaka and Starr (1959).

Physiological and Other Factors Influencing PBI Determination.

Effect of age and sex on normal range.

PBI values in the normal control series are shown in Table III according to age and sex. The values were statistically insufficient to define age group difference. No significant difference was apparent between the sexes in this group; the mean value in 84 females was 4.8 µg/100 ml (S.E. 0.12) and in 46 males 5.1 µg/100 ml (S.E. 0.19). A larger series will require to be studied in order to obtain statistically relevant data.

Excluding pregnancy, where a definite increase in PBI unassociated with any symptoms of hyperthyroidism occurs (Heineman et al. 1948; Dowling et al. 1956), no significant difference between the sexes has been demonstrated. Few statistically valid studies of the effect of age on PBI have been reported. Man et al. (1952) found that infants in the first few days had a distinctly higher normal PBI level than adults. This level gradually diminished to the adult range during childhood. This finding has not been statistically demonstrated in any large series. Tucker and Keys (1951) showed in a large group that middle-aged men (45 - 56) had a significantly lower PBI level than younger men. This finding has not been confirmed in the large series reported by Gaffney et al. (1960).

Table III. PBI VALUES IN 130 NORMAL SUBJECTS.

AGE IN YEARS	FEMALES NO. OF CASES	FEMALES PBI (µg/100 ml) RANGE	FEMALES PBI (µg/100 ml) MEAN	MALES NO. OF CASES	MALES PBI (µg/100 ml) RANGE	MALES PBI (µg/100 ml) MEAN
10 - 19	6	3.7 - 6.2	4.9	2	4.0 - 5.3	4.6
20 - 29	7	4.3 - 6.9	5.3	7	3.3 - 6.6	5.2
30 - 39	12	3.0 - 7.8	5.0	7	3.4 - 6.9	5.5
40 - 49	12	3.1 - 5.9	4.7	8	4.1 - 6.0	5.0
50 - 59	18	3.0 - 6.9	4.6	13	3.2 - 6.9	4.9
60 - 69	23	3.0 - 7.5	4.9	6	3.3 - 6.5	5.1
70 - 79	4	3.0 - 5.8	4.3	3	3.6 - 9.3	5.5
80 - 89	2	5.1 - 5.6	5.3			

Effect of organic iodinated drugs.

Many valuable therapeutic and diagnostic drugs contain iodine in organic combination. The contrast media used in radiography are the most commonly encountered substances which artifactually increase the concentration of PBI in serum, (Slingerland 1957; Cassidy, 1960). These compounds are apparently slowly metabolised, with the result that increased levels of inorganic iodine are frequently present in addition to elevated PBI concentrations which persist for 3 months or longer. Iodothiouracil, when used for the treatment of hyperthyroidism, renders measurement of PBI futile since elevated PBI values persist for some months, (Bondy, 1951).

Mercurial diuretics.

Falsely low values are obtained with these compounds (Meyers and Man, 1951) since mercury salts, even in extremely minute concentrations, inhibit iodide catalysis of the $Ce^{IV} - As^{III}$ system. The effect of diuretics is normally dissipated within 24 hours, but if excretion of the drug is delayed the effect may persist for 48 hours.

Summary and Conclusions.

A study has been made of the records of more than 1,500 patients in whom the level of serum PBI was determined. Thyroid function was assessed in each patient on the basis of data other than PBI.

The range of values for PBI and the mean values obtained in patients with different diseases of the thyroid gland are presented. Also determined was the range of values in a group of euthyroid persons without thyroid disease. The proportion of patients in the various diagnostic categories of thyroid disease who had values for PBI within the normal range was taken to be an index of the accuracy with which PBI delineates thyroid function.

It is concluded that a PBI range of 3.0 - 7.5 μg/100 ml provides a clear separation of normal cases from patients with hypo- and hyper-thyroidism. Estimation of PBI provides a very valuable laboratory test in the diagnosis of thyroid disease, but a small overlap occurs, especially within the hypothyroid group.

PART III.

INTAKE, EXCRETION AND STORAGE OF IODINE

Chapter 1.

DIETARY INTAKE OF IODINE.

Introduction.

The daily intake of iodine varies considerably from individual to individual depending on the amounts present in the food and water consumed. Published analytical figures (Chilean Iodine Education Bureau, 1952) reveal that wide variations may occur in the iodine content of similar foods marketed in different areas. In recent years several countries have ensured that an adequate dietary supply of iodine is available to the community by the provision of iodine supplements in the form of iodised salt. No legislation providing for compulsory salt iodisation has, however, been passed in Great Britain. Evidence is presented here in support of introducing iodised salt in Great Britain.

An investigation of the dietary supply of iodine in the West of Scotland was carried out. Since recent figures for the iodine content of fish, eggs and milk marketed in this area were not available, the iodine concentrations present in samples of these foods were determined. The iodine values so obtained, together with values for other foods taken from the Chilean Education Bureau (1952), were used to calculate the dietary

iodine intake in a control group of subjects without thyroid disease and in a series of patients with simple goitre, that is non-toxic goitre excluding frank cases of dyshormonogenesis, auto-immune thyroiditis and goitrogen administration. Additional sources of iodine investigated included drinking water and alginate food additives. The latter products were considered worthy of study since they are obtained by extraction from seaweed which is an extremely rich source of iodine.

Estimation of Iodine in Food and Water.

Materials and Methods.

Except where otherwise stated all samples were added to digestion tubes and analysed for iodine by the chloric acid digestion procedure previously described (Part II).

Milk: Samples were taken from bottles of pasteurised whole milk supplied by a Glasgow dairy. Analyses were carried out on 0.5 ml aliquots in duplicate.

Eggs: Government stamped 'Pool' hens eggs as supplied to the Western Infirmary were used. Whole eggs were diluted 1:1 with water and mixed thoroughly to give a homogenous solution. Approximately 0.5 gm aliquots were weighed into digestion tubes for analysis.

Fish: Filleted specimens of herring, haddock and whiting were

obtained from the Glasgow Fish Market and from Glasgow fish shops. Whole fresh fillets of each species were diluted with water and homogenised in a 'Kenmix' electric mixer. For the analysis of mussels, the entire contents of a jar were treated in this manner. All samples were diluted at least 1:3 and the high iodine content of haddock necessitated a dilution of 1:6. Homogenised material was transferred by pasteur pipette to weighed digestion tubes. The weight of fish taken for analysis ranged from 0.02 gm for haddock to 0.2 gm for herring.

The following modifications in analytical procedure were introduced in order to cope with iodine concentrations of more than 1,000 µg / Kg,

a the ceric sulphate-arsenious acid reaction temperature was reduced from 37°C to 20°C.

b the iodate standards used were doubled in concentration in order to cover the range 0 - 32 µg I %.

Alginate Food Additives. Food grade alginate samples were donated by Alginate Industries Ltd., Girvan, Ayrshire. Aqueous solutions of these products varying in strength from 0.2 - 1.0 % were analysed by the standard chloric acid method in 0.5 and 1.0 ml aliquots.

Drinking water. Samples were taken from 4 different cold water taps in the Western Infirmary. 100 ml aliquots containing

0.2 ml of 1% Na_2CO_3 were reduced by distillation to approximately 1 ml. The residue was taken up in 10 ml of chloric acid, which was divided into two equal portions and added to digestion tubes.

Recovery Experiments. 0.5, 0.75 and 1.0 ml aliquots of KIO_3 solution containing the equivalent of 40 - 80 µmg I were added to samples and the recovery of iodine determined. The recovery of iodine added as KI solution in 0.5 and 1.0 ml aliquots was measured for milk samples.

Assessment of the Accuracy of Analytical Procedures.

The iodine concentrations encountered in milk, eggs and water were of the same level as those normally measured in serum samples. Since the analytical technique used for serum was applied without modification the results obtained were of similar accuracy. The results of recovery experiments on two milk samples are given in Table I. The mean recovery of added iodine was 94 %. In a similar study the mean recovery of 40 µ mg I from an egg sample was found to be 100.6 %.

Table I.

Recovery of Added Iodine from Milk.

Iodine content of milk (µg / litre)	Iodine added as KI (µg/litre)	Total iodine found (µg/litre)	Iodine recovered %
124	50	177	106
		173	98
		173	98
133	50	173	80
		178	90
		-	-
124	100	222	98
		225	101
		215	91
133	100	223	90
		225	92
		222	89

Iodine loss during evaporation of water samples was shown to be less than 1.0 % by measuring the recovery of added NaI^{131}. It was found that the standard chloric acid method was not well suited to the analysis of fish samples by reason of the high iodine concentrations encountered. The use of small samples and the difficulty experienced in obtaining completely homogenious

material introduced a significant sampling error. The recovery of added iodine from a sample of herring ranged from 90 - 110% (Table II).

Table II.

Recovery of Iodine added as KIO_3 from a Sample of Herring.

Wt. Fish (x mg)	Iodine added to x mg (µmg)	Total Iodine in x mg (µ mg)	Recovery of Added Iodine µ mg	%
191.7	-	42.0	-	-
153.1	-	32.0	-	-
78.5	80	91.5	74.7	93.4
77.5	"	100.0	83.4	104.2
75.4	"	93.5	77.4	96.7
66.5	60	70.5	56.3	93.8
95.1	"	86.5	66.1	110.0
104.4	"	85.0	62.6	104.3
67.7	40	59.0	44.5	110.1
69.4	"	56.0	41.2	103.0
70.0	"	41.0	3 6.1	90.0

In the author's opinion the method best suited to the measurement of iodine concentrations of the order present in fish (1,000 - 10,000 µg/Kg) is that of alkaline incineration followed by starch - thiosulphate volumetric estimation. Harvey (1935) described a reliable method of this type. Unfortunately, however, while the present investigation was in progress a muffle furnace suitable for the alkaline incineration stage was not available. Attempts to combine chloric acid digestion with starch - thiosulphate volumetric determination of iodine were unsuccessful.

Results and Discussion.

The iodine concentrations found in eggs, milk and fish are shown in Table III. Average literature values quoted were obtained from the Chilean Iodine Education Bureau (1952). It must be stressed that few of the results included in the calculation of these averages were obtained on samples marketed in Great Britain and the figures merely serve to provide a rough means of comparison.

Table III. Iodine Content of Fish and Dairy Products.

	No. of Samples Analysed	Iodine content µg / Kg Found Range	Found Mean	Literature Average
FISH				
Haddock	4	6,590 - 9,860	8,250	3,180
Herring	3	210 - 710	400	520
Whiting	7	750 - 3,610	1,750	-
Mussels	1	-	850	2,660
DAIRY PROD.				
Eggs	5	142 - 373	247	93
Milk	10	26 - 133*	64*	35

* Values expressed as µg / litre.

Only two results for whiting have been reported - 300 µg/Kg by Bourcet (1899) and 34 µg/Kg by Zahoranszky (1924) - because of unsatisfactory analytical techniques these values cannot be compared with that obtained in the present study. The most recent figures for British market samples of herring and haddock were reported by Murray et al. (1948). These workers found concentrations of 2,200 and 9,320 µg/Kg respectively in dried samples. Assuming the water content of a wet sample to be 80 % of the total weight the above values on a wet basis are

approximately 450 and 1860 µg / Kg.

The iodine concentrations found in egg samples were high in comparison with published values. The most recent figures available on British samples were reported by Orr (1931), who found 14 - 194 µg/Kg in 58 samples from Scotland and England. It is probable that the high values obtained in this study reflect the present day use of feeding stuffs, such as fish meal derivatives, which provide poultry with a plentiful supply of iodine.

Glasgow milk samples also contained comparitively high iodine levels. The seasonal variation in milk iodine concentration noted by Young et al. (1936) was also observed in the present study. The following results illustrate the decrease in iodine content which occurred during the summer months:-

Date	Iodine Content of 2 Samples (µg / litre)
10/3/61	124, 133
26/4/61	78, 75
24/5/61	47, 46
11/9/61	26, 29

No previous study of the iodine content of alginate food additives is known to the author. All five samples analysed contained large quantities of iodine (Table IV). However, in

view of the high dilution at which these substances are used in the food undustry it appears that the dietary iodine supply from this source is small.

Table IV. Iodine Content of Alginate Food Additives.

TRADE NAME	USE IN FOOD INDUSTRY	APROX. CONC. OF ADDITIVE %	IODINE CONTENT µg/Kg
Manucol SS/HH/T	added to milk sets and water jellies	0.75	66,900
Manucol SS/CH/90	stabiliser in synthetic whipping cream	0.25	54,300
Manucol EA/KM	stabiliser in fruit squashes and salad dressings	0.25	5,300
Manucol SA/LN	toothpaste additive	1.0	21,000
Manulin SS/PJ	thickener and stabiliser	1.5	92,000

The iodine concentrations found in 4 samples of drinking water were 1.5, 1.6, 2.1 and 2.3 µg/litre. The mean value (1.9 µg/litre) is in good agreement with the figure of 1.5 µg/litre quoted by Murray et al. (1948) for a Glasgow sample. The small concentration of iodine found indicates that drinking water makes a negligible

contribution to the dietary iodine supply in the Glasgow area.

The iodine values obtained for fish, milk and eggs were used in a dietary survey of persons without known thyroid or other metabolic disease. Average values for other foodstuffs were taken from the Chilean Iodine Education Bureau (1952). In 44 female subjects studied the calculated daily dietary iodine intake was 268 ± 23 μg (mean ± S.E.). This figure is well in excess of the mean daily requirement of iodine which has been estimated as 160 μg / day (Koutras et al. 1961). There were, however, wide individual variations and values ranged from 38 - 1069 μg. These fluctuations were mainly due to the variable consumption of seafood (fish), among the subjects investigated.

In a similar study of 18 female patients with simple goitre dietary intake was 130 ± 19.6 μg / day (mean ± S.E.). The intake was lower (79 ± 20 μg / day) in 6 members of this series who had a high thyroid iodine clearance, than in the remaining members with normal thyroid clearance (164 ± 23 μg / day). Diminished iodine intake in this group was associated with a low 24 hr. urinary excretion of iodine (Chap. 1) and a low PII (Chap. 4).

This study in patients from the West of Scotland demonstrates that many patients with non-toxic goitre have a significantly smaller dietary iodine intake than normal subjects. Deficient dietary intake of iodine is possibly the most important single cause of non-toxic goitre.

Chapter 2.

IODINE EXCRETION

(a) Urinary Excretion of Iodine.

Introduction.

Daily excretion of iodine varies considerably from individual to individual depending on the dietary intake. It has therefore been widely used as an index of iodine deficiency (Stanbury, 1952; Lamberg et al. 1958; Koutras et al. 1960; de Visscher et al. 1961). In areas free from endemic goitre the 24 - hour urinary iodine may vary considerably from subject to subject, with an overall mean of 150.3 µg/day (Riggs, 1952).

Davison and Curtis (1939) and Elmer and Scheps (1934) investigated the composition of the urinary iodine using chemical methods and found that it was entirely inorganic. Radiothyroxine studies have confirmed the chemical findings (Albert et al. 1949; Albert and Keating, 1949; Myant and Pochin, 1950; Rall, 1950; Berger and Pegrin, 1957). It is, therefore, well recognised that all or almost all of the urinary iodine is in the form of iodide. (Riggs, 1952; Pitt-Rivers and Tata, 1959).

Small quantities of organic iodine compounds may be present, but normally they form an insignificant proportion of the total

iodine excreted. Greater quantities may be found in cases of dehalogenase deficiency (McGirr, 1959) and also to a lesser extent in thyrotoxicosis (Alexander et al. 1961). In addition, the injection or ingestion of non-hormonal organic iodine compounds, as for example in cholecystography or pyelography, leads to the appearance in urine of significant amounts of organically - bound iodine.

Determination of Urinary Iodine.

The chloric acid digestion method previously described for serum PBI analysis (Part II) was applied to the determination of urinary iodine. No alteration in technique was found necessary. Duplicate analyses were performed on 0.5 - 1.0 ml urine aliquots; diluted samples were used where high iodine concentrations were present. Urine was pipetted directly into the digestion tubes and chloric acid added. The pretreatment of samples in a column of ion exchange resin for the detection of organic iodine compounds will be discussed later.

The accuracy of the technique was assessed by determining the recovery of known quantities of iodide added as potassium iodide, from 0.5 ml aliquats of urine (Table I). The recovery of iodide from 9 specimens ranged from 90.0 - 106.6 %
mean 97.3 %.

Table I.

RECOVERY OF ADDED IODIDE FROM URINE SAMPLES.

SAMPLE	IODINE CONCN. OF URINE (µg I %)	IODIDE ADDED AS KI (µg I %)	TOTAL IODINE FOUND (µg I %)	RECOVERY OF ADDED IODIDE µg I%	%
1	4.5	3.0	7.4	2.9	96.6
2	4.5	4.0	8.1	3.6	90.0
3	9.5	6.0	15.4	5.9	98.3
4	9.5	9.0	18.0	8.5	94.4
5	6.3	6.0	12.0	5.7	95.0
6	4.3	6.0	10.2	5.9	98.3
7	8.0	6.0	14.0	6.0	100.0
8	4.1	6.0	10.5	6.4	106.6
9	2.2	6.0	8.0	5.8	96.6

The Validity of Urinary Iodine Values.

Stanbury et al. (1954) using a modification of the method of Barker (1948) and Rodgers and Poole (1958) using a modification of the method of Elles and Duncan (1953) found that the recovery of iodine from urine decreased as the volume of urine taken decreased. Kilpatrick (1960) using an alkaline ashing procedure also found that consistent results could not be obtained on different volumes of the same specimen, and suggested that substances in urine affect the $Ce^{IV} - As^{III}$ iodide catalysed reaction giving false results. He observed that the presence of nitrite gave elevated iodine values.

In order to assess the effect of variations in sample volume on the present method, iodine estimations were carried out on 4 volumes of 8 different urine specimens. The results are shown in Table II. Iodine values obtained on a volume of 0.25 ml showed a significant difference from those found on volumes of 0.5 - 1.0 ml. The standard error of a single observation was 0.3 and the coefficient of variation 6.6 %. By comparing the mean values obtained on each volume a very slight increase in iodine recovery with increasing volume was apparent. However, since determinations were normally performed on either 0.5 ml or 1.0 ml aliquots this effect was not significant.

Table II. Effect of Sample Volume on Urinary Iodine Estimation.

SAMPLE	URINARY IODINE CONCENTRATION (μg I %)			
	0.25 ml	0.5 ml	0.75 ml	1.0 ml
1	6.0	6.4	6.5	6.5
2	5.6	5.9	6.1	6.1
3	2.0	1.8	1.6	1.7
4	5.4	6.3	6.7	6.9
5	5.4	6.7	6.5	6.4
6	5.0	5.1	5.1	5.2
7	4.8	4.8	4.9	4.9
8	2.6	2.6	2.7	2.8
MEAN	4.60	4.95	5.01	5.06

Detection of Organic Iodine Compounds in Urine.

Urinary iodine values obtained by the present method were used in the calculation of the PII level (see Chap. III). It is assumed in this calculation that these values express the concentration of inorganic iodine in urine. If significant amounts of organically - bound iodine were present false PII values would be obtained. A method of screening urine samples for organic iodine compounds was therefore required in order to eliminate such cases.

Radiochromatography of I^{131} - labelled iodine compounds has been used to detect iodinated amino acids in urine (Albert and Keating, 1951; Stanbury, 1956). However, it is not practical to employ chromatography to investigate every urine specimen prior to iodine estimation. Accordingly an alternative method of testing samples was sought. The removal of inorganic iodine from urine by treatment with an ion exchange medium was considered the most suitable procedure. Fletcher (1958) used a column of silver chloride for this purpose. In the present case anion exchange resin treatment as used for serum PBI analysis was found to provide a quick and efficient separation of the organic and inorganic iodine fractions in urine. Columns of resin were prepared as previously described (Part II) and 2.5 ml urine aliquots were treated.

The validity of the separation procedure was investigated in

two ways. The efficiency of removal of inorganic iodine was shown to be 99.1 - 100 % by treating samples containing added NaI^{131}. Separation of organic iodine containing compounds was tested by adding known concentrations of monoiodotyrosine, diiodotyrosine and triiodothyronine to urine samples and measuring the recovery of iodine after resin treatment. 0.5 ml of a solution of the amino acid in 0.0001 N NaOH was added to 9.5 ml of urine, and 4 determinations carried out on 0.5 ml aliquots before and after treatment in each case. The results, given below, show that more than 80 % of organic iodine was recovered.

	Recovery of Iodine after Resin Treatment			
	µg I %		%	
	RANGE	MEAN	RANGE	MEAN
Addition of 7.6 µg I % as MIT	6.7 - 7.1	6.9	88.2 - 93.4	90.8
" " 5.5 µg I % as DIT	4.1 - 4.7	4.5	74.5 - 85.5	81.8
" " 2.8 µg I % as T_3	2.0 - 2.6	2.3	71.4 - 92.9	82.1

Results of Urinary Iodine Analysis.

Forty urine samples from normal persons and patients with simple goitre were tested for the presence of organic iodine. In 22 cases no iodine was detected following resin treatment

and in the remainder the amounts of iodine left were very small compared to that found in the untreated samples. This finding suggests that neither organic iodine compounds nor other substances giving the chemical reaction of iodide are excreted in the urine in amounts capable of interfering significantly with iodide estimation by the present method.

The urinary excretion of iodine in normal cases ranged from 39 - 171 μg / day, but one apparently normal subject with a low PII level (0.06 μg / 100 ml) had a 24 hr. excretion of 25 μg. Excluding that case, the mean excretion in 15 cases was 77 μg / day.

In contrast, patients with simple goitre showed a significantly decreased excretion of iodine in the urine. Those with a high radioiodine uptake had a mean value of 44 ± 5.5 μg / day ($p < 0.001$) and those with a normal uptake of radioiodine a mean of 45 ± 11.2. These values indicated that both groups of goitrous subjects were iodine deficient.

(b) Faecal Excretion of Iodine.

Introduction.

In contrast to urinary iodine the iodine excreted in faeces has been shown to be almost entirely organic in nature (Albert et al. 1949, Myant, 1946). Faecal iodine is derived from thyroxine, which is excreted with the bile as thyroxine glucuronide. Part is reabsorbed, but the remainder is excreted with the faeces. Its absolute quantity has been indirectly calculated as 6 µg daily (Riggs, 1952) or 12 µg daily (Berson and Yalow, 1954). From measurements of the faecal clearance of radiothyroxine (Triantaphyllidis et al. 1955; Myant, 1956) the faecal excretion can be calculated as between 9 and 20 µg of iodine daily.

Early attempts to determine chemically the quantity of iodine excreted in faeces are reviewed by Salter (1940). It is doubtful if any of these analyses were sufficiently accurate to be trustworthy. At the present time few reliable estimates of faecal iodine excretion are available. In a recent study Van Middlesworth (1960) using the method of Astwood (1957) reported values of 10 - 57 µg / day in 4 euthyroid adults, and 13 - 33 µg / day in 3 children.

Further investigation of the faecal excretion of iodine by direct chemical estimation was considered necessary in order to verify the above results and determine the significance of the iodine loss by this route. For this purpose the method of iodine analysis already described (Part II) was modified and applied to

faecal samples from euthyroid subjects and from patients with thyroid disease.

Chemical Estimation of Iodine in Faeces.

Method.

Faecal samples were diluted with water and homogenised in a 'Kenmix' electric mixer.

Digestion of samples: The efficiency of chloric acid as a digestion medium for the destruction of faecal matter was first studied. Unsatisfactory results were obtained when 0.5 - 1.0 gm aliquots of homogenised faeces were treated by the standard method. In most cases digestion was incomplete and a tarry residue remained which prevented colorimetric determination of iodine. Further experiments showed that complete digestion of organic material required reduction in the sample size to 0.2 - 0.4 gm and an increase in the volume of chloric acid to 8 ml.

Determination of iodine: Initial studies revealed that colorimetric measurement of ceric ion following faecal digestion was not possible in many samples due to the development of turbidity in the ceric sulphate - arsenious acid solution. This effect was eliminated and satisfactory results obtained by the addition of 1 ml of 50 % H_2SO_4 to the digestion residue prior to the addition of arsenious acid solution.

Table I.

Recovery of Iodine (Added as KIO_3) from Faecal Samples.

Faecal Sample	Mean Wt. of Faeces	Iodine Added to x mg Faeces	Mean total Iodine in (x mg) Faeces	Mean Recovery of Added Iodine		Mean Total Iodine in Faecal Sample
	x mg	μ mg	μ mg	μ mg	%	μg/Kg
A	516	-	9.0	-	-	17.4
A	448	50	60.2	52.4	104.8	-
B	403	-	30.3	-	-	75.2
B	333	40	67.3	42.3	105.8	-
C	306	-	35.8	-	-	117.0
C	299	40	76.3	41.3	103.3	-
D	290	-	31.7	-	-	109.3
D	294	40	69.0	36.9	92.3	-
E	310	-	54.7	-	-	176.5
E	304	40	91.5	37.9	94.8	-
F	302	-	15.5	-	-	51.3
F	292	40	58.7	43.7	109.2	-
G	286	-	13.7	-	-	47.9
G	279	40	52.5	39.1	97.8	-
H	292	-	120.0	-	-	411.0
H	312	40	162.2	34.0	85.0	-

The Validity of Faecal Iodine Values.

The results of recovery tests carried out by adding 0.5 ml of KIO_3 solution containing the equivalent of 40 or 50 µg I to faecal specimens are shown in Table I. The mean recovery obtained was 99.1 % (S.E. 2.7).

The reproducibility of the analytical technique was checked by carrying out 14 separate estimations on a single faecal specimen. The results showed a range of 35.3 - 79.2 µ g / Kg (S.E. 4.3). Although the range of values appears rather wide, only one result of less than 40 µg / Kg was obtained (35.3) and three values (56.4, 79.2. and 62.0) were greater than 54.

It can be seen from the above results that the degree of accuracy attainable in faecal iodine analysis by the present method is less than that obtained for serum and urine. In practice, however, the accuracy of faecal results was improved by a carrying out each analysis in triplicate and b determining the recovery of added iodine for each specimen and applying a correction to the result where recoveries were outwith the range 90 - 110 %.

Results of Faecal Iodine Analyses.

The mean faecal excretion of iodine in 5 normal subjects was 18 µg / day. Values ranging from 4 - 33 µg / day were obtained. These results are in good agreement with the values

quoted by Van Middlesworth (1960).

The faecal iodine loss was also measured in 5 patients with thyrotoxicosis. In 4 cases values of 35, 91, 172 and 276 µg / day were obtained, all of which were considerably higher than the normal mean. Although an excretion of only 8 µg / day was noted in the remaining case, the above results demonstrate that an increased faecal iodine loss occurs in thyrotoxicosis. Berson and Yalow (1954) on the basis of radioiodine measurements first predicted that this was the case, however, the only experimental verification prior to the present study was provided by Van Middlesworth (1960), who found a faecal excretion of 83 µg / day in one thyrotoxic subject.

Hydovitz (1960) and Van Middlesworth (1960) postulated that increased faecal iodine loss may be an important factor in iodine deficiency goitre, but no experimental evidence has so far been published. In one of three patients with iodine deficiency goitre studied in Glasgow a high faecal excretion of 139 µg / day was found.

Chapter 3.

PLASMA INORGANIC IODINE (P.I.I.)

Introduction.

The PII concentration is normally too low to be measured directly by chemical techniques. An alternative method has been suggested by Stanley (1949) based on the principle of isotope dilution.

Since the body is unable to distinguish between stable and radioactive atoms of iodine, and since urinary iodine is derived from the PII, the specific activity of the urinary iodine is presumably the same as that of the PII, that is:

$$\frac{I^{131} \text{ (or } I^{132}\text{) plasma}}{\text{PII}} = \frac{I^{131} \text{ (or } I^{132}\text{) urine}}{\text{I - urine}}$$

The three parameters in the above equation can be estimated by standard techniques and the PII calculated.

Methods and Materials.

The PII level was determined following the oral administration of 50 μC of I^{132} (Koutras et al. 1960; Alexander et al. 1961). After allowing 1 hr. for absorption and equilibration, a 90 min. urine collection was taken and the radioactive and stable iodine

concentrations measured. A plasma sample was taken at the mid point of the collection and the radioiodine also measured. In this way the PII was calculated from the above formula.

It is obvious that, were significant amounts of organic iodine present in the urine, the above calculation would be invalid, since the assumption is made that all the urinary iodine is inorganic and in equilibrium with the PII. To check this the resin column screening procedure already described (Chapter 2) was applied to all samples.

In subjects with high PII levels due to exogenous iodine administration direct chemical determination of PII was carried out using the method described in Part II. The iodine content of serum samples was determined before and after resin column treatment and the difference taken to represent PII.

Results and Discussion.

The validity of PII determinations was checked in two ways. Firstly, simultaneous estimation by the method described and by chemical analysis was carried out in cases where the PII, following iodide administration, was sufficiently high to be measured directly as the difference between the total serum iodine and the PBI. The results (Fig I) showed a close correlation between the two techniques ($r = 0.95$, $p < 0.001$). The regression equation; $y = 0.02 + 1.13x$, indicated that the specific activity method gave systematically higher values than direct measurement.

Fig I.

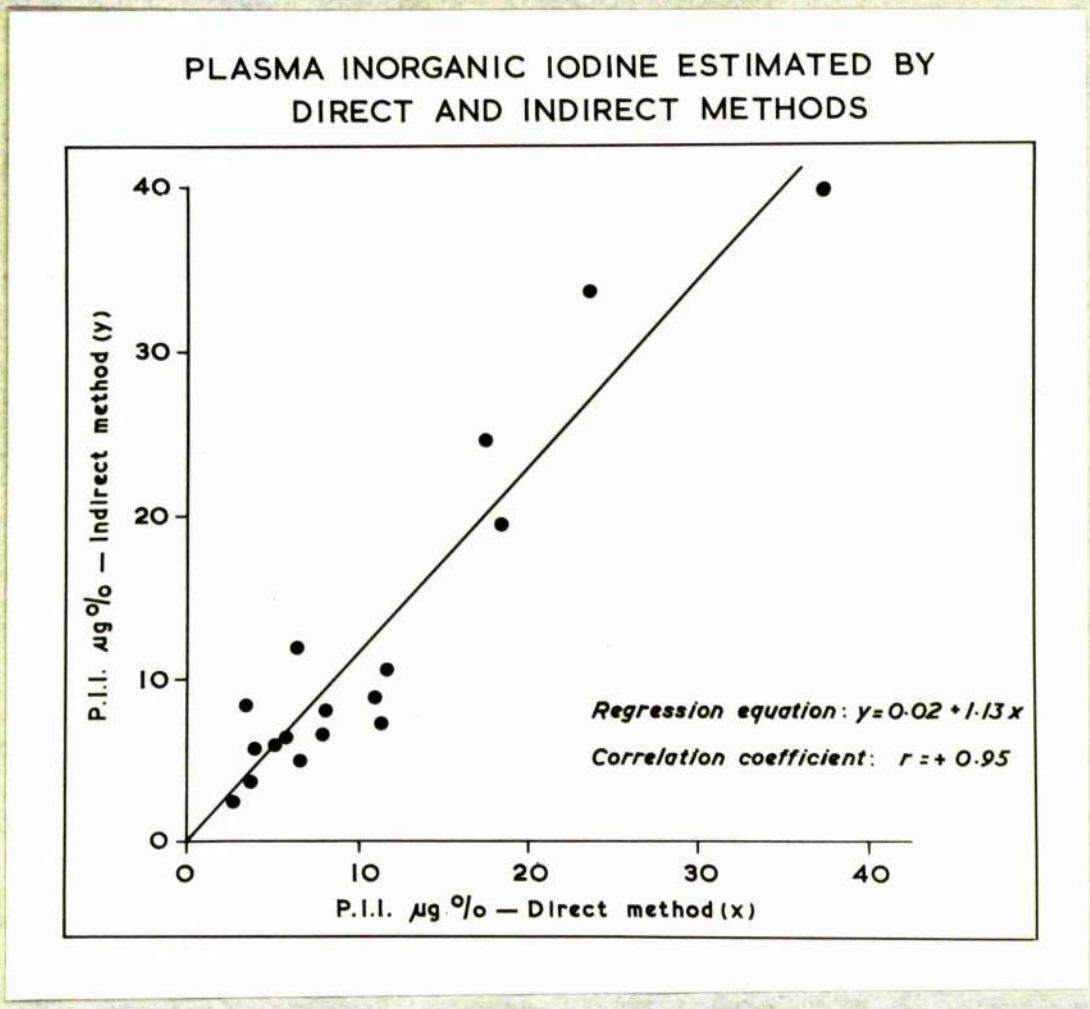
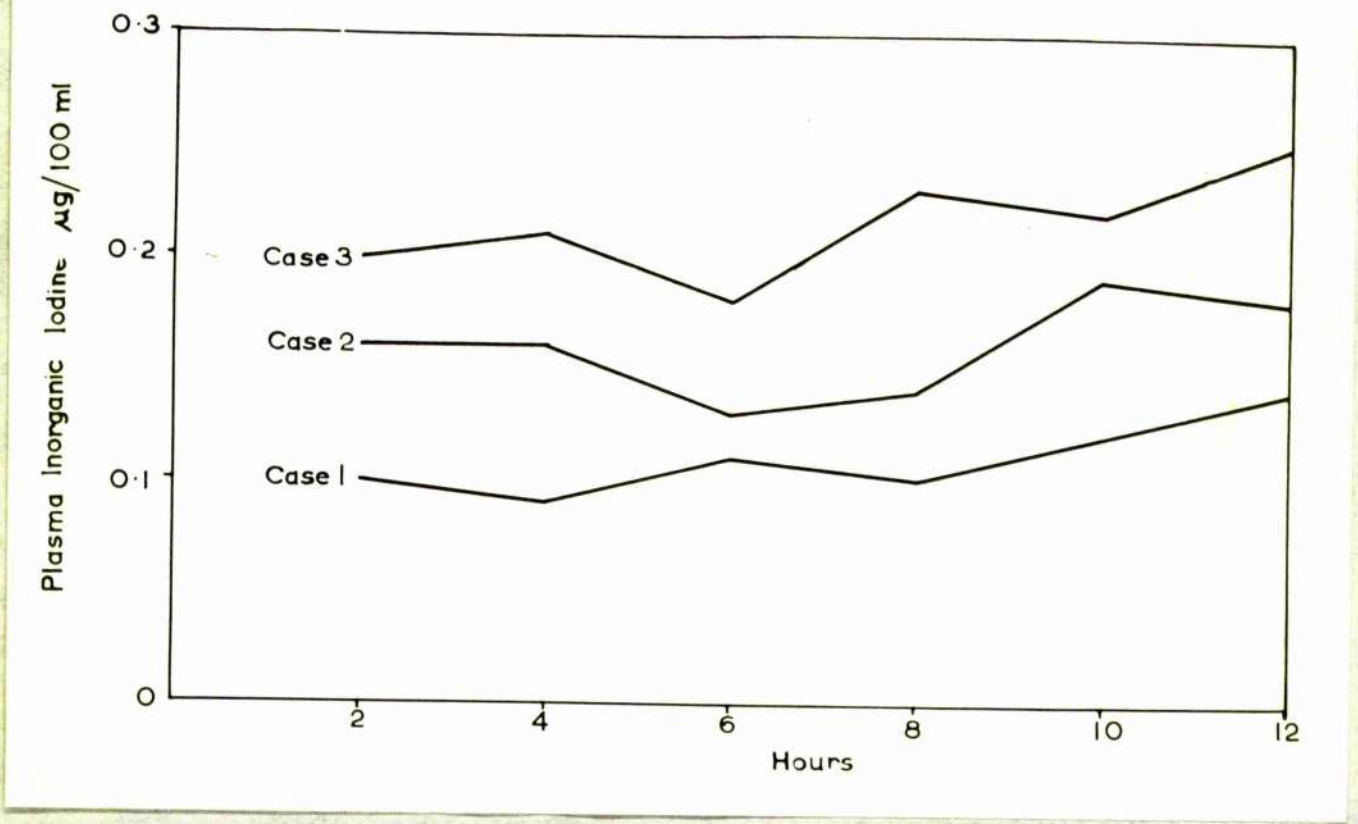


Fig II. DIURNAL VARIATION IN PII IN THREE SUBJECTS.



Secondly, repeated estimation of the PII level was performed in 3 subjects, by giving a tracer dose of I^{131} (25 μC) and collecting urine specimens at 2 hr. intervals, with plasma samples being taken at the mid point of the urinary collections. The results (Fig II) showed that the PII remained relatively constant throughout the 12 hr. period of observation. The values in the 3 patients studied were 0.16 ± 0.02, 2.1 ± 0.02 and 0.11 ± 0.02 μg/100 ml (mean ± S.D.). It is probable that the variations in PII observed throughout this time period were due partly to technical error and partly to actual biological variation, since the patients had normal meals excluding fish.

The PII results obtained in normal subjects and in patients with thyroid disease are shown in Table I. Values ranging from 0.04 - 0.57 μg / 100 ml were found in normal cases.

Table I. Plasma Inorganic Iodine (μg / 100 ml)

	No. of Cases	Mean	S.D.	Obs. range
Normal	17	0.19	0.1	0.04 - 0.39
Thyrotoxicosis	23	0.13	0.08	0.02 - 0.33
Hypothyroidism	3	0.11	-	-
Simple goitre with high R.I. uptake	18	0.05	0.02	0.01 - 0.08
Simple goitre with normal R.I. uptake	18	0.10	0.05	0.03 - 0.22
Dyshormonogenesis	5	0.22	0.15	0.09 - 0.46
Auto-immune thyroiditis	16	0.20	0.15	0.02 - 0.58

Patients with simple goitre, irrespective of whether the radioiodine

uptake was high or normal, had a low PII significantly different from the normal group ($p < 0.001$). This provides clear e idence of iodine deficiency in these patients and correlates with the finding of decreased 24 hr. urinary excretion of iodine.

A low PII of borderline statistical significance ($p = 0.05$) was also found in the thyrotoxic patients studied. Factors which may contribute to this finding include an increased faecal excretion of iodine (Berson and Yalow 1954; See also Chap. 2) and an increased renal clearance of iodide (Hlad and Bricken, 1954; Cassano et al. 1957).

The distribution ofvalues in the normal group studied is not a statistically normal distribution, nor does it become one after logarithmic conversion. Therefore the definition of the normal range in terms of mean $\pm$ 2 S.D. is not permissable. The lower and upper limit of the ormal range were established in terms of best separation between normal cases and high uptake goitres on the one side and cases of iodine administration on the other. Thus the normal range can be defined as 0.08 - 0.60 µg / 100 ml by setting 0.08 µg / 100 ml as the lower limit. The best separation can be made between the normal group and the high radioiodine uptake goitres, though a small overlap does occur. By taking0.60 µg / 100 ml best separation is made between normal cases and those given iodine in other than physiological amounts. Therefore values above 0.60 µg /100 ml are not included in Table I.

The normal PII values found in the present study do not differ greatly from the figure 0.17 ± 0.005 found by Perry and Hughes (1952). Reilly et al. (1958) recorded a value of 0.55 ± 0.06; this higher value may be due to the widespread use of iodised salt in the U.S.A. or to the inclusion of some patients who had previously taken iodide. Stanley (1949) recorded values of 1 µg / 100 ml or less, but it has been pointed out (Riggs, 1952) that Stanley's values are too high.

Chapter 4.

INTRATHYROIDAL EXCHANGEABLE IODINE.

Introduction.

The normal thyroid gland has been shown to contain in the region of 8 mg of iodine (Riggs, 1952). No evidence exists to show that all the iodine in the thyroid is uniformly metabolised. It may be that some is readily exchangeable and is in continuous turnover, whereas some is comparitively static and is more or less in a storage form (Triantaphyllidis, 1958 a and b).

Total thyroidal iodine can only be measured by chemical methods following excision of the gland. The quantity of exchangeable iodine in the thyroid (I.E.I.) can, however, be estimated in vivo by the specific activity procedure devised by Nodine et al. (1957).

The technique of Nodine et al. was used to measure the I.E.I. in patients with thyroid disease and in a control series.

Method.

Each patient was given 75 μC of I^{131} and after allowing 9 days for equilibration of the dose, the radioiodine uptake, the PBI and the PBI^{131} were measured. Immediately thereafter an intramuscular injection of 10 units of TSH was given in each case and the same 3 parameters measured 24 hours later. The

rise in PBI and PBI^{131} observed enabled the specific activity of the newly released thyroid hormone to be calculated. Using this value and the radioiodine uptake figure before TSH injection the I.E.I. was calculated from the formula

$$\text{I.E.I.} = \frac{\text{rise in PBI} \times \text{thyroid } I^{131} \text{ uptake}}{\text{rise in } PBI^{131}}$$

The following example illustrates the method of calculation:

	Before TSH	After TSH
Thyroid radioiodine uptake (%)	32.8	30.2
PBI^{131} (% of litre)	0.5	0.62
PBI (µg/100 ml)	7.8	13.3

Net rise in PBI - 55 µg / litre

Net rise in PBI^{131} - 0.12 % / litre

i.e. Specific activity of hormonal iodine released by TSH is $\frac{55}{0.12}$

∴ Thyroid uptake of 32.8 % corresponds to

$$\frac{32.8 \times 55}{0.12} = 15033 \text{ µg I} = \text{I.E.I.}$$

The above value expressed in mg (15.033) represents the Intrathyroidal Exchangeable Iodine.

Values for I.E.I. obtained by the above method refer only to the quantities of iodine with which the tracer dose has equilibrated after 9 days. Different values would be

obtained if TSH injections were given at any other time.

Results and Discussion.

The results obtained in normal subjects and in patients with auto-immune thyroiditis and simple goitre are given in Table I.

Table I. Intrathyroidal Exchangeable Iodine Results.

Diagnosis	No. of Cases	Intrathyroidal Exchangeable Iodine (mg) range	mean	S.E.
Normal	9	0.9 - 15.7	7.5	1.9
Auto-immune thyroiditis	12	0.1 - 3.8	1.0	0.36
Simple goitre	6	6.3 - 29.6	13.7	-

It can be seen that patients with auto-immune thyroiditis have a much smaller I.E.I. than normal subjects or patients with simple goitre.

It was not possible to measure I.E.I. in patients with hypo or hyper-thyroidism because of a lack of any measurable rise in PBI^{131}.

The results obtained in cases with auto-immune thyroiditis and simple goitre explain why in the former case there is a

high PBI^{131} although PBI is normal. Assuming a constant production of thyroid hormone daily and a constant degree of utilisation of iodide taken up by the gland, the PBI^{131} values are expected to be proportional to the thyroidal radioiodine uptake and inversely proportional to the I.E.I. That this is so has been shown by Koutras et al. (1961).

In cases of simple goitre for which iodine deficiency has been implicated as the main aeteological factor, the I.E.I. is normal or high normal, and this accounts for the fact that these patients have normal PBI^{131} values in spite of their high radioiodine uptake.

It is concluded that the finding of a low I.E.I. by this technique explains the high PBI^{131} values found in auto-immune thyroiditis. The measurement of I.E.I. is recommended for the investigation of the significance of unexpectedly high PBI^{131} values in apparently euthyroid subjects.

The Determination of Iodine in Biological Material by Neutron - Activation Analysis.

Introduction.

The chemical methods previously discussed, for the microdetermination of iodine are highly sensitive and susceptible to contamination by substances which effect the analytical procedure. This applies in particular to the use of the $Ce^{IV} - As^{III}$ catalytic technique. A method is required which gives direct measurement of iodine and which does not rely on oxidation - reduction mechanisms for its fulfilment. The recently developed technique of radioactivation analysis may provide the solution to this problem. Attempts have already been made to determine trace quantities of iodine by this procedure, (Spencer et al, 1958; Leddicotte et al. 1958; Bowen, 1959). An investigation of the application of activation analysis to the measurement of iodine in biological material was carried out.

Principles of Activation Analysis

Activation analysis consists of the nuclear bombardment of a weighed sample together with a standard which contains a known weight of the element to be determined. The radioactivity from the element concerned, in the sample, is then compared with that

in the standard. Since the intensity of radiation emitted after bombardment is directly proportional to the weight of stable isotope activated, a measure of its concentration is obtained. The contribution of a specific element to the total induced activity may be isolated in two ways:

1. Chemical separation followed by radioactive assay,
2. Analysis of the emitted radiation by spectroscopy or coincidence counting.

Normally a chemical separation is carried out in order to purify the radioisotopes of the element sought and remove all other induced radioactivity. The amount of the element in the sample is calculated from the ratio of the separated activities. Since the quantities being determined are frequently very minute, chemical separations are carried out usually after the addition of a few milligrammes of the normal inactive form of the element ('carrier') to the activated sample and standard. The addition of carrier after activation serves two purposes: a. it facilitates recovery of the unknown, b. it permits any losses in the separation process to be calculated.

The method of activation used at present is mainly that of neutron bombardment. Most of the radioisotopes likely to be useful in activation analysis can be induced by irradiation with thermal neutrons. To gain the greatest sensitivity the neutron

flux must be of the order of 10^{12} neutrons / cm^2 / sec. The reaction which takes place is that of neutron capture in which the neutron collides with the nucleus and is absorbed. When this occurs a ray is emitted. The process is described as an n, γ reaction. Other reactions may take place, but the above is most common where thermal neutrons are involved. As a result of the n, γ reaction the atomic weight increases by one unit, the atomic number remaining unchanged; thus forming an isotope of the parent species. The reaction is represented as follows: -

$$X^{A}_{Z} \; (n, \gamma) \; X^{A+1}_{Z}$$

where A is atomic weight and Z is atomic number.

On neutron irradiation the radioisotope is formed at a steady rate depending on the intensity of irradiation. The product begins to decay when it is formed, till finally a saturation activity is reached where rate of decay is equal to rate of formation. The activity (A_t) after time t is given by:-

$$A_t = A_{sat.} \; (1 - e^{-\lambda t})$$

where A_{sat} = saturation activity

λ = radioactive decay constant.

The saturation activity also depends on the "activation cross section" of the target element; this can be regarded as a

measure of the avidity of the target nucleus for neutrons and is a physical constant for a given element and a given neutron energy.

If the target element is irradiated for $T_{\frac{1}{2}}$ of the isotope then 50% of the saturation activity will be reached. Longer periods of irradiation are decreasingly profitable since only 75% of the maximum possible activity will be achieved after irradiation for two half-life periods.

Neutron Irradiation of Iodine.

The nuclear reaction occurring on irradiation of iodine with thermal neutrons is

$$I_{53}^{127} \quad (n,\gamma) \quad I_{53}^{128}$$

for which the cross section to thermal neutrons is high (5.5 barns). The I^{128} formed has a half life of 25 mins. and is β and γ active. The short half life provides a major problem in the application of activation analysis to iodine. Because of this, the entire analytical procedure must be carried out in close proximity to the neutron source. The time available for chemical separation of iodine after irradiation is thus very short, and speed is essential.

The estimated order of sensitivity attainable by activation analysis for iodine using a flux of 10^{12} neutrons / cm^2 / sec.

until saturation is 5×10^{-9} gm (Jenkins and Smales, 1956). This assumes that a 2 hr. period is permitted for radiochemistry and that the yield is quantitative. Leddicotte et al. (1958) observed 1 - 10 p.p.m. of iodine in tissue and quote 0.05 p.p.m. as the limit of sensitivity for its detection.

Iodine has been determined in the protein thyroglobulin by a short neutron irradiation of samples weighing a few mg. (Brues and Robertson). Since pure samples were used interference from other isotopes was reduced and it was possible to measure the activity present after irradiation without chemical separation, using an end-window Geiger counter.

Bowen (1959) described a method which was suitable for activation analysis of iodine in biological material. Chemical separation of iodine was performed after irradiation. The limit of detection of the method was given as 10^{-10} gm.

Isolation of Iodine from Interfering Elements.

In analysis for iodine, I^{128} must be isolated in a radiochemically pure state before activity measurements are made. Irradiation of biological material leads to the formation of many radioactive isotopes in the matrix, making efficient chemical separation essential. The isotopes Na^{24}, Cl^{38}, and Br^{80} in particular, interfere in the measurement of the γ spectrum of I^{128} and must be removed. These elements are normally present

in biological material in huge quantities in comparison with iodine and complete separation is difficult.

Isolation of iodine from interfering elements may be performed either before or after sample irradiation. In view of the short half-life of I^{128}, separation prior to activation is the procedure which offers the most advantage, since the induced activity may then be measured with minimum delay. The alternative procedure, however is superior in the respect that isolation of iodine following activation may be greatly simplified by the addition of inactive carrier iodine. Both the above techniques were investigated with a view to devising a suitable method for analysis of iodine in biological material and in particular, serum-protein bound iodine.

(a) Separation of Iodine Before Irradiation.

Isolation of sub-microquantities of iodine in a pure form from serum is not feasible by normal chemical procedures. The separation of interfering elements by either dialysis or ion-exchange resin treatment was considered. Dialysis, however, is a lengthy procedure and the number of samples which can be treated is restricted. First consideration was given to ion-exchange resin treatment which has already proved effective in serum PBI analysis.

Ion exchange resin separation

The separation of inorganic iodine from serum PBI by treatment with Amberlite IRA 400 (Cl) anion exchange resin has already been described. As a result of this treatment the halide ions, iodide and bromide, are removed by the resin and replaced by chloride. Since chloride is not removed, however, this treatment cannot be used prior to neutron activation. A similar resin with hydroxyl functional groups is required for removal of all halogens. Cations, in particular sodium, can readily be removed with resins containing phenolic, carboxylic or sulphonic functional groups. By using a combination of anion and cation exchangers a separation of all interfering ions may be obtained. Experiments were carried out to assess the efficiency of ion exchange resin treatment of serum for this purpose.

The mixed bed resin Amberlite MB 3 was used. This resin contains a hydroxyl exchanger similar to IRA 400 in combination with the cation exchanger IR 120 which has sulphonic acid functional groups. The efficiency of resin treatment was assessed using NaI^{131}, $K^{42}Cl$ and thyroxine - I^{131} as radiotracers. Resin was prepared and used by the method previously described for IRA 400 (Cl). 2.5 ml aliquots of serum were passed through 120 x 6 mm columns of MB 3 resin. Serum was counted for activity

in 0.5 ml samples before and after treatment, using a well-type scintillation counter for I^{131} and a Geiger Muller tube for K^{42}.

Using serum labelled with thyroxine I^{131} it was observed that 3.6 - 6.3 % of organically bound iodine was retained in the resin column. The results are given in Table I, each value quoted being the mean of triplicate measurements corrected for background activity.

TABLE I

Sample No.	Activity of Serum C/100sec. Untreated	Resin treated	Recovery of Thyroxine %
1	28,638	27,329	95.43
2	27,329	25,816	94.46
3	20,032	19,180	95.75
4	19,180	17,974	93.71
5	18,054	17,427	96.53

The loss of organic iodine was higher than that obtained using IRA 400 Cl anion exchange resin. This was due to protein denaturation occurring in the presence of the sulphonic acid functional groups of the cation exchange component in MB 3.

Tracer studies using serum containing iodide labelled with NaI^{131} showed that 92.5 - 97.2 % of halide ion was removed on passage through the column (Table II).

TABLE II

Sample No.	Activity due to NaI^{131} in Serum C/100 sec Untreated	Resin treated	NaI^{131} Removed %
1	70,124	1,982	97.17
2	3 7,156	2,102	94.34
3	24,200	1,815	92.5

Resin treatment proved effective in removing potassium as $K^{42}Cl$ from serum. Less than 1 % of the K^{42} originally present in the serum appeared in the eluate (Table III).

TABLE III

Sample No.	Activity from K^{42} in Serum C/5 min. Before treatment	After treatment	K^{42} removed %
1	35,556	325	99.09
2	35,248	318	99.10

Conclusion. The above results indicate that the resin treatment used removes more than 90 % of interfering ions. In view of the high concentration of Na, Cl and Br present in biological material, however, the separation requires to be virtually 100 % efficient to ensure accurate measurement of I^{128} following irradiation. It is apparent that such a complete separation cannot be achieved by resin treatment. The alternative activation analysis technique of chemical separation following irradiation was therefore investigated.

(b) Separation of Iodine After Irradiation.

The short half life of I^{128} makes it essential that chemical separation be accomplished with the least possible delay. A maximum of 1 hour may normally be allowed when dealing with serum levels of iodine. Since relatively large concentrations of carrier iodine may be added, the separation and purification of iodine by conventional chemical methods is facilitated. For serum and most biological samples which contain iodine in organic combination, destruction of organic matter, followed by separation and purification of the inorganic iodine released is necessary.

Separation by Solvent Extraction and Precipitation as Silver Iodide:

Bowen (1959) describes a method for the activation analysis of iodine in blood which involves digestion of organic material

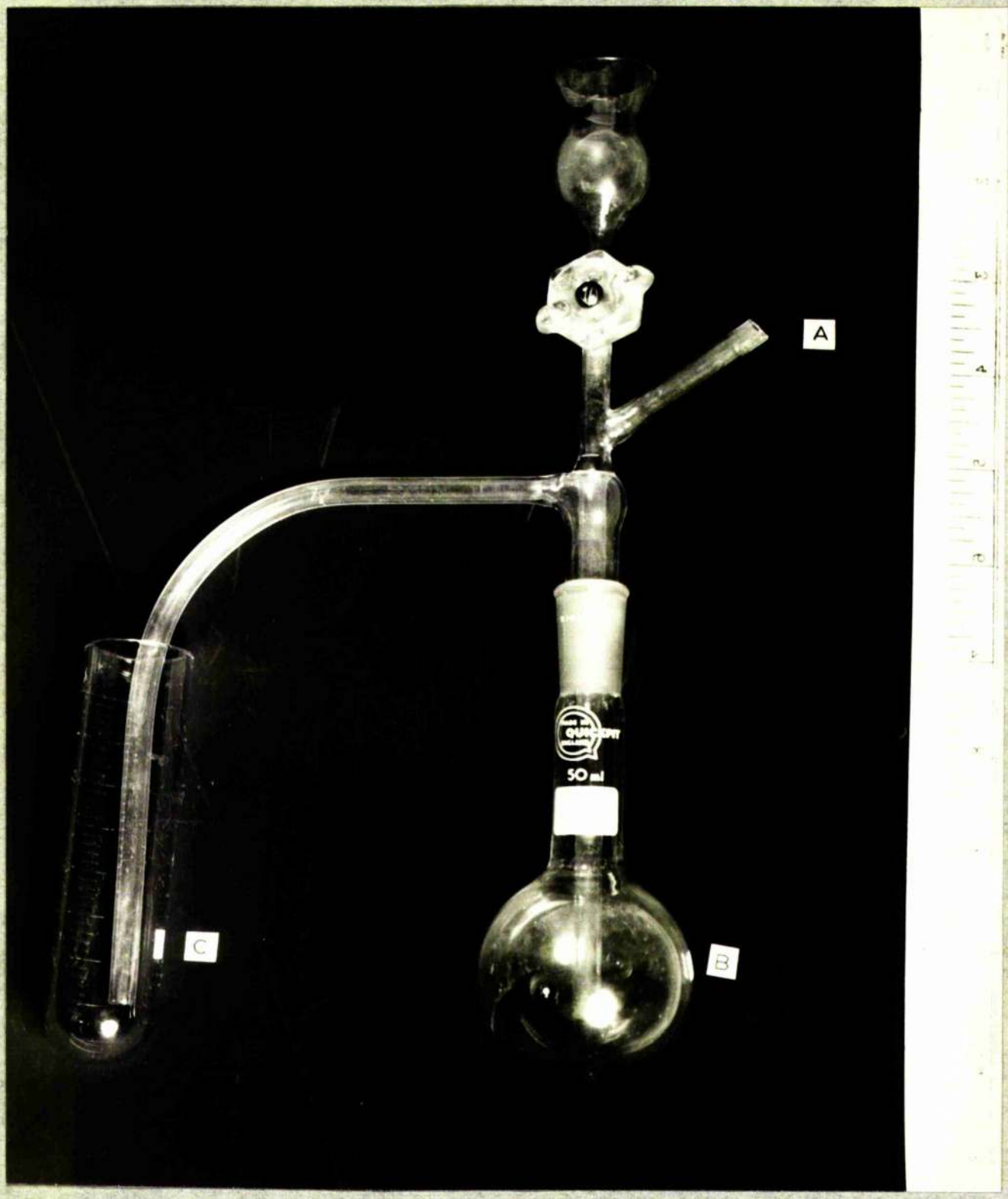
and distillation of iodine with phosphoric and nitric acids, followed by solvent extraction and precipitation of iodine as silver iodide. An investigation of this method was conducted using I^{131} and Cl^{36} as radiotracers to assess its efficiency.

The procedure described by Bowen is as follows:-

The activated sample (1gm) is added to a 50 ml flask containing 20 mg of iodine as NH_4I, 1 ml of 90 % H_3PO_4 and 1 ml of 16 N. HNO_3. A distillation head is fitted and distillation carried out for 5 min. at 150°C in a slow current of air. The distillate is collected in 5 ml of 5 % NaOH in a 50 ml separating funnel.

The distillate is made N with respect to nitric acid, and bromide and chloride 'holdback carriers' are added (2 drops of 5 % ammonium salt in each case). The iodine is twice extracted by shaking with sodium nitrite solution and CCl_4, and then re-extracted into 10 ml of 0.5 % NaOH in a fresh separating funnel. This extraction and re-extraction procedure is carried out twice for samples of high iodine content and three times for samples containing much chlorine. The final solution in aq. NaOH is treated successively with 2 drops of 5 % NH_4Cl, 1 ml of NH_4OH, and 3 ml of 4 % $AgNO_3$. The precipitate is centrifuged and the supernatant discarded. Washing is carried out with 2N. HNO_3, H_2O (twice) and acetone. The silver iodide is transferred to an aluminium planchet and dried, then weighed and counted.

Fig I. DIGESTION - DISTILLATION APPARATUS.



A slow flow of air is pumped through the apparatus at the inlet 'A'. Digestion is carried out in the flask 'B'. Iodine distilled out is collected in dilute alkali contained in the graduated flask 'C'.

Experiments were first conducted to determine the recovery of iodine both as carrier and as added NaI^{131}. The separation procedure was carried out as described by Bowen. The distillation apparatus used is shown in Fig 1. Fine mesh carborundum was added to the distillation flask to prevent bumping. Silver iodide precipitates in acetone were transferred with pasteur pipettes to $\frac{1}{2}$" diameter aluminium planchets, dried under an infra-red lamp and weighed without delay.

The yield of iodine added as I^{131} to 20 mg of carrier iodide in 4 analyses was 49, 72, 73 and 97 %. In two instances the weight yield exceeded 100 %. It was apparent that the silver iodide precipitate was decomposing on the aluminium surface of the planchet. In an attempt to prevent this decomposition the precipitate was given an additional wash with acetone and thoroughly dried. No improvement was apparent as a result of this treatment.

Serum obtained from a hyperthyroid patient who had recently received a therapy dose of I^{131} was used to measure the recovery of organically bound iodine (PBI^{131}). Inorganic iodine was removed from the sample by treatment with IRA 400 (Cl) resin. 0.5 ml samples were then digested and the iodine separated. The recovery of I^{131} was only 33 %.

In order to determine the efficiency of the method for removing interfering halogens, 0.5 ml of a solution of $NaCl^{36}$ was added to the ammonium chloride carrier. The initial activity

from Cl^{36} as measured in a Geiger Muller tube was 11,600 C/100 sec. After processing, the activity was identical with background, demonstrating that a complete separation of iodine was obtained.

The low and inconsistent radiochemical recovery of iodine indicates that a substantial loss occurs during the separation procedure. It is possible that the discrepancy between weight and radiochemical recoveries may be eliminated by using planchets of more stable material such as stainless steel, nickel or plastic. The time required to carry out a complete iodine separation was considerably longer than expected, only three samples could be processed by one person in 1 hour.

It is concluded from the above studies that Bowen's separation procedure is not suitable for the purposes of the present investigation. Accordingly, an attempt was made to develop an alternative separation technique which did not rely on a lengthy solvent extraction process. The direct precipitation of iodine, following distillation, as palladous iodide was considered to be a possible solution to this problem.

Separation of Iodine by Precipitation as Palladous Iodide, (PdI_2).

Iodide is selectively precipitated in the presence of chloride and bromide by palladous chloride ($PdCl_2$) in dilute hydrochloric acid (Vogel, 1953). The brownish - black precipitate of PdI_2 is insoluble in water and acetone, but readily soluble in alkali.

Substances such as alcohol which cause reduction to metallic palladium must be absent.

The following precipitation technique was employed to test the separation of iodine as PdI_2 from distillates:-
To 10 ml of 5 % NaOH containing 20 mg of iodide as NH_4I was added 5 ml of 1 % (w. v.) $PdCl_2$ in 1 % HCl. Since the PdI_2 precipitate was soluble in excess HCl the acidity was carefully controlled during precipitation. Conc. HCl was added initially until the solution was nearly neutral, then 5 % HCl was added dropwise until precipitation was complete. The precipitate was spun down and separated, then washed twice with water, twice with acetone and transferred in acetone to an aluminium planchet. After drying under an infra red lamp the PdI_2 was weighed and the activity measured.

The recovery of iodine by weight as PdI_2 was 95 - 99 % using this method. The separation of iodide from chloride was tested by adding a solution of $NaCl^{36}$ (6,060 C/ 100 sec) to the iodide in alkali and carrying out the precipitation procedure. The separated PdI_2 was counted and found to be without activity demonstrating that chloride is removed with 100 % efficiency.

Nitric - phosphoric acid digestion and distillation (as described by Bowen) was then combined with PdI_2 separation of distilled iodine and the efficiency of the procedure determined by both chemical and radiochemical recovery of iodine. NaI^{131} was added to iodide carrier in the distillation flask,

and the distillate collected in 10 ml of 5 % NaOH in a 50 ml graduated centrifuge tube. The PdI_2^{131} precipitate was counted by placing the planchet on the crystal head of the EKCO N550 scintillation counter.

Table IV. Chemical and Radiochemical Recovery of Iodine.

No.	Wt. PdI_2 Recovered	Initial - I^{131}	PdI_2^{131}	Recovery of Iodine %	
	mg	C/400 sec	C/400sec	By Wt.	As I^{131}
1	21.22	14,856	8,255	74.7	55.6
2	9.48	14,856	4,325	34.6	29.1
3	17.20	29,606	17,402	60.6	58.8
4	12.52	29,606	12,827	44.1	43.3

Theor. wt. recovery on 20 mg I is 28.4 mg PdI_2

The results obtained in 4 analyses (Table IV) showed that an appreciable loss of iodine occurred during separation. The discrepancy between weight and activity yields was found to be due to decomposition of the PdI_2 precipitate on the aluminium counting tray. It was believed that this error would be eliminated by using counting trays of different material. The principal

cause of iodine loss was found to be incomplete precipitation of PdI_2 due to the presence of nitrite in the alkaline trapping medium. This sounce of error cannot be avoided when nitric acid is used for distillation. Accordingly an alternative digestion - distillation procedure using acid permanganate was tested.

Acid Permanganate Digestion- Distillation.

Digestion of iodine-containing biological material with excess of acid permanganate destroys organic material and releases iodine as iodic acid. Iodine is then isolated by reducing the iodic acid and remaining permanganate, and distilling the iodine into an alkaline absorbing medium.

The distillation apparatus used for the nitric - phosphoric acid procedure proved suitable for acid permanganate digestion and distillation. The amounts of sulphuric acid and permanganate were adjusted to allow digestion of 0.2 - 0.5 ml samples of serum in the presence of 20 mg of carrier iodine. The following procedure was employed.

To the digestion flask containing serum and carrier iodine was added 10 ml of 18N H_2SO_4 . The addition of a few grains of carborundum powder prevented bumping. 5 ml of 2N $KMnO_4$ were then introduced slowly from the dropping funnel and the flask heated to 140°C. After digestion at this temperature for 4 mins. satd. oxalic acid solution was added slowly to reduce excess $KMnO_4$. The iodine in the digest was distilled at 140°C into

10 ml of 5 % NaOH. A slow current of air was pumped through the apparatus during digestion and distillation. The entire procedure was completed in less than 10 mins.

Precipitation of iodine as PdI_2 following permanganate distillation was more efficient than by the previous distillation technique. Radiochemical recoveries on I^{131} - labelled serum ranged from 60.7 - 88.5 % (Table V). The use of stainless steel planchets improved considerably the agreement between weight and radiochemical yields, the maximum difference being only 5.5 %.

Table V. Chemical and Radiochemical Recovery of Iodine.

SAMPLE NO.	RECOVERY OF IODINE % BY WEIGHT (1)	AS I^{131} (2)	DIFFERENCE (1)-(2)
1	65.2	63.5	+ 1.7
2	62.0	60.7	+ 1.3
3	81.5	81.0	+ 0.5
4	62.4	67.7	- 5.3
5	74.3	71.9	+ 2.4
6	69.0	74.5	- 5.5
7	67.2	70.3	- 3.1
8	87.3	88.5	- 1.2
9	72.0	73.2	- 1.2
Mean	71.2	72.4	

Iodine loss in the distillation and PdI_2 precipitation stages was measured radiochemically using 0.5 ml samples of I^{131}- labelled serum. Four determinations were carried out. I^{131} remaining in the digestion flask following distillation was washed out with water and measured in a scintillation counter accepting liquid samples. The mean loss in distillation was 6.1 % (range 3.8 - 8.2 %) and in precipitation 22.8 % (range 15.8 - 27.7 %).

At this stage it was considered that the separation procedure was sufficiently reliable for application to neutron irradiated samples.

Neutron irradiation of samples.

Weighed serum samples of about 0.2 gm were sealed in silica ampoules which were then packed in an aluminium irradiation can. Standard ammonium iodide solution containing a known weight of iodine was included with each batch of serum samples. Irradiation was carried out at A.E.R.E. Harwell in the BEPO reactor at a flux of approximately 10^{12} neutrons /cm^2 / sec. for 1 hr. Two serum samples and a standard were treated at the one time. Chemical separation was started approximately 10 mins after irradiation was completed.

The yields by weight of carrier iodine obtained after PdI_2 separation ranged from 60 - 80 %. Measurement of I^{128} activity was unsatisfactory, however, due to the presence of radioactive

impurities in PdI_2 precipitates. This interference occurred only in samples and was due to chlorine and another source. Repeated washing of precititates with acetone removed the chlorine contamination but an additional source remained which could not be identified. The $t_{\frac{1}{2}}$ values obtained on 4 serum samples before removal of chlorine contamination were 37, 35, 33 and 30 mins. After processing the values were 27, 27, 26 and 26 mins. respectively. The true I^{128} half life of 25 mins. was found for all standards. PBI values showed no agreement with the figures obtained by chemical analysis (Table VI.).

Table VI. Analysis of Serum Samples.

SPECIMEN	P.B.I. µg I / 100 ml	
	Act. Anal.	Chem. Anal.
S.1.	12.4	6.6
S.2.	6.4	6.6
K.1.	8.8	5.1
K.2.	5.4	7.6
S.N.	20.6	2.6
M.Cl.	23.1	1.7

In an attempt to obtain more efficient separation of I^{128} the PdI_2 precipitate was dissolved in NaOH, reprecipitated and

washed with acetone. The additional time required to carry out this process allowed the isotope to decay beyond a measureable activity.

Conclusion. The sensitivity of the method is so much reduced by the lengthy processing required for radiochemical purification that the purpose of using activation analysis is defeated. It is apparent to the author that chemical methods of analysis are more accurate and reliable than the activation procedure used. Other workers in A.E.R.E., Harwell (Cousins, 1961; Smith, 1961) have used different separation techniques and reached the same conclusion.

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