ANALYSIS OF IODOAMINO ACIDS IN SERUM

THE SEPARATION AND ANALYSIS OF IODOAMINO ACIDS IN HUMAN SERUM

By

MERVYN WARREN BILLINGHURST

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

September, 1970

DOCTOR OF PHILOSOPHY (1970) McMASTER UNIVERSITY
(Chemistry) Hamilton, Ontario

TITLE: The Separation and Analysis of Iodoamino Acids in Human Serum

AUTHOR: Mervyn Warren Billinghurst, B.Sc. (Auckland University)
M.Sc. (Auckland University)

SUPERVISOR: Professor R. H. Tomlinson

NUMBER OF PAGES: x, 164

SCOPE AND CONTENTS:

This project was undertaken with the aim of developing a method for the assay of iodoamino acids in human serum. The iodoamino acids were separated by an ion exchange technique, and the resulting solutions analyzed for iodine using neutron activation analysis. This analytical system was applied to the analysis of a number of serum samples from hypothyroid and hyperthyroid subjects. This allowed the division of each of these conditions into a number of classes in terms of the deviation of the individual iodoamino acid concentration from their normal ranges.

ACKNOWLEDGEMENTS

The author wishes to express his grateful thanks to his Research Director, Dr. R. H. Tomlinson, for his advice and encouragement during the course of this work.

The author is indebted to the members of his Supervisory Committee, Dr. K. Fritze and Dr. K. Freeman, for their many helpful comments.

The author also wishes to thank Dr. S. Garnett for many helpful discussions on the medical aspects of this work and the laboratory staff of the Hamilton General and St. Joseph's Hospitals for supplying the serum samples together with the pertinent clerical data.

The financial support of this research by the National Research Council of Canada is gratefully acknowledged.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS USED

P. B. I.	protein bound iodine
B. E. I.	butyl alcohol extractable iodine
MIT.	monoiodotyrosine
DIT.	diiodotyrosine
T_3	triiodothyronine
T_4	thyroxine
DEAE	diethylamino ethyl
B.M.R.	basal metabolic rate
Tris	Trihydroxymethylamino methane

INTRODUCTION

1.1 General Introduction

The first unequivocal description of the thyroid gland is found in the work of Thomas Wharton[1] in 1656. Although earlier authors were evidently aware of its existence they did not distinguish it clearly from the other glands in the region of the neck. As early as the second millenium B.C. the Chinese recommended seaweed preparations as a remedy for the disease now known as goitre. This is characterized by the enlargement of the thyroid gland, frequently resulting from a lack of dietary iodine. Although iodine was isolated from seaweed in 1812 by the French Chemist Courtois, the element was not associated with the thyroid until 1896 when Baumann[2] discovered that the thyroid contained considerable amounts of it. Baumann[3] also showed that the bulk of the iodine in the thyroid was contained in the protein fraction and, on hydrolysis of this protein, in a substance he called "iodothyrin", Kendall[4] in 1915 further purified this "iodothyrin" and obtained a crystalline substance which he called "Thyroxin". This compound was found to be 3,5,3',5'-tetraiodothyronine by Harington and Barger[5] in 1927. It was not until 1952 that the second thyroid hormone, 3,5,3'-triiodothyronine was identified by Gross and Pitt-Rivers[6].

Most of the early work on the organic iodine compounds synthesised by the thyroid gland was carried out on extracts of the thyroid, however routine clinical evaluation of the thyroid function can obviously not be carried out in this way. As the hormones are transported from the gland in the blood, clinical tests are usually carried out on blood samples. The low concentration of the iodine in the blood (4-8 μg/100 ml of serum in a euthyroid person) has precluded the accurate analysis of the individual iodinated compounds, by classical methods, on a routine basis. Recently a number of methods using activation analysis have been reported[7,8,9] for the total organic iodine, but there has been no reported study of the individual iodoamino acids based on this technique.

Fermi[10] in 1934 embarked on a systematic production of radioactive isotopes and succeeded in producing, among others, radioactive I^{131}. In 1938, Hertz et al[11] used I^{131} in a study of the iodine metabolism in experimental animals and the following year Hamilton and Soley[12] used it on human subjects. Radioactive iodine, used as a tracer, has led to the analysis of the relative concentrations of the various iodine containing compounds. A number of workers[13-20] have fractionated serum, taken from both humans and animals following treatment with radioactive iodide ion, and concluded that neither monoiodotyrosine (3-iodotyrosine) nor

diiodotyrosine (3,5 diiodotyrosine) are in blood even though careful chemical analyses using classical techniques [21-26] have indicated that significant proportions do exist.

This thesis describes the development of an activation analysis technique for the assay of the individual iodoamino acids which has a greater sensitivity than the classical analytical methods. It has made possible the first detailed study of the amounts of monoiodotyrosine and diiodotyrosine in serum. The results obtained in this work support the general result obtained by those workers using chemical analysis. A possible reconciliation of the results obtained by those workers using radioactive tracers with these results is discussed. The concentrations of the individual iodoamino acids in 12 euthyroid people were determined, so ascertaining a normal concentration range of these compounds in serum. Serum samples from 26 hyperthyroid and 25 hypothyroid patients were analysed and the resulting iodoamino acid concentrations compared to the normal values. This comparison clearly indicates that there are a number of unique sub-divisions within these two broad categories. A study of the effect of a large dose of iodine on the concentration of iodoamino acids in the serum of six euthyroid people was also carried out.

1.2 Methods of Separation of Organic Iodides

It is the normal practice for the organic iodine

content of serum to be reported as a single value under the heading of "protein bound iodine". However, depending on the method used to separate the organic iodine from the inorganic iodide and the serum proteins, this so called "protein bound iodine" consists of thyroxine and varying proportions of triiodothyronine, diiodotyrosine and monoiodotyrosine. Academically, it is obviously desirable to determine each fraction on its own and then relate the variation of each, in conjunction with the others, to the various thyroid disorders. This, together with the need to be able to separate these four compounds in thyroid hydrolysates, has led to a large number of possible separation systems being investigated. One of the earliest used in the study of serum iodine was based on butyl alcohol extraction[27]. Taurog and Chaikoff found that the iodotyrosines, which were extracted with the iodothyronines from the serum by butyl alcohol, could be removed by back extraction with 4N sodium hydroxide and 5% sodium carbonate.

Methods which have been applied to the separation of these four organic iodine compounds include many separation techniques.

1. Ion Exchange. Both anionic and cationic exchange columns have been investigated. In 1959, Galton and Pitt-Rivers[28] reported a method using an anion exchange resin, Dowex 1 x 2, mesh 200-400, from which they were able to elute monoiodotyrosine, diiodotyrosine and the iodothyronines using

different concentrations of acetic acid. Monoiodotyrosine came off first at the highest pH. However, they were unable to separate thyroxine and triiodothyronine. This process was further developed in its application to serum by Pitt-Rivers and Sacks[29] in 1962. In this paper they discuss the problem caused by the binding interaction between the thyroxine and the serum proteins. This interaction causes some of the thyroxine and triiodothyronine to be eluted with the serum proteins, instead of being retained more strongly than either of the iodotyrosines, if the serum is not left in contact with the resin for a relatively long period of time. In 1960 Wynn[21] published a method involving a gradient elution of the iodoamino acids from an anion exchange column with 5-88% formic acid. This method gave a good separation of the iodotyrosines from the iodothyronines, however monoiodotyrosine and diiodotyrosine were only just resolved as were thyroxine and triiodothyronine. The separation of these four iodoamino acids on a cation exchange resin was reported by Block and Mandl[30] who continuously varied the eluant from 0.2M oxalic acid in 30% ethanol to ethanolic 0.05M ammonium hydroxide (on Dowex 50W x 4, 20-40 mμ). This method is suitable for the much larger quantities found in thyroid extracts but not for those present in serum.

2. Paper Chromatography. A very large number of publications have appeared in which the separation of the iodinated amino acids has been attempted using paper

chromatography. Bjorksten, Grasbeck and Lamberg[31] published a useful review of these methods in which they grouped the various solvent systems and their efficiencies as follows.

(A) Alkaline Organic Systems. These normally have three constituents.

(i) An aliphatic C_3 - C_5 alcohol, the main constituent.

(ii) Occasionally a smaller amount of a C_1 - C_4 alcohol or some other solvent is added, e.g. acetone or dioxane.

(iii) The organic solvent(s) is (are) saturated with aqueous ammonia. The molarities of these ammonia solutions vary within wide limits.

For these systems the resolving efficiency (and the R_f values) is highly dependent on the ammonia concentration in the atmosphere of the chromatographic tank, and reproducibility was difficult to achieve. In general, it may be said that an increase in the concentration of (ii) or a higher concentration of ammonia will counteract trailing and increase the R_f values, particularly of the more highly iodinated species. However, this group fails to separate monoiodotyrosine from diiodotyrosine. As the relative mobilities are similar throughout the group, two such systems do not provide a check on each other, or form a suitable pair for two dimensional chromatography.

(B) Acidic Organic Systems. These are generally composed of n-butanol with varying amounts of acetic acid and water. However, while these provide a good separation of monoiodotyrosine and diiodotyrosine, triiodothyronine and thyroxine are not separated. With these systems, a very annoying artifact arises, especially when radioactive tracers are used. In acid solution, any iodine molecules, which may arise from the oxidation of iodide, exchange quite readily with the organic iodine and, to complicate things still further, they will also iodinate sites which were not previously iodinated, i.e. triiodothyronine may be converted to thyroxine. Thus, acidic organic systems are undesirable.

(C) Acidic Aqueous Systems. A system of water and formic acid containing 0.3 g/litre of sodium thiosulfate has been shown to separate all four iodoamino acids with relative mobilities quite different from those of systems A and B. The exchange and iodination problems which arise in the acidic organic system are minimized in this case by the speed of the method. However, it is only suitable for very low concentrations owing to the low solubility of the iodothyronines in aqueous solution.

(D) Neutral Organic, Alkaline and Neutral Aqueous Systems. These have all been tried but they have proved to be less useful.

An example of how these paper chromatographic techniques may be used for the identification of an organic

constituent is found in the paper published by Wynn[21] in which he used three different solvent systems to identify the organic iodides in the eluant from an ion exchange column.

A variation on this method of separation was reported in 1965 by Brown et al[32] in which they first formed the dinitrophenyl derivatives of the iodoamino acids and then chromatographed them using as the aqueous phase a mixture of chloroform, propan-1-ol, and aqueous ammonia (600: 600: 360 by volume).

A general problem in paper chromatographic, paper electrophoretic and thin layer chromatographic separations is that in drying the paper chromatogram the iodoamino acids, and especially thyroxine, are subject to rapid deiodination. A second problem arises from the fact that normally only several drops of sample are used. Thus, to determine the monoiodotyrosine, diiodotyrosine and triiodothyronine in the blood, a sensitivity in the range of 0.01μg would be required unless the sample had been concentrated prior to running the chromatogram.

3. Paper Electrophoresis. Bjorksten et al[31] have reported a paper electrophoretic separation of monoiodotyrosine, diiodotyrosine, triiodothyronine and thyroxine. In a diethylbarbiturate buffer, pH 8.0 and ionic strength 0.06, the electrophoretic mobilities of monoiodotyrosine, diiodotyrosine, triiodothyronine and thyroxine are 0.59, 1.09, 0.80 and 1.15 respectively, on Whatman No. 1 paper when a 7 volt/cm potential is used. These mobilities indicate that

this technique has considerable potential in the separation of iodoamino acids, especially when used in conjunction with paper chromatography, as the tendency to group diiodotyrosine and thyroxine is in direct contrast to the paper chromatographic tendency to group monoiodotyrosine and diiodotyrosine, and triiodothyronine and thyroxine. However, there is still the problem of deiodination of these iodoamino acids while the paper is being dried.

4. Gel Chromatography is a modern technique which was primarily characterized as a method of separation based on the size and shape of the molecules. The larger the molecule, the earlier it should be eluted, as it can only pass through a smaller volume of the column, the remainder of the column being taken up with beads which have a pore size too small for the larger molecules to enter. Accordingly, with careful gradation of the pore size, a corresponding spread of the molecular size of the molecules in the effluent can be obtained. In 1963 Mougey and Mason[33] reported that slightly acidic conditions favour the separation of iodide, monoiodotyrosine, and diiodotyrosine on "Gel" columns, while alkaline conditions favour the separation of triiodothyronine and thyroxine. Hocman[34] used a 1 x 10 cm column of "Sephadex" G-25 (medium) to separate organic iodine from serum. He found that most of the thyroxine was collected after elution with 10-12 column volumes of trihydroxymethylaminomethane hydrochloride buffer of pH 7.4 and ionic strength of 0.05. However, some of the thyroxine remained with the high mole-

cular weight proteins, and a small portion was irreversibly bound to the column. Hocman did not attempt to determine any of the other organic iodides. Makowetz et al[35], using a 110 x 0.27 cm column of Sephadex G-25 (fine) and 0.015N sodium hydroxide as an eluant, were able to separate and assay thyroxine and triiodothyronine present in 0.5 ml of serum. The separation took about 10 hours and the assay relied on the prior administration of I^{131} to the subject. The results on some specimens are shown below.

Specimen	P.B.I.	B.E.I.	T_3	T_4
1	5.8	5.3	0.5	8.0
2	-	5.3	0.4	8.0
3	2.5	18.0	0.1	3.0
4	11.3	-	0.9	16.1

N.B. Results are given in μg I/100 ml of serum.

Obviously the agreement between P.B.I. (protein bound iodine) or B.E.I. (butyl alcohol extractable iodine) and the value of triiodothyronine (T_3) and thyroxine (T_4) is poor.

Blasi and DeMasi[36] determined the distribution coefficients for monoiodotyrosine, diiodotyrosine, triiodothyronine and thyroxine in an aqueous solution when eluted with 0.02N sodium hydroxide from Sephadex G-25, and Brown et al[32] studied the elution of dinitrophlenyl derivatives of

monoiodotyrosine and diiodotyrosine from Sephadex G-25 with chloroform saturated with 0.1N ammonia in 10% propan-2-ol.

It is of interest to note that generally the triiodothyronine and the thyroxine are eluted after the "ionic fraction", that is to say, the process by which these two are separated is an absorption or a binding phenomenon, and not a molecular filtration.

5. Starch Columns. In 1953, Dobyns and Barry[37] attempted to use starch columns with an eluant of butanol-propanol-water (1:2:1). In acidic eluants, when water was replaced by 1N hydrochloric acid, the iodothyronines were well separated but monoiodotyrosine and diiodotyrosine were not resolved. The problems of iodine exchange and iodination which occur in acidic solution on paper chromatograms was also found to occur on these columns. In alkaline eluants, where water was replaced by 0.05N sodium carbonate, a good separation was obtained, but it was very difficult to remove the diiodotyrosine from the column. The best method of removing the diiodotyrosine was to change to an acidic eluant after the monoiodotyrosine had been eluted.

Other chromatographic separations such as the use of Kieselguhr and DEAE cellulose have also been studied but have not proved to be very useful. A good review of the investigation of these methods has been published by Roche and Michel[38].

Gas chromatography of various derivatives of these

iodoamino acids have proved to be very sensitive and to have good resolution[118, 119]. However, attempts to isolate the iodoamino acids from the serum proteins in extracts which are suitable for the formation of these derivatives have not been successful.

1.3 Distribution of Organic Iodine Compounds in Serum

It is difficult, if not impossible, to draw any conclusions from the literature on the distribution of the organic iodine compounds in serum. The major controversy revolves around the question of whether or not iodotyrosines are present in normal sera. The reported assays basically fall into two categories: those employing radioactive I^{125} or I^{131} as a tracer, and those which depend on a colorimetric reaction, such as the catalytic effect of iodide on the rate of decolorization of ceric ion by arsenious ion. It is possible to make the generalization that methods employing radioactive tracers find little or no iodotyrosines, while those based on colorimetric assay find up to 69% of the organic iodine as iodotyrosines. However, there are a number of exceptions in both groups. The following is a selective review of some of the papers which have been published.

(A) Investigation Employing Colorimetric Techniques

1959 Mandl and Block[22]. Separation: Paper chromatography with an ammonia butanol solvent. Both iodotyrosines and iodothyronines were found in the two samples they analyzed.

1.	MIT	5.9%	DIT	6.6%	T_3	1%	T_4	78.3%	I^-	9.2%
2.	MIT	20.6%	DIT	17.6%	T_3	6.1%	T_4	39.6%	I^-	16.1%

1960 Wynn[21]. Separation: Paper chromatography and anion exchange.

No iodotyrosines were observed using either method, although an unidentified iodoprotein appeared just ahead of the iodotyrosines region and accounted for about 4% of the total iodide.

1960 Kono et al[23]. Separation: Paper chromatography. Only thyroxine was found in human serum.

1962 Wellby and Hetzel[24]. Separation: Paper chromatography. No iodotyrosines were observed in normal plasma; however, diiodotyrosine was found in thyrotoxic cases.

Diiodotyrosine was also found in a few cases which had abnormally high "protein bound iodine" values.

1964 Dimitriadou et al[25]. Separation: Butanol extraction followed by anion exchange and paper chromatography. About half the organic iodine was identified as iodotyrosines. This analysis by the ceric/arsenious colourimetric method was confirmed by neutron activation analysis. However, it was observed that the value ob-

tained for the iodotyrosines was dependent on the method of separation and could not be explained in terms of the efficiencies of the separation methods. This led the authors to suggest that the iodotyrosines are present as organic iodine conjugates.

1967 Backer et al[26]. Separation: Cation exchange column separated the iodotyrosines from the iodothyronines. They obtained an average value of 0.8 µg/100 ml for iodotyrosines in healthy patients with a standard deviation of 0.5 µg/100 ml.

(B) Comparative Studies between the Distribution of Organic I^{127} and Organic I^{131}

1948 Taurog and Chaikoff[27]. Separation: Butyl alcohol extraction followed by back extraction with 4N sodium hydroxide and 5% sodium carbonate. Radioactive counting showed 5% present as iodotyrosines; however, chemical measurements showed 13% of the total iodine was present as iodotyrosines.

1959 Werner[13]. Separation: Paper chromatography. The following results were obtained.

I^{127}	T_4 20-60%	T_3 2-26%	DIT 15-36%	MIT 8-30%	I^- 1-9%

^{131}I - T_4 40-70% T_3 8-50% DIT 1-4% MIT 1-5% I^- 16-48%

1960 Block et al[14]. Separation: Paper chromatography. Chemical analysis showed 1/3 - 1/2 of the organic iodine was present as iodotyrosines. However, they could find no radioactivity at all in these iodotyrosines and suggested that the iodotyrosines could be present in the blood in some sort of labile combination which prevents their iodine from exchanging with the labelled iodide. This suggestion was questioned by Lissitzki et al[15] who injected rats with labelled diiodotyrosine (I^{131}) and observed a biological half-life of only 1.5 hrs. This means that within a short time most of the iodotyrosine present in the serum will have been freshly synthesised and should therefore contain radioactive iodine.

1963 Werner and Radichevich[16]. Separation: Paper chromatography in three different solvents. About one-third of the iodine was present as iodotyrosines, although, when radioactive serum was used, no radioactivity was observed in the iodotyrosine spots.

1967 Glöbel[17]. Separation: Paper chromatography.

About 60% of the unlabelled organic iodine was found to be present as iodotyrosines and most of the remainder as thyroxine. The incorporation of I^{131} after a 25 μCi dose of I^{131} was followed as a function of time for 26 days. Radioactivity in the iodotyrosines was first observed after eight days. Their percentage, with respect to the total radioactivity of the organic iodine was found to increase in a logarithmic manner until by the 26th day, when about 10% of the remaining radioactivity of the organic iodine was in the form of iodotyrosines. This indicates very clearly that the incorporation of iodine into the iodotyrosines in the blood is not a rapid process.

(C) Studies on Laboratory Animals Using Radioactive Isotopes

It is possible to introduce greater amounts of radioactivity into laboratory animals than is acceptable in humans, but such a practice may affect the thyroid and lead to an altered distribution of the iodoamino acids. However, greater specific activity may be obtained, making it easier to achieve a high degree of sensitivity in radioactive analysis. For this reason, a large number of studies have been carried out on laboratory animals, especially rats, either by using a radioactive iodine diet for some time

before taking blood samples or by injecting radioactive iodide.

1961 Pitt-Rivers and Rall[18]. Separation: Paper chromatography. Established that iodine in rat serum was distributed as follows: 80% T_4, 3.5% T_3, and less than 0.4% iodotyrosines.

1966 Taurog *et al*[3,19]. Separation: Paper chromatography. Studied rabbits, dogs and cats and were unable to detect any iodotyrosines even after treatment with thyroid stimulating hormone. They observed that the amount of triiodothyronine present in the blood serum rose with the thyroid stimulating hormone treatment.

1965 Matsuda and Greer[20]. Separation: Butanol extraction followed by paper chromatography. Reported that with rats only thyroxine and a small inconsistent amount of triiodothyronine were observed under normal basal conditions. However, when rats were treated with thyroid stimulating hormone, the quantity of triiodothyronine observed rose, both absolutely, and relative to thyroxine. At the same time monoiodotyrosine and diiodotyrosine were observed in the serum.

1966 Rhodes and Wagner[39]. Separation: Butanol extraction followed by paper chromatography.

A diet of ^{129}I was fed to rats for three to six months. Thermal neutron irradiation of the ^{129}I resulted in the formation of ^{130}I, a γ emitting isotope. The induced radioactivity was then used for the detection and assay of the iodine compounds. Only 1-2% of the iodine was detected as iodotyrosines. The authors suggested that the quantity of iodotyrosines decreased during storage, although they offered no data to support this suggestion.

1966 Lissitzky *et al*[125]. Separation: Gel filtration. ^{125}I was used to show that there were no "free" iodotyrosines in the plasma of rats although this does not exclude the possibility that the iodotyrosines may be present and bound to the plasma proteins.

1966 O'Halloran and Wellby[40]. These workers approached the question indirectly by studying the recoveries of the various iodotyrosines and iodothyronines from serum when separated and analysed for "protein bound iodine" by two of the commonly used methods. They obtained the following results.

Precipitation Method	Percentage Recoveries			
	Thyroxine tyrosine	Triiodo-tyronine	Diiodo-tyrosine	Monoiodo-tyrosine
Somogyi (Zn^{++}/NaOH)	94	99	93	88
Trichloro-acetic Acid	94	95	8	4

From this they concluded that, as there was no difference in the euthyroid range between these two methods, the iodotryosines could not be present in any significant concentration in normal sera.

The results from these reports are summed in the following chart from which it can be seen that it is not possible to draw a definite conclusion as to the presence or absence of iodotyrosine in normal sera.

1.4 Clinical Evaluation of the Thyroid Function

A number of different tests have been used to evaluate the thyroid function. These include the basal metabolic rate (B.M.R.), the so called "neck uptake test", the serum tyrosine level[41], the protein bound iodine (P.B.I.) determination[42] and the so called "Saturation" analysis[43]. The two types of tests which are of most interest in connection with this project are the "protein bound iodine" determinations in which the organic iodine compounds

SUMMARY OF ORGANIC IODINE DISTRIBUTION

	Isotope Used	Iodiotyrosines	Triiodothyronine	Thyroxine
Mandl and Block[22]	I^{125}	absent	present	present
Wynn[21]	I^{127}	absent		
Koro et al[23]	I^{127}	absent	absent	present
Wellby and Hetzel[24]	I^{127}	absent		
Dimitriadou et al[25]	I^{127}	present	present	present
Backer et al[26]	I^{127}	present		
Taurog and Chaikoff[27]	I^{127}	present	present	present
Werner[13]	I^{127}	present	present	present
	I^{131}	less observed		
Block et al[14]	I^{127}	present	present	present
	I^{131}	absent	present	present
Werner and Radichevich[16]	I^{127}	present	present	present
	I^{131}	absent	present	present
Globel[17]	I^{127}	present	present	present
	I^{131}	present	present	present
Pitt-Rivers and Rall[18]	I^{131}	very little	present	present
Taurog et al[19]	I^{131}	absent	present	present
Matsuda and Greer[20]	I^{131}	absent	uncertain	present
Rhodes and Wagner[39]	I^{129}	very little	present	present
Lissitzki et al[15]	I^{125}	absent		

in the blood are analysed as a group, and the "saturation" analysis tests in which the degree of saturation of the binding sites for organic iodine compounds, such as thyroxine, is measured.

Protein Bound Iodine Determinations

In determining the protein bound iodine concentration, it is first necessary to separate the protein bound iodine which is present in the blood serum from the inorganic iodide and the serum proteins. This may be done by a number of methods.

(a) Butyl alcohol extraction. Taurog and Chaikoff[27] reported a separation of organic iodides from serum proteins and the inorganic iodide. The serum was first extracted with butan-1-ol, and then the butan-1-ol was back extracted with 4N sodium hydroxide and 5% sodium carbonate to remove any inorganic iodide and iodotyrosines which might be present.

(b) Precipitation. The most commonly employed method of separating organic iodine compounds from the inorganic iodide is by precipitation of the proteins. There are two systems commonly used for precipitation.

(i) A solution of zinc sulphate in sulphuric acid followed by sodium hydroxide solution.

(ii) A solution of trichloroacetic acid.

The efficiencies of these two systems with respect to carrying down monoiodotyrosine, diiodotyrosine, triiodothyronine and thyroxine with the precipitate was investigated by O'Halloran and Wellby[40] who found that system (i) had high efficiencies in excess of 90% except MIT $\simeq$ 88%, while system (ii) precipitated about 95% of the triiodothyronine and thyroxine, but only 4 and 8% of monoiodotyrosine and diiodotyrosine respectively. A variation of system (i) was used by Moran[44] in which sodium tungstate replaced the zinc sulphate and the sodium hydroxide was omitted.

After precipitation, the serum proteins are ashed. The most common methods used are:

(i) Wet ashing: a sulphuric-chromic acid digestion and distillation of elemental iodine[45].

(ii) Dry ashing: a potassium carbonate fusion, leaving the iodine as iodide[42].

A modification of the sulphuric-chromic acid technique which avoided the need for distilling the iodine was reported by Zak et al[46] with chloric acid in the presence of sodium chromate to digest the organic material and convert the iodine directly to iodate. The iodate is converted

to iodide by a measured excess of arsenious acid. Stolc and Knapp[47] developed a more rapid method to handle small quantities. Up to 0.1 ml was spotted on filter paper, inserted in a platinum coil, and burnt in an atmosphere of of oxygen over a sodium carbonate solution. The ash was then immersed in a sodium carbonate solution and analyzed by the ceric/arsenious acid decolorization method[48].

(c) Ion Exchange Methods. Both anion and cation methods have been used to remove the organic iodide. An anion exchange method was developed by Pitt-Rivers and Sacks[29], and further advanced by Pileggi and Kessler[49], to remove organic iodine contaminants as well. The cation exchange resin technique has been developed and reported in two papers by Backer et al[26,50]. This method enables the determination of either the total iodoamino acid content of serum, or just the hormonal iodine (triiodothyrone and thyroxine) if a preliminary wash of borate buffer pH8.5 is used to remove the iodotyrosines. It was found that the hormonal iodine value obtained using this method was relatively free from interference from iodinated medication.

(d) Gel Filtration. This method has been studied by Jones and Schultz[51]. They were able to separate not only the thyroxine from the serum and inorganic iodide but also

to remove interfering organic iodides. However, the method was designed on the assumption that only thyroxine is of interest in the evaluation of the thyroid function. It is of interest to note that the removal of other organic iodides is totally dependent upon the fact that once the thyroxine is absorbed onto the column and separated from the protein, it is chemically bound and cannot be eluted except at high pH. Thus, a wash of several column volumes of Tris-HCl buffer pH8.6 can be used to remove contamination prior to the elution of the thyroxine with ammonium hydroxide.

The next step in the procedure is the assay. The most common method for the assay utilizes the catalytic effect of iodine, iodotyrosine and iodothyronine on the decolorization of ceric sulphate by arsenious acid[48]. The reaction is made pseudo first order by using a large excess of arsenious acid. However, the reaction has a fairly high temperature coefficient, and is influenced by visible light and a number of other ions, so that considerable care must be taken.

Another method dependent on the catalytic influence of these iodine compounds was developed by Gmelin and Virtanen[52]. The system is composed of acidified ferric chloride, ferricyanide and sodium arsenite. Reduction of ferric or ferricyanide ion by the arsenite yields ferrous or ferro-

cyanide ions respectively. These then react with the reactants, ferricyanide or ferric ion respectively, to form the insoluble ferriferrocyanide commonly referred to as "Prussian Blue". This method has a greater sensitivity than the ceric/arsenious acid system, but has the disadvantage of being sensitive to reducing agents. Another colorimetric method which is normally used only with paper chromatographic or paper electrophoretic methods is the reaction of the phenolic groups with diazotized sulphanilic acid to give a red or violet coloration[31]. The obvious problem with this technique is that it is non-specific and the reaction is dependent on the presence of the phenolic group rather than the iodine. It is also rather insensitive.

Activation analysis has also been used as an assay method. Two different types of activation have been used: thermal neutron activation[7,8,9] and Bremsstrahlung activation[53]. In thermal neutron activation analysis, the purified sample is irradiated with thermal neutrons at a flux of about 10^{12}-10^{13} neutrons cm^{-2} sec^{-1} for 10 minutes to one hour, and the induced radiactivity is then counted. The nuclear reaction occurring is:

$$I^{127}(n,\gamma)I^{128}.$$

The I^{128} has a half life of 25 minutes and emits a β particle and a γ ray of energy 442 keV.

Smith et al[7] found that they obtained a systematically higher value by this method than the value obtained using the Barker method of chemical analysis, involving precipitation, digestion and ceric/arsenious colorimetric analysis. The results were systematically higher by an average value of 1.8 μg/100 ml. This, they suggest, could be because they may have been measuring the total organically bound iodine while the chemical analysis may have only been measuring the iodotyrosines. This should be compared with the method developed by Feldman et al[54] who measured the thyroxine in 90-100% yield, possibly with some triiodothyronine, and obtained values slightly lower (0.3 μg/100 ml lower) than those obtained by the Barker method. In Bremsstrahlung activation analysis[53] the sample is irradiated for about 90 minutes in a 22 MeV linear accelerator with the sample placed in a 6 cm aluminum block. The nuclear reaction is

$$I^{127}(\gamma,n)I^{126}$$

which has a threshhold energy of 10 MeV and a cross-section of 0.04 barns.

The values obtained for protein bound iodine are normally reported in micrograms of iodine per 100 ml of serum. For an euthroid person, a range from 4-8 μg/100 ml, with slight variation in the so-called normal range, depending on

the individual refinements of the methods of separation and analysis used.

"Saturation" Analysis Methods of Evaluating Thyroid Function

This type of analysis is based on the competitive binding of the iodoamino acids between the normal binding site in the serum proteins and another substance. Substances which have been used for this purpose include red cells, charcoal, and ion exchange resins. The basic idea is to add labelled iodoamino acids, usually triiodothyronine or thyroxine to the serum sample. The radioactive iodothyronine then establishes the position of the equilibrium distribution between the "free" and protein bound iodothyronine. This may then be related to the distribution in euthyroid people.

One of the earliest reports of this type of thyroid function evaluation was that of Scott et al[43]. These workers found that inorganic iodide passed readily into the red cells with the result that the red cell iodide/plasma iodide ratio was approximately 0.5. However, the cell membrane was relatively impermeable to large organic molecules such as the iodine hormones, with the result that as the I^{131} was incorporated into the triiodothyronine and thyroxine, the ratio dropped. The rate of this drop of the red cell I^{131}/plasma I^{131} ratio was then an expression of the thyroid function. However, the red blood cells or

erythrocytes are able to bind the iodine hormones to some extent, and Hamolsky et al[55] developed a test based on the distribution between the serum and the red cells of a tracer amount of I^{131} labelled triiodothyronine. It soon became apparent that the red cells were far from ideal for this purpose[56], and a more suitable adsorbent was sought. One adsorbent that was tried was charcoal coated with haemoglobin[57,58]. A trace amount of labelled triiodothyronine was added to the serum, incubated with it for two hours, and then left in contact with haemoglobin-coated charcoal for ten minutes. Irvine and Standeven[58] compared the use of this coated charcoal with ion exchange resin and concluded that the charcoal method was equally as accurate and had the advantage of being more rapid. A number of variations of the resin adsorption have been used, including resin paper and a resin sponge[59]. This latter is marketed in special kits by Abbott Laboratories under the name of "Trisorb Resin Sponge". McAdams and Reinfrank[59] claim that this method is easier to use than the erythrocyte method and gives a better differentiation between hyperthyroid and euthyroid conditions. Tabern et al[60] have made a detailed study of this system and offer three very important observations which apply to "saturation studies".

1. The adsorption is not a simple procedure, but rather is the summation of a number of reactions , some of

which are easily reversible while some are unidirectional. Thus it is necessary that the temperature and the concentrations be carefully controlled. It is also preferable that a single long term standard should be used; the authors suggest that sheep serum is ideal for the purpose.

2. What is actually being measured is the degree of blood protein saturation by thyroxine and triiodothyronine, so that the presence of abnormal binding proteins produced by physiological stimuli interfere with the interpretation of these results.

3. Steroids,especially those used in contraceptives, introduce a large error into this method.

An interesting variation on this saturation analysis procedure was reported by Scholer[61] who treated the resin with labelled triiodothyronine so as to obtain a resin to which was bound a specific amount of labelled triiodothyronine. This was then equilibrated with the test serum. Such a method is very easy to handle on a routine basis and the method was found to give satisfactory clinical results. Foeckler et al[62] conducted a comparative study of this triiodothyronine labelled resin test and the red cell uptake test and reported that they found good agreement. However, Tauxe and Kusakabe[63] claim that these two tests do not give the

same information and offer the following evidence to support this claim.

1. Some patients with relatively high erythrocyte uptake do not show concomitantly high resin uptake. The presence of salicylates causes a decrease in the protein bound iodine, presumably because the circulating hormone is shifted away from the plasma proteins. Salicylates also cause the erythrocyte triiodothyronine uptake to be increased but the resin triiodothyronine uptake is not increased.
2. The thyroxine is not significantly bound to the erythrocytes,but binds readily to the resin.

Both of these facts suggest that the resin triiodothyronine uptake test would be superior to the erythrocyte uptake test, as it is not as sensitive to salicylate effects and is also an index of the total iodothyronine content of the serum.

The observation that thyroxine is readily adsorbed by the resin and by the serum proteins immediately suggested the possibility of replacing the labelled triiodothyronine by labelled thyroxine in the uptake test. A comparison of the use of these two labelled iodothyronines was reported by Oliver and Harris[64] who found that the distinction between hyperthyroid and euthyroid subjects is much clearer when the thyroxine uptake is used instead of the triiodothyronine

uptake. This, they suggested, was possibly due to the fact that the thyroxine binds to thyroxine-binding prealbumin as well as to thyroxine-binding globulin and albumin, while the triiodothyronine does not bind to the thyroxine-binding prealbumin. Accordingly, the triiodothyronine test does not measure the degree of unsaturation in the prealbumin fraction.

Another method of studying the competitive binding of triiodothyronine is to use "Sephadex" columns. This approach was applied to the triiodothyronine equilibrium by Shapiro et al[65]. In essence the method consists of incubating the tracer with the serum, then absorbing it onto a "Sephadex" column, with the protein bound fraction being eluted first, followed by the iodide. The "free" triiodothyronine can later be removed from the column by washing with fresh serum. This method has the considerable advantage of having a "built-in" estimate of the amount of the original activity which is present as iodide, thus removing one of the sources of error inherent in the use of these radioactive tracers. (See Section 1.6).

All this work has been done using iodine-131 as the radioactive tracer; however, iodine-125, besides having a longer half-life, has also been found to be subject to a lesser amount of self-induced radiation decomposition. Thus it would appear to be preferable to use I^{125} as a label in

the triiodothyronine and thyroxine used in these experiments. Jenkinson and Young[66] found that this was indeed the case when they compared the results obtained with I^{125} and I^{131} labelled triiodothyronine in the triiodothyronine resin uptake test.

Another technique for the evaluation of the thyroid function, sometimes referred to as "Competitive Protein Binding Analysis", is closely akin to radioimmuno-assay. The essence of this technique is described by Murphy[67].

(i) Deproteinisation
- (A) Precipitation of the protein with ethanol. 77% of the thyroxine is left in the supernatant.
- (B) Evaporation of an aliquot of the supernatant to dryness.

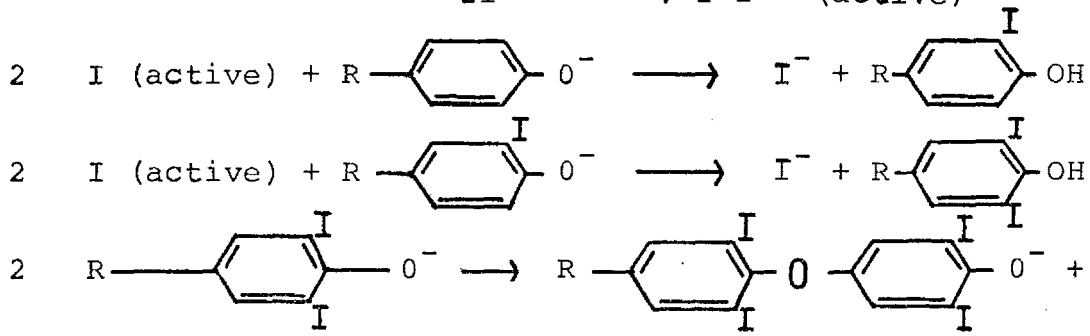
(ii) Assay
- (C) Equilibration of the dried thyroxine at 0°C with a solution containing radiothyroxine and thyroxine-binding globulin.
- (D) Separation of the protein bound fraction.
- (E) Calculation of the percentage of radiothyroxine in the protein bound fraction.
- (F) Determination of the amount of thyroxine in the serum from a calibration curve.

In this method the thyroxine-binding globulin may be replaced by a standard serum[68]. The radiothyroxine may be replaced by radiotriiodothyronine and the resin by "Gel"[69] if desired. While this method overcomes the obvious deficiencies of the "saturation tests", e.g. the dependence on the individual composition of the test sera, it does not remove the necessity for careful control of the temperature at which the separation of the "free" and the protein bound iodothyronines is carried out.

1.5 Biochemistry of the Iodoamino Acids

Biosynthesis: The biosynthesis of the iodotyrosines and iodothyronines occurs principally in the thyroid gland. It appears to be accepted that the overall synthesis of thyroxine and triiodothyronine may be represented in the following manner[70,71]

$$2I^- \xrightarrow{-2e} 2\ I \quad \text{(active)} \qquad (1)$$



(2)

(3)

If reaction (3) were to take place between a monoiodotyrosine and a diiodotyrosine molecule, then the product would be triiodothyronine. Blasi[72] reported that the direct

reaction between two iodinated tyrosines is slow, and that a more rapid synthesis occurs via 4-hydroxy-3, 5-diiodophenylpyruvic acid. Nishinaga et al[73] described this reaction between 4-hydroxy-3, 5-diiodophenylpyruvic acid and diiodotyrosine as a two step process.

(i) An aerobic reaction in which the keto acid in the enol form is oxidized to a thyroxine precursor.

(ii) A nonaerobic reaction in which this precursor couples with diiodotyrosine to form thyroxine.

Deiodination. In their review[71] Robbins and Rall reported that the use of labelled thyroxine injected in man has been unable to reveal any iodinated metabolites of thyroxine in the blood stream. Thus, when thyroxine and, by analogy, triiodothyronine, are metabolised, all the iodines must be metabolised. Stanbury[74] found that the iodotyrosines are rapidly deiodinated by a widely distributed deiodinase which requires the presence of the reducing triphosphoropyridine nucleotide as a cofactor. He also noted that monoidodotyrosine is more rapidly deiodinated than diiodotyrosine. Robbins and Rall[71] noted in their review that the more rapid turnover of triiodothyronine, as compared to that of thyroxine, is probably due to the weaker binding of triiodothyronine to the thyroxine-binding prealbumin. This more rapid turnover means that small changes in triiodo-

thyronine serum concentration have a disproportionately high hormonal effect. Robbins and Rall suggested that triiodothyronine may be responsible for about half the biological effect of the thyroid hormones. If this were the case, then small changes in the concentration of triiodothyronine in the blood would be of considerable significance.

Binding Between Serum Proteins and Iodoamino Acids.

In 1956, Robbins[75] used reversed flow electrophoresis to estimate the binding capacity of serum proteins and obtained values between 0.16 and 0.25 µg/ml with an average of 0.19 µg/ml. Werner[76] fractionated the various proteins in serum and was able to determine, to some extent, which iodoamino acids were bound to which proteins. They observed that thyroxine was bound to the α-globulins, triiodothyronine to the α-globulin and albumin, while the iodotyrosines were basically bound to the γ-globulins. More recently Miyia et al[77] using radioimmuno electrophoretic methods were able to show that thyroxine is bound to the prealbumin, albumin, thyroxine-binding globulin and the α- and β-liproproteins.

(A) Globulins. Deiss et al[78], using paper electrophoresis, compared the binding of thyroxine and triiodothyronine to the α-globulin. They found that the thyroxine was more firmly bound and capable of displacing the triiodothyro-

nine. If the thyroxine was displaced from the α-globulin, it migrated with the albumin fraction, while when the triiodothyronine was displaced from the α-globulin, it just trailed the α-globulin band. Larsen and Albright[79] continued this work and reported that the amino group was most important in the binding with α-globulin, while the 3' and 5' iodo groups had an effect which paralleled their effect on the phenolic proton. This explains the weaker binding of triiodothyronine. However, they were unable to determine the exact nature of the binding site of the iodothyronines on the α-globulins. In 1959, Tanaka and Starr[80] reported the case of a man who had no thyroxine-binding globulin but was still euthyroid. In this case the thyroxine was found to migrate with the albumin and prealbumin proteins.

(B) Thyroxine-Binding Prealbumins. Robbins and Rall have published three papers[81-83] dealing with the binding of thyroxine to the prealbumin proteins. Although they experienced some difficulty in determining the capacity of the prealbumin for thyroxine, because of the presence of other serum proteins, they were eventually able to ascertain that normal serum contains sufficient thyroxine-binding prealbumin to bind 0.2 μg/ml of thyroxine. Investigation of the relative affinity of the thyroxine-binding prealbumin for thyroxine, triiodothyronine, and their analogues showed that only D-thyroxine had a greater binding affinity than

L-thyroxine. Lesser degrees of binding were shown by substances with changes in the 3' and 5' positions; for example, triiodothyronine. Brauerman and Ingbar[84] have shown that triiodothyronine does not bind to thyroxine-binding prealbumin.

(C) Serum Albumin. Sterling and Tabachnick[85], in a study of the binding of thyroxine and its analogues to serum albumin, evaluated the association constant between thyroxine and albumin as 110,000, and that between triiodothyronine and albumin as 11,000 at pH 7.4. As the pH was increased, the affinity of triiodothyronine for the albumin increased as would be expected if its lower degree of association, compared to that of thyroxine, were attributable to the lesser degree of ionisation of the phenolic group. Thus, the authors suggest that the anionic groups of triiodothyronine and thyroxine react with the free cationic groups of the protein. Tritoch studied the binding sites of thyroxine on albumin and was able to show that the principal site is in the region of the amino terminus, but that there are also five secondary sites[86].

Thus it is obvious that the equilibria between "free" and bound organic iodides in serum are far from simple, as there are a number of different proteins involved and, at least in the case of albumin, more than one binding site in each protein molecule. If a competitor for these binding

sites is added, possibly in the form of medication, then its overall effect will be the sum of its effect on all the binding sites, and the effects on the individual binding sites may vary widely. Osario[87] injected sodium salicylate intravenously and observed that this caused an immediate drop in the I^{131} labelled thyroxine in the blood. He suggested that this was due to a reduction in the protein binding. Bandy and Hagewood[88] observed that cortisone would reduce the rate of disappearance of injected thyroxine; that is, there was a decrease in the rate of metabolism of the thyroxine. It is possible that this could be due to an increase in the binding of the thyroxine to the proteins.

Bondy and Hagewood also reported that prolonged fasting, exhaustive exercise, and exposure to the cold caused a drop in the protein bound iodine value, accompanied by an increase in the rate of metabolism of injected thyroxine. Hetzel et al[89] reported that protein bound iodine values were elevated by emotional stress, while Hetzel and Wellby[24] suggested that the occasional appearance of diiodotyrosine in the plasma of euthyroid patients could be related to stress.

Yamada and Lewis[90] considered that the ratio of triiodothyronine to thyroxine, rather than the total overall concentration of thyroid hormone might be the major factor in goiter control. Support for this idea is found in Heninger and Albright's report[91] which stated that in rats fed on an

iodine deficient diet, the ratio of triiodothyronine to thyroxine changed from 0.01 to 0.06 as a result of the increased concentration of triiodothyronine and the decreased concentration of thyroxine. Thus it would appear that if one could analyse routinely for each iodine component of serum separately, a much more informative and accurate diagnosis would be possible.

1.6 The Use of Isotopically Labelled Iodoamino Acids

Preparation. The preparation of I^{131}-labelled iodotyrosines may be carried out by the iodination of tyrosines with I^{131} iodide[92]. It has also been observed that diiodotyrosine exchanges iodine atoms with iodine molecules present in solution, especially in alkaline conditions[93]. Thus, labelled iodotyrosine can be prepared by, either iodination or exchange. Similarly, the preparation of iodothyronines by both exchange[92,94,95] and by iodination[94,96,97] has been reported. It should be noted that the exchange reactions with elementary iodine normally occur most rapidly in solutions of high pH. Exchange reactions with iodide are considerably slower; for example Lemmon et al[92] reported only 11% incorporation of I^{131} into thyroxine after refluxing it for six hours at pH 5-6. The existence of these exchange reactions are convenient in the preparation of the labelled compounds, but are also responsible for the existence of difficulties encountered when they are used

in tracer experiments because of the reversal of this exchange process.

Problems with the Use of Labelled Iodoamino Acids. When labelled compounds, such as I^{131} labelled thyroxine, are used as tracers for non-labelled compounds in blood, it is necessary that the labelled thyroxine reach equilibrium with the thyroxine bound to the protein in the blood. At the same time, the radioactive iodine must not be allowed to exchange with other iodine compounds in the system. Thus in the time required for the tracer experiment, ideally the exchange of the "free" with the protein bound thyroxine should be rapid, while exchange of the iodine label should be slow. Typically, *in vivo* experiments last 24 hours or longer, whereas *in vitro* tests, such as saturation analyses, are conducted in about an hour. The exchange of "free" and protein bound thyroxine and triiodotyronine is rapid in relation to both *in vivo* and *in vitro* experiments[98]. As it is possible to form the labelled compounds by iodine exchange in solution under certain conditions, as discussed above, there is obviously a certain amount of lability of the iodine bond. This is also indicated in the problems which arise with paper chromatographic separations in acidic systems (see section 1.2). In 1944, Miller *et al*[93] reported a study on the exchange which occurs between labelled diiodotyrosine and both iodine and iodide in solution. Using a concentration of 0.016

µg/ml of diiodotyrosine and five times as much iodine, they observed an exchange of 72% in five minutes at pH 5, but only 8% at pH2. This increase in the rate of exchange with increase of pH was observed to continue to even higher pH. The exchange between diiodotyrosine and iodide was observed to be much slower and to have a maximum rate in the region of pH 4-5.5. At pH 4.3 there was only a 62% exchange between iodide and diiodotyrosine in six hours at 37°C. At pH 7.5 no exchange was observed even after two days. However, the addition of a small amount of iodine greatly accelerated the rate of exchange. This work would seem to suggest that the use of diiodotyrosine as a tracer, for *in vivo* studies taking a day or more, would cause considerable problems in interpretation. However Miller *et al*[93] said that preliminary experiments led them to believe that these problems may not occur in the presence of serum proteins. Lippert and Meintjes[99] found that no exchange occurred between iodine and thyroxine in the blood. Thus the problem of exchanging the iodine label from the iodoamino acids may give rise to complications in both *in vivo* and *in vitro* studies.

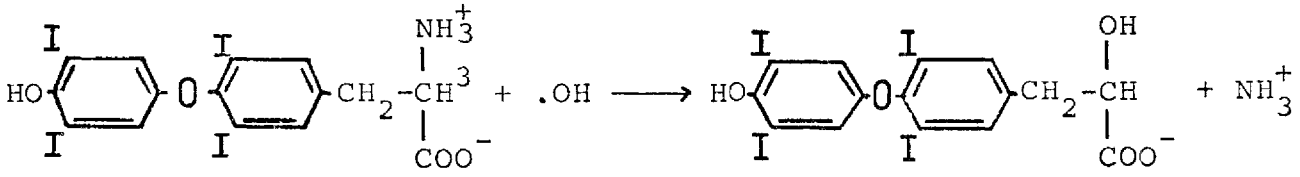
It is known that a number of compounds when labelled with radioisotopes decompose on storage[100]. This may be due to the radiation energy being absorbed by the molecule, causing it to break up, or by free radicals, or other reactive species being formed and reacting with the labelled

compound. There is general agreement in the literature that I^{131} labelled iodothyronines and iodotyrosines do decompose on storage; however, there are some differences of opinion as to what the decomposition products are in the case of iodothyronines. The Radiochemical Centre, Amersham, England, in its Technical Bulletin 68/9 "Labelled Thyroid Hormones" claims that the major route of radiation decomposition is via reductive deiodination, in which the iodine atom is replaced by a hydrogen atom. This view is supported by Volpert et al[101] who found that one week after the receipt of samples of thyroxine, 79-88% of the activity was present as thyroxine, 2-8% as 3,5,3' triiodothyronine, 5-7% as 3,3,5' triiodothyronine, and 3-7% as iodide. In the other hand, Tata[102], in a systematic study of I^{131} labelled triiodothyronine and thyroxine, did not observe the formation of any I^{131} triiodothyronine from the thyroxine. Instead, he observed that after a period of relative stability, of 18-27 days, there was a sudden drop in the amount of thyroxine (or triiodothyronine) coincident with the appearance of tetraiodothyrolactic acid (or triiodothyrolactic acid in the case of triiodothyronine), the amount of which rose to a maximum in a few days and then dropped off again, with an increase in the amount of free iodide being formed. For this he proposed the following decomposition scheme:

$$H_2O \longrightarrow H_2O^+ + e$$

$$H_2O^+ \longrightarrow .OH + H^+$$

$$H_2O^+ \longrightarrow .OH + H^+$$



$$NH_3^+ + H_2O \longrightarrow NH_4^+ + .OH$$

Jenkinson and Young[66] compared the composition of samples of I^{131} triiodothyronine that had been stored for two to eight weeks, and noted a drop of about one third in the radioactivity of the triiodothyronine fraction. They found that this radioactivity appeared as an increased radioactivity in the diiodotyrosine fraction and in a fraction which was eluted with the serum proteins. The following two statements suggest that the fraction might be triiodothyrolactic acid:

(i) Its similarity to triiodothyronine would probably allow it to associate with protein molecules in the same way as triiodothyronine, although to a lesser extent because of the replacement of the amino group with the hydroxide group. Thus the portion associated with the serum proteins would give rise to the radioactivity seen to migrate with the serum proteins.

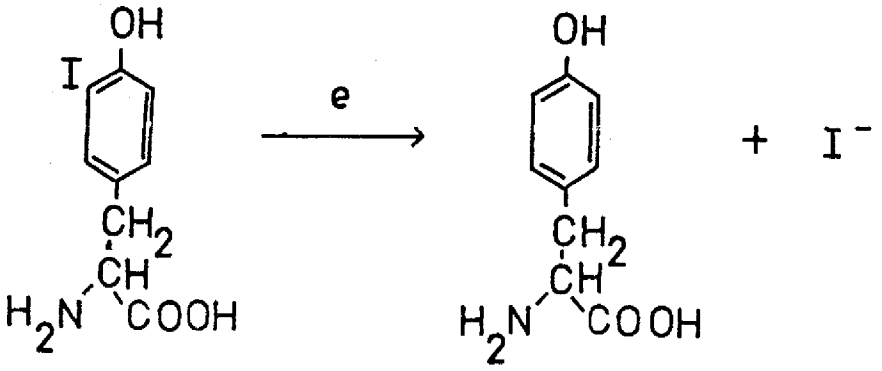
(ii) The substitution of the amino group with the hydroxide group would raise the pKa, and thereby reduce its affinity for the anion exchange resin used in this separation. Thus the portion removed from the serum proteins by the competitive binding of the resin would be eluted more readily than the triiodothyronine, and might appear with the diiodotyrosine fraction.

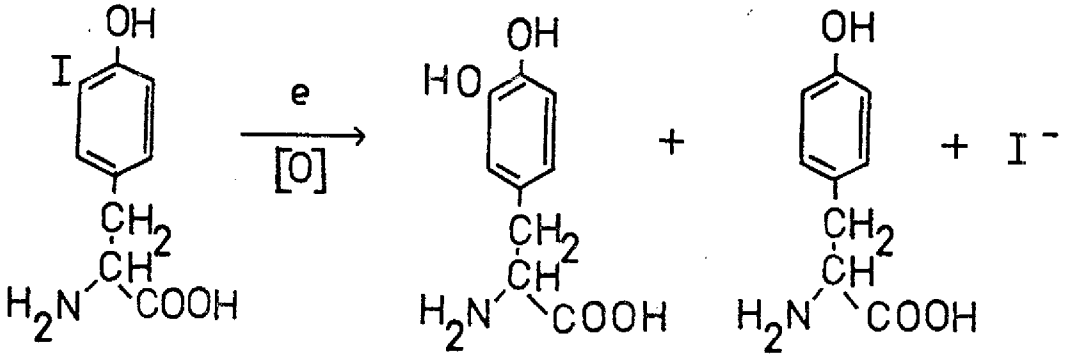
Jenkinson and Young[66] also observed that there was no increase in the amount of iodide present in triiodothyronine samples, while Tata[102] did not observe any diidotyrosine. Thus, the only agreement which is reached is that I^{131} labelled iodothyronines do decompose on storage.

Hoye[95] investigated the decomposition of iodothyronine as a function of specific activity and found a relatively stable period followed by a rapid decrease in the purity of the sample, and then by a second relatively stable period. In general, the greater the specific activity, the sooner the rapid decomposition took place and the greater the percentage decomposition. A preparation of thirty millicuries per milligram was subject to a 15% decomposition in one week, 23% in two weeks, and 26% in five weeks. (The normal specific activity supplied by the Radiochemical Centre is in the region of 20-40 mCi/mg.).

Much less work has been done on iodotyrosines. Liebster and Kopoldova[103] reported that the radiation-induced de-

composition of the iodotyrosines follows one of the two overall reaction pathways indicated below, depending on the presence or absence of oxygen.





Corresponding transformations occur for diiodotyrosine.

Precautions Against Decomposition. Bayly and Evans[100] recommend the following general precautions in storing any labelled compounds.

(i) Disperse the active molecules.

(ii) Reduce the storage temperature. (The Radiochemical Centre recommends storage of I^{131} labelled iodothyronines at 4°C in the dark.)

(iii) Scavenge reactive species such as radicals.

Jenkinson and Young[66] also investigated the instability of I^{125} labelled iodothyronines over an eight week period and found only 2-3% decomposition. This has also been observed by other research workers[90]. This is to be expected because of the lack of beta emission from I^{125}. I^{125} decays via a K capture of 0.11 MeV, forming a daughter product with a γ ray energy of 0.035 MeV, while the major decay route of I^{131} is via an 0.61 MeV β^- and an 0.36 MeV γ ray from the daughter product. Thus, for equivalent specific activities I^{131} delivers nearly seven times the radiation dose of I^{125}.

DEVELOPMENT OF THE SEPARATION TECHNIQUE

2.1 Introduction

The method chosen for the assay of the organic iodine compounds was thermal neutron activation analysis. This technique involves the bombardment of the sample with thermal neutrons. Neutron capture by iodine atoms in the sample produces the radioactive iodine I^{128} which can be identified by its half-life and the energy of the gamma ray emitted. This method of analysis is independent of the chemical form in which the iodine exists, so that it is necessary, when analysing for an individual iodine compound. to separate that compound from all other compounds containing iodine. This separation of the iodine compounds may, in principle, be done before or after the irradiation. The post irradiation treatment has the advantage that contamination by ion-irradiated impurities during the separation do not interfere with the final analysis. However, in 1934 Szilard and Chalmers showed that the carbon-iodine bond in ethyl iodide was broken when the I^{127} was transformed by neutron capture to I^{128}. Many similar reactions have since been observed and are known as Szilard-Chalmers reactions. Thus, after irradiation of the iodoamino acid, one cannot be

sure that the iodine is in the same chemical form; therefore, the separation procedure must precede the irradiation. As the irradiation is to be carried out after the separation, care must be taken to ensure that the materials used in the separation procedure do not contain iodine in any form, or any other element which, when irradiated, will produce radioactive species that could interfere with the iodine analysis. It is also necessary that the volumes of the fractions which contain the iodoamino acids be kept to a minimum, not only because of the limited capacity of the irradiation containers for the McMaster Nuclear Reactor, but also to minimize radiation produced from the oxygen in the water and from other background radioactivity dependent on the size of the irradiated sample. A consideration of the various separation procedures available (section 1.2) suggests that an ion exchange procedure is the most likely method which could be developed to meet the specific requirements of this analytical technique.

The general procedure which was adopted was to remove the inorganic iodide and, simultaneously, the sodium ions and chloride which, as they are present in considerable concentrations in serum, contribute by virtue of their Compton radiation, to the background of the gamma spectrum in the region of the iodine photopeak and reduce the analytical sensitivity. This was carried out using a mixed bed resin.

Preliminary experiments showed that iodothyronines, in the absence of serum proteins, when absorbed onto a mixed resin column could not be readily eluted. Thus, to avoid the loss of the iodothyronines it is essential that they remain bound to the serum proteins while the serum is passed over the mixed resin column which removes the iodotyrosines and the inorganic iodide. Thyroxine is bound to thyroxine-binding globulin, thyroxine-binding prealbumin, albumin and possibly the α- and β-lipoproteins[77]. The association constants for all these complexes are very high. Those for albumin are 110,000 and 11,000 for thyroxine and triiodothyronine respectively[85], while those for the other proteins are thought to be even higher[98], but are difficult to measure owing to the difficulty in obtaining pure protein preparations. Normal serum has a capacity of about 0.2 μg/ml so that, except possibly in cases of extreme thyrotoxicosis, the serum proteins are not saturated with iodothyronines. Robbins and Rall[82] suggest that saturation occurs at two to three times the normal concentrations. Thus, the actual concentration of free iodothyronines at any time is very low. However, the "free" iodothyronine and the protein-bound iodothyronine are in a dynamic equilibrium so that all the iodothyronines can be removed by passing the serum slowly over an ion exchange column. Therefore, the time for which the serum is in contact with the mixed resin must

be short compared with the time required to reestablish this equilibrium. Andreoli et al[98] reported that at room temperature thyroxine is completely dissociated from thyroxine-binding globulin and albumin in two minutes if the free thyroxine is continuously removed. Barnes[104] observed that the percentage uptake of radiothyroxine from serum by resin increased linearly with temperatures between 25° and 35°C, while Abbott Laboratories[105] find no difference in the distribution of of thyroxine between resin sponge and serum at temperatures up to 14°; but, at temperatures above this, they find a sharp increase in the proportion bound to the resin sponge. These facts would seem to suggest that the most successful operating temperature for the mixed resin column would be below 14°.

The iodotyrosines which are retained by the mixed resin column can be separated by elution at different pH's. Cationic exchange columns were used to separate the iodothyronines. To achieve this, at least one of the iodothyronines must be completely dissociated from the serum proteins and absorbed onto the cation exchange column. The reports mentioned above would seem to indicate that the best method for doing this would be to allow a longer time in contact with the ion exchange resin, and to use as high a column temperature as would be technically convenient, the evolution of gas on the resin column from the wash solution and the denaturation of the proteins being the limiting factors.

In the separation technique there are a number of variables: the temperature of the ion exchange columns, the mesh size of the ion exchange resins, the crosslinkages of the resins, the pH of elution of the individual iodoamino acids from the ion exchange columns, and the length of the ion exchange columns. The success of the separation is dependent upon the correct choice of each of these variables, so that a large number of preliminary experiments were needed to establish an approximate set of conditions for the separation before each variable could be systematically studied and optimized. This optimization was carried out using serum which was "doped" with the individual iodoamino acids labelled with I^{131} as a tracer. The amounts added were small in comparison with those naturally present and it is assumed that these additions did not seriously disturb the equilibria.

2.2 Materials and Counting Equipment

Ion Exchange Resins. All ion exchange resins used in this work were Bio-Rad Analytical Grade resins obtained from Bio-Rad Laboratories, California. The cation resins were obtained in the hydrogen form and the anion resins in the chloride form. In cases where other forms of the resins were required, batch conversions were carried out by percolating the appropriate dilute solution through a resin column until the conversion was complete. The excess reagent was then washed off with two column volumes of distilled water.

I^{131} Labelled Iodoamino Acids. The I^{131} labelled iodoamino acids were obtained from the Radiochemical Centre, Amersham, as solutions in 50% aqueous propylene glycol. The iodothyronines, 3,5,3'-triiodo-L-thyronine and 3,5,3'5'-L-thyroxine, labelled in the labile 3', and 3' and 5' positions respectively, had specific activities between 20 and 40 mCi/mg. The iodotyrosines, 3-iodo-L-tyrosine and 3,5-diiodo-L-tyrosine had specific activities between 6 and 8 mCi/mg. The results reported here were all obtained within one week to ten days of receipt of the radioactive samples. Excessive decomposition of the labelled compounds on storage was therefore avoided.

Eluting Reagents. The solutions used to elute the iodoamino acids from the ion exchange resins were prepared from acetic acid, ammonium acetate, and ammonium hydroxide. Where separations were being studied using I^{131}-labelled iodoamino acids, no special purity of the reagents was required; however, for neutron activation, it was essential to avoid introducing contamination prior to irradiation. It was found that the reagent grade ammonium acetate contained a small amount of iodide. Thus the eluting solutions where neutron activation analysis was to be employed were prepared entirely from acetic acid and ammonium hydroxide, both of which were found to be free of interfering contaminants by neutron activation analysis.

Electronic Counting Equipment. In the experiments using the I^{131}- labelled iodoamino acids a 2 inch Harshaw NaI(Tl) crystal coupled to a Nuclear Chicago model 8725 single channel analyzer was used to follow the elution of the radioactivity from the ion exchange columns. The gamma ray spectra of the fractionated samples following neutron activation were obtained using an 8 cc planar Lithium-drifted Germanium detector with a 2.54 keV resolution and a 10/1 peak/Compton ratio for the 661.6 keV Cs^{137} photopeak. This Ge(Li) detector was coupled, through a Canberra 1408C preamplifier and an Ortec 410 linear amplifier, to a Nuclear Data model 160 4096 channel analyzer.

Preparation of Ion Exchange Columns.

(a) Mixed Resin Columns. The cation exchange resin, Bio-Rad Ag 50W × 2 mesh 100-200, was converted to the ammonium form by washing with 0.2M ammonium hydroxide. Then the excess ammonium hydroxide was removed with a two column volume wash of distilled water. The anion exchange resin, Bio-Rad Ag 1 × 2 mesh 100-200, was converted to the acetate form by washing with 0.2M acetic acid until no further chloride in the effluent could be detected with silver nitrate. Then the excess acid was removed from the resin with two column volumes of distilled water. Equal weights of the cation and anion resins were mixed in molar ammonium acetate solution. This slurry was used to pack the resin

columns of 0.6 cm diameter to a height of 4 cm. These columns were enclosed in water jackets for temperature control.

(b) Cation Resin Columns. Bio-Rad Ag 50W ×2 mesh 200-400 was converted to the ammonium form as described above. Columns of 0.6 cm were packed to a height of 5 cm with a slurry of this resin in distilled water. These columns were also enclosed in water jackets for temperature control.

Preparation of Serum Samples for the Development of the Separation Procedure.

In experiments conducted to determine the set of optimum conditions for the separation of the iodoamino acids, I^{131}-labelled iodoamino acids were used as tracers. A 2 ml serum sample was treated with 1-2 drops of the solution of one of the I^{131}-labelled iodoamino acids and allowed to equilibrate for half an hour at 10°C.

2.3 Optimization of the Separation Procedure

After a number of preliminary experiments the following conditions were established as a starting point from which each of the separation variables could be individually optimized.

Mixed Resin Column: Mesh 100-200

Ammonium Acetate form

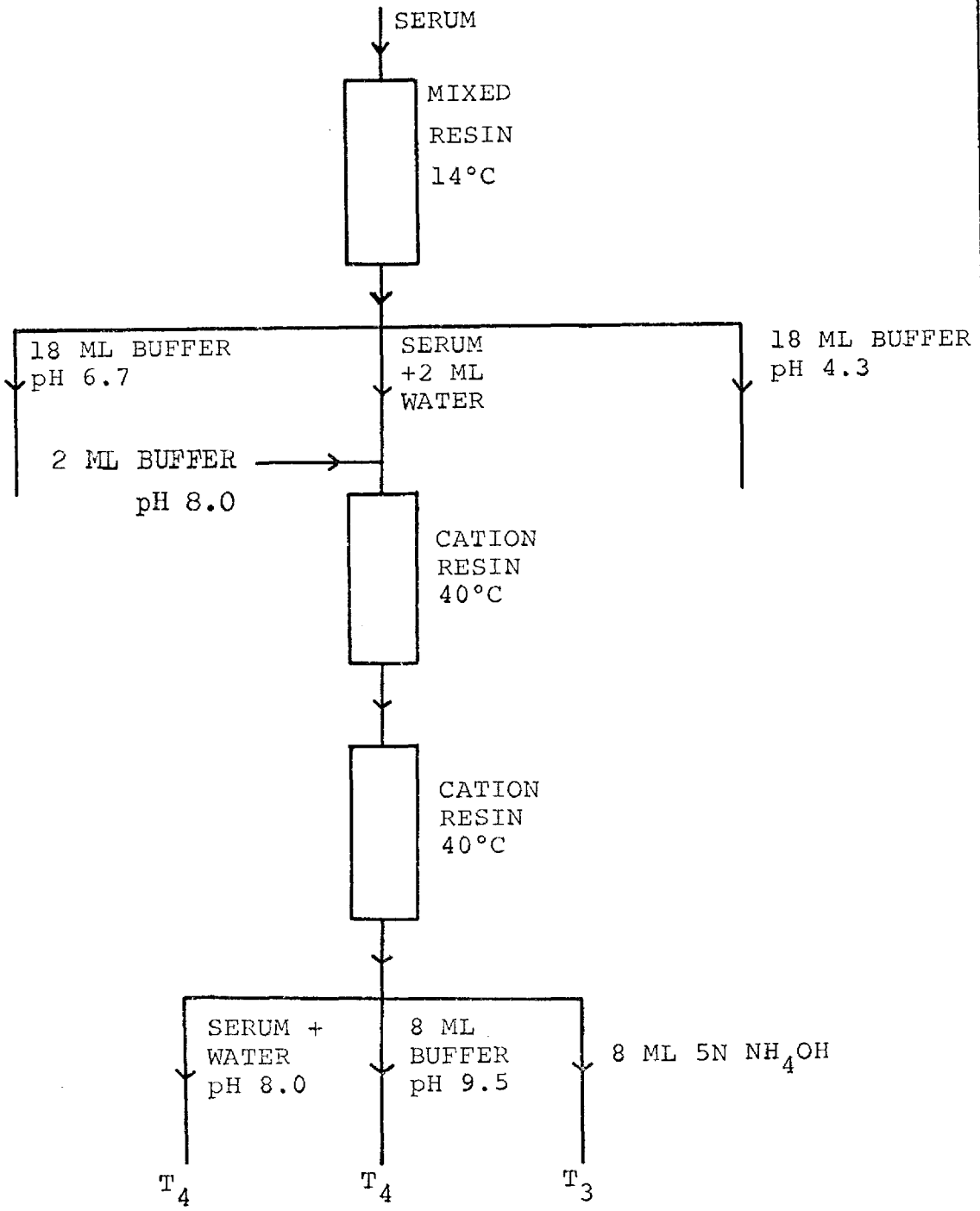
	Crosslinkage 2
	pH of elution of monoiodotyrosine 7.0
	pH of elution of diiodotyrosine 4.3
	Length of column 4 cm
Cation Resin Column:	Mesh 200-400
	Ammonium form
	Crosslinkage 2
	pH of elution of thyroxine 9.5
	Triiodothyronine eluted with 5M ammonium hydroxide
	Length of column 5 cm

Optimum Temperature for the Mixed Resin Column

The effect of the temperature on the elution efficiency from the mixed resin columns was studied using columns maintained at 40°, 20°, and 0°C. The column temperatures were controlled by passing water of the appropriate temperature through the water jacket surrounding the ion exchange column. The serum samples were prepared as described previously. The results obtained appear in Table 1. These results indicate that it is necessary to cool the mixed resin columns to get good recoveries of thyroxine and triiodothyronine with the serum proteins. As suggested by Abbott Laboratories[105], it appears that the recoveries of thyroxine and

Figure 1

FLOW DIAGRAM



triiodothyronine at 14°C are just as good as at 0°C. It will also be observed that the radioactivity of the monoiodotyrosine is eluted more rapidly at 14° and 0° than at 20°. However, this is of little importance, as it may be controlled by varying the pH of the eluting solution. Thus the temperature chosen for the mixed resin column was between 10° and 14°C.

Optimum pH for the Separation of Monoidothyrosine and Diiodotyrosine on a Mixed Resin Column.

To elute an iodoamino acid from a mixed resin column, it would appear the the most likely pH would be the one close to the isoelectric point. Dalton et al[106] and Winnek et al[107] both obtained a value of 4.29 for the isoelectric pH of diiodotyrosine with individual pK's of 2.12, 6.48 and 7.82. Winnek et al[107] also reported a value of 5.63 for the isolectric pH of tyrosine, the pK's being 2.20, 9.11 and 10.07. Thus, the isolectric pH of monoiodotyrosine should be between 4.29 and 5.63. As the resin column and the serum samples are both of approximately neutral pH, the monoiodotyrosine should be eluted before the diiodotyrosine. Preliminary experiments showed, that while monoiodotyrosine was eluted more rapidly than diiodotyrosine at pH 5.6, a good separation was not obtained, so higher pH's, 6.5, 6.7 and 7.0, were studied. The serum samples were prepared as previously described, absorbed onto mixed resin columns

Table 1

The effect of temperature on the elution of Iodoamino Acids from mixed resin columns

Temperature	Percentage of Radioactivity Eluted 0°C	14°C	20°C	40°C
(i) Monoiodotyrosine				
Eluted volume Sample + 2 ml water	2.7	3.0	2.6	
Ammonium Acetate pH7.0				
1st 6 ml.	32.3	43.5	9.4	
2nd 6 ml.	37.1	23.6	37.7	
3rd 6 ml.	5.3	6.4	22.0	
4th 6 ml.	1.2	1.2	5.5	
	78.5	77.7	77.2	
(ii) Diiodotyrosine				
Eluted volume Sample + 2 ml water	1.9	1.1	1.0	
Ammonium Acetate pH7.0				
1st 6 ml.	2.0	1.2	1.0	
2nd 6 ml.	2.8	1.2	1.7	
3rd 6 ml.	1.2	1.6	1.5	
4th 6 ml.	1.2	1.3	1.1	
	9.1	6.4	6.3	
(iii) Triiodothyronine				
Eluted volume Sample + 2ml water	84	81	51	31
(iv) Thyroxine				
Eluted volume Sample + 2 ml water	92	92	77	56

maintained between 10° and 14° and eluted with solutions of 0.2M ammonium acetate adjusted to the required pH with ammonium hydroxide. The results obtained are recorded in Table 2. These results show that at pH 6.5 and pH 6.7, 18 mls of eluting solution were sufficient to give recoveries of 84% and 91% of the monoiodotyrosine respectively, and 14% and 9% of the diodotyrosine. At pH 7.0, the elution of monoiodotyrosine showed considerably more tailing, and even after 24 mls of eluant only 79% had been eluted. Thus it appears that pH 6.7 is the best for separating the iodotyrosine. Under these conditions, 9% of the diiodotyrosine radioactivity was recovered in the monoiodotyrosine fraction. However, this is probably not all diiodotyrosine, as radiation-induced decomposition yields monoiodotyrosine and inorganic iodide[103]. Tata[108] observed that when I^{131}-labelled iodotyrosines were dissolved in aqueous solution, they lost some of their radioactivity. This could possibly be another dissociation process, possibly yielding monoiodotyrosine from diiodotyrosine, and this then would account for some of the 9% of the diiodotyrosine radioactivity eluted in the monoiodotyrosine fraction.

Recovery of Diiodotyrosine from the Mixed Resin

In the preliminary experiments, it was observed that if an eluting solution of ionic strength higher than 0.2M was used, the recovery of any iodoamino acid left on the

Table 2
The effect of pH on the separation of
Moniodotyrosine and Diiodotyrosine

	Moniodotyrosine		Diiodotyrosine	
	Radioactivity c/m	% of Total Radioactivity	Radioactivity c/m	% of Total Radioactivity
(i) pH 6.5				
Radioactivity added	14204		17418	
Eluted volume sample + 2 ml water	491	3.5	129	0.7
Ammonium Acetate pH 6.5				
1st 6 ml	7169	50.5	276	1.6
2nd 6 ml	3736	26.3	596	3.5
3rd 6 ml	496	3.5	1433	8.2
4th 6 ml	82	0.6	1135	6.5
		80.9		19.8
(ii) pH 6.7				
Radioactivity added	12572		12847	
Eluted volume sample + 2 ml water	366	2.9	93	0.8
Ammonium Acetate pH 6.7				
1st 6 ml	6302	50.1	164	1.3
2nd 6 ml	4382	34.8	383	3.0
3rd 6 ml	387	3.1	510	4.0
4th 6 ml	78	0.7	586	4.6
		88.7		12.9

	Moniodotyrosine		Diiodotyrosine	
	Radioactivity c/m	% of Total Radioactivity	Radioactivity c/m	% of Total Radioactivity
(iii) pH 7.0				
Radioactivity added	13116		14404	
Eluted volume sample + 2 ml water	595	4.5	156	1.1
Ammonium Hydroxide pH7.0				
1st 6 ml	5715	43.5	172	1.2
2nd 6 ml	3095	23.6	161	1.2
3rd 6 ml	833	6.4	226	1.6
4th 6 ml	154	1.2	186	1.3
		74.7		5.3

column after that solution had been used was considerably decreased, although the higher ionic strength did not appear to reduce the recovery of the iodoamino acid that it was eluting. Thus, 0.2M solutions must be used to elute the monoiodotyrosine, however, diiodotyrosine may be eluted with a more concentrated solution if it is found to be desirable. The decrease in the recovery of the iodoamino acid held on the resin during the elution with more concentrated solutions is probably due to the shrinkage of the resin, causing entrapment of the iodoamino acid inside the resin bead. The failure of this entrapment to occur with the iodoamino acid being eluted is probably due to the fact that it is eluted from the resin bead before it shrinks. In cases where higher ionic concentrations are used, the elution peak occurs in the first fraction collected, while a certain amount of time is necessary for the higher ionic concentration to diffuse into the resin bead and cause it to shrink. Thus, shrinkage probably does not occur until after the iodoamino acid has been eluted.

For the elution of diiodotyrosine from the mixed resin, 0.2M and 1.0M solutions of acetic acid adjusted to pH 4.3 with ammonium acetate were used. The results obtained are recorded in Table 3. From these results, it is seen that in the first 18 mls of pH 4.3 solution, there is a 4% difference in the percentages of diiodotyrosine recovered

Table 3

The Recovery of Diiodotyrosine from the Mixed Resin Column

	Radioactivity c/m	% of Total Radioactivity
(i) 0.2 Molar Acetic Acid		
Adjusted to pH 4.3 Radioactivity added	20196	
Eluted volume Sample + 2 ml water	182	0.9
18 ml Ammonium Acetate PH 6.7	1772	8.5
Acetic Acid 0.2 M pH 4.3		
1st 6 ml	7969	39.5
2nd 6 ml	4505	22.4
3rd 6 ml	2161	10.7
		72.6
(ii) Molar Acetic Acid		
Ajusted to pH 4.3 Radioactivity added	25204	
Eluted volume Sample + 2 ml water	202	0.8
18 ml Ammonium Acetate pH 6.7	2110	8.4
Acetic Acid M pH 4.3		
1st 6 ml	12897	51.2
2nd 6 ml	5202	20.6
3rd 6 ml	1273	5.0
		76.8

but with molar solutions, the peak occurs much earlier in the elution than with the 0.2M eluting reagent. In both cases, about 10% more radioactivity could be recovered by continued elution, but a large volume of eluant was required. This tailing occurs because of the zwitterion nature of the iodoamino acid at the isoelectric point.

Optimum Length for the Mixed Resin Column

It was previously observed that it appears that a complete separation of the iodotyrosines was not obtained on a 4 cm column of mixed resin. The effect of lengthening the column was studied, not only with respect to improving the resolution of the iodotyrosines, but also with respect to the absorption losses of the iodothyronines. Samples, prepared as described previously, were passed through cooled mixed resin columns of 4 cm and 5 cm lengths. The results obtained are recorded in Table 4. By increasing the column length, the separation of the iodotyrosines is improved, but the recovery of the triiodothyronine dropped from about 84% to 77%, and that of the thyroxine from 93% to 89%. This trend clearly indicates that if the mixed resin column is lengthened sufficiently to obtain a complete resolution of the iodotyrosines, a fairly large percentage of the iodothyronines, especially the triiodothyronine, will be adsorbed on the mixed resin. Thus, for the routine separation procedure, a 4 cm column was chosen.

Table 4

The effect of column length on the elution of Iodoamino Acids from mixed resin columns

Length of Columns	4 cm		5 cm	
	Radioactivity c/m	% of Total Radioactivity	Radioactivity c/m	% of Total Radioactivity
(i) Monoiodotyrosine				
Radioactivity added	12572		26378	
Eluted volume Sample + 2 ml water	366	2.9	540	2.0
Ammonium Acetate pH 6.7				
1st 6 ml	6302	50.1	5478	20.8
2nd 6 ml	4382	34.8	12468	47.2
3rd 6 ml	387	3.1	5071	19.2
4th 6 ml	78	0.7	1344	5.1
		88.7		92.3
Acetic Acid 0.2M pH4.3				
1st 6 ml	400	3.2	244	0.9
2nd 6 ml	165	1.3	309	1.2
3rd 6 ml	47	0.3	268	1.0
4th 6 ml			250	0.9
		4.8		4.0

	4 cm		5 cm	
	Radioactivity c/m	% of Total Radioactivity	Radioactivity c/m	% of Total Radioactivity
(ii) Diiodotyrosine				
Radioactivity added	25204		26905	
Eluted volume Sample + 2 ml water	202	0.8	1009	3.7
Ammonium Acetate pH 6.7				
1st 6 ml	353	1.4	299	1.1
2nd 6 ml	731	2.9	383	1.4
3rd 6 ml	1032	4.1	463	1.7
4th 6 ml			821	3.1
		8.4		7.3
Acetic Acid 0.2M pH4.3				
1st 6 ml	12897	51.2	2371	8.8
2nd 6 ml	5202	20.6	8072	30.0
3rd 6 ml	1273	5.0	5837	21.6
4th 6 ml			3277	12.2
		76.8		72.6
(iii) Triiodothyronine				
Radioactivity added	8784		10433	
Eluted volume Sample + 2 ml water	7034	84.3	7937	77
(iv) Thyroxine				
Radioactivity added	12066		8078	
Eluted volume Sample + 2 ml water	11284	93.5	7150	89

Optimum Crosslinkage of the Mixed Resin

The "crosslinkage" is the percentage of divinylbenzene incorporated into the polymer beads prior to the attachment of the ionic groups.

Resins of crosslinkages 2 and 4 were investigated. Preliminary experiments with the mixed resin of crosslinkage 4, mesh 100-200, showed that the optimum pH for the separation of monoiodotyrosine from diiodotyrosine was 6.3 rather than 6.7 when using this crosslinkage. From the results recorded in Table 5 it will be observed that there is not much difference between the two crosslinkages with respect to the separation of monoiodotyrosine from diiodotyrosine. The real distinction between the efficiencies of the two crosslinkages is shown in the elution of the diiodotyrosine. The resin column with the crosslinkage of 4 shows a very wide elution band for the diiodotyrosine and even after elution with 24 ml of acetic acid solution only 70% has been recovered, whereas, with a crosslinkage of 2, 84% is eluted with 18 ml of acetic acid solution. These clearly indicate that a mixed resin column of crosslinkage 2 is more suitable for the separation and recovery of iodotyrosines.

Optimum Mesh of the Mixed Resin

The results recorded in Table 6 show that with a resin of mesh 200-400 the diiodotyrosine was being eluted too early. To try to overcome this, a solution of higher pH

Table 5

The effect of resin crosslinkage on the separation of iodotyrosine

	Monoiodotyrosine		Diiodotyrosine	
	Radioactivity c/m	% of Total Radioactivity	Radioactivity c/m	% of Total Radioactivity
(i) Crosslinkage 4				
Radioactivity added	298262		317372	
Eluted volume Sample + 2 ml water	1702	0.6	860	0.3
Ammonium Acetate pH 6.3				
1st 6 ml	64686	21.6	3637	1.1
2nd 6 ml	115786	38.7	6235	2.0
3rd 6 ml	58006	19.3	4212	1.3
		79.6		4.4
Acetic Acid pH 4.3				
1st 6 ml			47090	14.6
2nd 6 ml			82173	25.7
3rd 6 ml			57561	18.0
4th 6 ml			33802	10.7
				69.0
(ii) Crosslinkage 2				
Radioactivity added	28876		25204	
Eluted volume Sample + 2 ml water	540	1.9	449	1.8
Ammonium Acetate pH 6.7				
1st 6 ml	10480	36.1	299	1.2
2nd 6 ml	11477	39.6	842	3.3
3rd 6 ml	2404	8.3	821	3.3
		84.0		7.8
Acetate Acid pH 4.3				
1st 6 ml			10628	42.2
2nd 6 ml			8138	32.4
3rd 6 ml			2333	9.3
				83.9

Table 6

The effect of the mesh of the resin on the separation of the Iodotyrosines on mixed resin columns

	Monoiodotyrosine		Diiodotyrosine	
	Radioactivity c/m	% of Total Radioactivity	Radioactivity c/m	% of Total Radioactivity
(i) Mesh 200-400				
Radioactivity added	14450		13036	
Eluted volume				
Sample + 2 ml water	533	3.7	314	2.4
Ammonium Acetate pH 6.7				
1st 6 ml	7573	52.3	585	4.5
2nd 6 ml	3351	23.2	1671	12.8
		75.5		17.3
(ii) Mesh 100-200				
Radioactivity added	12572		12847	
Eluted volume				
Sample + 2 ml water	366	2.9	93	0.8
Ammonium Acetate pH 6.7				
1st 6 ml	6302	50.1	164	1.3
2nd 6 ml	4382	34.8	383	3.0
3rd 6 ml	387	3.1	510	4.0
		88.0		8.3

was tried. The elution of the iodotyrosines at pH 7.0 from a 200-400 mesh mixed resin column is shown in Table 7. While the percentage of diiodotyrosine was decreased, the yield of monoiodotyrosine was only 74%. Thus it is seen that a better resolution was obtained using a mixed resin of mesh 100-200 and an eluting solution of pH 6.7.

Optimum Dilution of Sample Before Being Passed Over the Cation Exchange Resin

Preliminary experiments showed that a better separation of the iodothyronines was obtained if the 2 ml water wash, used to remove the proteins from the mixed resin column, was mixed with the serum sample before it was passed over the cation exchange resin. This raised the question of whether further dilution would help and, if so, what would be the best diluent. Serum samples were prepared as described previously and mixed with various quantities of water and aqueous solutions of ammonium hydroxide and ammonium acetate. The results obtained are recorded in Table 8. From these results it may seem that as the serum samples are diluted, the percentage of triiodothyronine eluted with the thyroxine decreases to about 20% with a 4 ml water dilution; however, the percentage of thyroxine recovered dropped to 63% from 89%. To try to increase the amount of thyroxine recovered without increasing the amount of triiodothyronine, three 0.2M solutions of pH 7.0, 8.0 and 9.0, composed of ammonium acetate

Table 7

The separation of Iodotyrosines on mixed resin mesh 200-400 at PH 7.0

	Monoiodotyrosine		Diiodyrosine	
	Radioactivity c/m	% of Total Radioactivity	Radioactivity c/m	% of Total Radioactivity
Radioactivity added	14730		15008	
Eluted volume				
Sample + 2 ml water	358	2.4	212	1.4
Ammonium Acetate pH 7.0				
1st 6 ml	6322	42.9	391	2.6
2nd 6 ml	4271	29.0	345	2.3
3rd 6 ml	325	2.2	603	4.0
		74.1		8.9

Table 8
The effect of dilution of the serum sample
on the separation of the Iodothyronine

	Thyroxine		Triiodothyronine	
	Radioactivity c/m	% of Total Radioactivity	Radioactivity c/m	% of Total Radioactivity
(i) No dilution				
Radioactivity added	83496		76778	
Eluted volume				
Sample	42968	51.5	27054	35.0
Ammonium Hydroxide pH 9.1				
1st 2 ml	15414	18.5	15071	19.5
2nd 2 ml	8929	10.7	10674	13.8
3rd 2 ml	4747	5.7	3434	4.5
4th 2 ml	1979	2.4	1513	3.0
		88.8		74.8
(ii) 2 ml water dilution				
Radioactivity added	81299		74831	
Eluted volume				
Sample	30898	37.9	15634	20.8
Ammonium Hydroxide pH 9.1				
1st 2 ml	15774	19.5	7394	9.9
2nd 2 ml	10322	12.7	3816	5.1
3rd 2 ml	2176	2.7	2133	2.8
4th 2 ml	1529	1.9	1444	1.9
		74.7		40.5

	Thyroxine		Triiodothyronine	
	Radioactivity c/m	% of Total Radioactivity	Radioactivity c/m	% of Total Radioactivity
(iii) 4 ml water dilution				
Radioactivity added	61430		37600	
Eluted volume				
Sample	21746	35.4	5371	14.2
Ammonium Hydroxide pH 9.1				
1st 2 ml	4046	6.6	774	2.0
2nd 2 ml	7488	12.2	767	2.0
3rd 2 ml	4084	6.7	661	1.7
4th 2 ml	1356	2.2	322	0.9
		63.1		20.8
(iv) 2 ml water + 2 ml pH 7.0 dilution				
Radioactivity added	65634		31606	
Eluted volume				
Sample	32310	49.3	8817	27.9
Ammonium Hydroxide pH 9.1				
1st 2 ml	11114	17.0	3361	10.6
2nd 2 ml	1389	2.1	268	0.8
3rd 2 ml	4013	6.1	678	2.1
4th 2 ml	2041	3.1	538	1.7
		77.6		43.1
(v) 2 ml water + 2 ml pH 8.0 dilution				
Radioactivity added	82102		75141	
Eluted volume				
Sample	19130	23.3	7496	10.0
Ammonium Hydroxide pH 9.1				
1st 2 ml	18308	22.3	3509	4.6
2nd 2 ml	13013	15.9	2880	4.0
3rd 2 ml	7373	9.0	2636	3.5
4th 2 ml	1940	2.4	1831	2.5
		72.9		23.2

	Thyroxine		Triiodothyronine	
	Radioactivity c/m	% of Total Radioactivity	Radioactivity c/m	% of Total Radioactivity
(vi) 2 ml water + 2 ml pH 9.0 dilution				
Radioactivity added	66152		30623	
Eluted volume				
Sample	43827	66.3	11093	36.3
Ammonium Hydroxide pH 9.1				
1st 2 ml	11875	18.0	6460	21.1
2nd 2 ml	2008	3.4	992	3.2
3rd 2 ml	952	1.4	505	1.7
4th 2 ml	626	0.9	384	1.3
		90.0		63.6
(vii) 2 ml water + 4 ml pH 8.0 dilution				
Radioactivity added	80847		75610	
Eluted volume				
Sample	4947	6.1	2295	3.0
Ammonium Hydroxide pH 9.1				
1st 2 ml	26826	33.1	11063	14.7
2nd 2 ml	12993	16.2	2526	3.3
3rd 2 ml	8882	10.9	2708	3.6
4th 2 ml	1833	2.3	810	1.1
		68.6		25.7

and ammonium hydroxide, were used in place of the second 2 ml of water. The use of pH 7.0 and pH 9.0 caused the amount of triiodothyronine eluted to be increased to 43% and 63% respectively. However, when the solution of pH 8.0 was used, a 73% recovery of thyroxine was obtained with only a 23% recovery of triiodothyronine. Part (vii) of the table shows the effect of increasing the dilution to 2 ml of water and 4 ml of the pH 8.0 solution. Although there appears to have been a slight deterioration in the separation,this is within the statistical counting error of the two experiments. However, it does indicate that further dilution of the sample does not improve the separation.

If the ionic strength of the diluent is raised from zero to decimolar, the amount of unfolding of the protein chain will be decreased and, as shown by part (iv) of Table 8 the amount of iodothyronine retained by the resin column is decreased. Parts (v) and (vi) of Table 8 show the effect of increasing the pH of the diluent. As increasing the pH would increase the amount of unfolding of the protein chains, an increase in the amount of the iodothyronines retained by the resin would be expected. However, this must be coupled with the fact that thyroxine is eluted from the resin at pH 9.1. Thus, when a diluent of pH 9.0 is used, the thyroxine should remain with the serum, while the triiodothyronine will

be retained by the ion exchange resin. Unfortunately, this was not the case, as it appears that the affinity of the resin for triiodothyronine is not sufficiently greater than that of the serum proteins at this pH. Thus, a dilution of 2 ml of a solution of pH 8.0 and 2 ml of water (from the mixed resin column wash) was used, giving a recovery of 73% of the thyroxine with only 23% of the triiodothyronine at room temperature.

Optimum Temperature for the Cation Exchange Column

The effect of the temperature on the separation efficiency of the cation columns was studied using columns maintained at 14°, 20° and 40°C. The serum samples, pre-equilibrated with one of the iodothyronines, were first passed over a mixed resin column at 14° and the radioactivity counted before being passed through a cation column. The results obtained are recorded in Table 9. In this experiment, an 0.2M ammonium hydroxide solution adjusted to pH 9.5 rather than pH 9.1, was used to elute the thyroxine. From these results it can be seen that higher temperatures give better separations. However, even at 40°, about 36% of the triiodothyronine remains with the thyroxine fraction, principally with the serum proteins. Higher temperatures could not be studied, as the serum tended to coagulate on the column, causing it to become blocked.

Table 9

The effect of temperature on the elution of Iodothyronines from cation columns

Temperature	Percentage of Radioactivity Eluted 14°C	20°C	40°C
(i) Triiodothyronine			
Eluted volume Initial 4 ml	32.4	38.0	21.4
Ammonium Hydroxide pH 9.5			
1st 4 ml.	20.2	9.7	8.0
2nd 4 ml.	10.5	3.8	7.1
	63.1	51.5	36.5
(ii) Thyroxine			
Eluted volume Initial 4 ml.	63.8	63.3	80.6
Ammonium Hydroxide 9.5			
1st 4 ml.	35.6	28.8	13.2
2nd 4 ml.	4.5	6.4	1.7
	103.9	98.5	95.5

Thus, to obtain a better resolution of the iodothyronines it appears to be necessary for the serum proteins to be in contact with the resin for a longer period of time so as to complete the removal of triiodothyronine from the serum proteins. This could be achieved by one of two methods:

(i) a slower flow rate, As the flow rate is already very slow the accurate control of a slower flow rate proved to be difficult;

(ii) a longer column. Since the optimum column length is dependent on the pH of the eluting solution, the effect of the pH on the elution of the iodoamino acids will be considered prior to the optimization of the column lengths.

Optimum pH for the Separation of Triiodothyronine and Thyroxine on the Cation Exchange Column

The efficiency of the separation of the thyroxine from the triiodothyronine on the cation resin columns was studied using solutions of various hydrogen ion concentrations. the serum samples were prepared as described previously and passed over a mixed resin column, together with a column wash of 2 ml of water. These samples were further diluted with 2 ml of 0.2M ammonium acetate adjusted to pH 8.0 with ammonium hydroxide. The eluting solutions were 0.2M ammonium hydroxide adjusted to the required pH with ammonium acetate. The triiodothyronine was later eluted with 5M ammonium hydroxide.

The results obtained are recorded in Table 10. From these results it is clear that, under these conditions, the best separation is obtained using a pH 9.5 solution. An eluting solution of lower pH, i.e. 9.3, does not give a good recovery of thyroxine, while a solution of higher pH, i.e. 9.6, elutes much more triiodothyronine. However, the separation obtained of 95% of the thyroxine and 37% of the triiodothyronine using the pH 9.5 solution is not satisfactory and further improvement must be obtained by either lengthening the column or using a second cation exchange column.

Optimum Length of the Cation Column

A series of cation columns of lengths 5, 6, 7 and 8 cm were prepared. The serum samples were prepared as described previously, passed through mixed resin columns, combined with their 2 ml water washes from the mixed resin columns, and further diluted with 2 ml of pH 8.0 solution before being passed through the cation column. The results are recorded in Table 11. From these results it may be seen that nothing is gained by increasing the length of the cation column. The percentage of triiodothyronine eluted with the thyroxine remains virtually the same, and there is a small drop in the percentage of thyroxine recovered. This immediately raises the question of whether possibly the 35% of the triiodothyronine radioactivity may be actually thyroxine

Table 10

The effect of the pH on the separation of Thyroxine and Triiodothyronine

	Thyroxine		Triiodothyronine	
	Radioactivity c/m	% of Total Radioactivity	Radioactivity c/m	% of Total Radioactivity
(i) pH 9.3				
Radioactivity added	10537		10344	
Eluted volume				
Initial 6 ml	7451	70.5	2252	21.8
Ammonium Hydroxide pH 9.3				
1st 4 ml	591	5.6	498	4.8
2nd 4 ml	34	0.3	188	1.8
		75.9		28.4
4 M Ammonium Hydroxide				
1st 4 ml	2180	20.6	6814	65.9
2nd 4 ml	62	0.6	51	0.5
		21.2		66.4
(ii) pH 9.5				
Radioactivity added	11284		7034	
Eluted volume				
Initial 6 ml	8334	74.0	1498	21.3
Ammonium Hydroxide pH 9.5				
1st 4 ml	2314	20.3	660	9.4
2nd 4 ml	138	1.2	485	6.9
		95.5		37.6
5 M Ammonium Hydroxide				
1st 4 ml	256	2.3	4224	60.1
2nd 4 ml	100	0.9	41	0.6
		3.2		60.7

		Thyroxine		Triiodothyronine	
		Radioactivity c/m	% of Total Radioactivity	Radioactivity c/m	% of Total Radioactivity
(iii)	pH 9.6				
	Radioactivity added	12892		10424	
	Eluted volume				
	Initial 6 ml	10079	78.0	2733	25.9
	Ammonium Hydroxide pH 9.6				
	1st 4 ml	1856	14.4	1730	16.6
	2nd 4 ml	383	3.0	922	8.8
			95.4		51.3
	5M Ammonium Hydroxide				
	1st 4 ml	180	1.4	4457	42.7
	2nd 4 ml	123	1.0	29	0.3
			2.4		43.0

Table 11

The effect of the length of the cation column on the separation of the Iodothyronines

	Thyroxine		Triiodothyronine	
	Radioactivity c/m	% of Total Radioactivity	Radioactivity c/m	% of Total Radioactivity
(i) 5 cm column				
Radioactivity	11284		7034	
Eluted volume				
Initial 6 ml	8334	74.0	1498	21.3
Ammonium Hydroxide pH 9.5				
1st 4 ml	2314	20.3	660	9.4
2nd 4 ml	138	1.2	485	6.9
		95.5		37.6
(ii) 6 cm column				
Radioactivity added	13457		9596	
Eluted volume				
Initial 6 ml	6122	45.5	1786	18.5
Ammonium Hydroxide pH 9.5				
1st 4 ml	4533	35.5	868	9.0
2nd 4 ml	1438	10.7	627	6.0
		91.7		33.5

	Thyroxine		Triiodothyronine	
	Radioactivity c/m	% of Total Radioactivity	Radioactivity c/m	% of Total Radioactivity
(iii) 7 cm column				
Radioactivity added	7210		8856	
Eluted volume				
Initial 6 ml	4873	66.7	2086	23.5
Ammonium Hydroxide pH 9.5				
1st 4 ml	1083	15.1	670	7.5
2nd 4 ml	274	3.8	332	3.7
		85.6		33.8
(iv) 8 cm column				
Radioactivity added	18524		13286	
Eluted volume				
Initial 6 ml	12651	68.5	1736	13.0
Ammonium Hydroxide pH 9.5				
1st 4 ml	2813	15.2	2669	20.0
2nd 4 ml	312	1.7	656	4.9
		85.4		37.9

formed by iodine exchange during the half hour incubation of the serum and tracer. To test this hypothesis it was decided to pass the three fractions of effluent, the initial 6 ml, the pH 9.5 and the 5M ammonium hydroxide fractions, from the first column, consecutively over two more identical columns. All three columns were 5 cm in length. The results obtained are shown in Table 12. The hypothesis that the 35% of the radioactivity of the triiodothyronine eluted with the thyroxine from the first cation column is actually thyroxine is clearly disproved by these results. They show that a constant percentage ie.about 35% of triiodothyronine applied to the top of the column with the thyroxine is eluted with the thyroxine. As increasing the length of the column and consequently increasing the time for which the serum proteins are in contact with the resin does not decrease the percentage of triiodothyronine eluted with the thyroxine, the reason for this improvement in the separation efficiency using consecutive columns must be related to the time between the protein's being eluted from one resin column and being absorbed onto the next. Such a situation could occur if some, but not all, of the dissociation rates for the protein-triiodothyronine complexes were high as compared to the time for which the protein was in contact with the resin. The other dissociation rates must be relatively low, so that virtually no dissociation of these complexes takes

Table 12

The effect of consecutive columns on the separation of Iodothyronines

	Throxine		Triiodothyronine	
	Radioactivity c/m	% of Total Radioactivity	Radioactivity c/m	% of Total Radioactivity
Radioactivity added	11284		7034	
(i) 1st column				
Eluted volume Initial 6 ml	10334	91.5	1498	21.3
Ammonium Hydroxide pH 9.5				
1st 4 ml	314	2.8	660	9.4
2nd 4 ml	138	1.2	485	6.9
		95.5		32.6
5m Ammonium Hydroxide				
1st 4 ml	256	2.3	4224	60.1
2nd 4 ml	100	0.9	41	0.6
		3.2		60.7
(ii) 2nd column				
Eluted volume Initial 6 ml	8969	77.0	624	8.9
Ammonium Hydroxide pH 9.5				
1st 4 ml	1693	15.0	158	2.2
2nd 4 ml	164	1.4	74	1.1
		93.4		12.2
5m Ammonium Hydroxide				
1st 4 ml	426	3.8	5756	82.0
2nd 4 ml	121	1.1	564	8.0
		4.9		90.0

		Thyroxine		Triiodothyronine	
		Radioactivity c/m	% of Total Radioactivity	Radioactivity c/m	% of Total Radioactivity
(iii) 3rd column					
Eluted volume					
Initial 6 ml		7299	64.8	260	3.7
Ammonium Hydroxide	9.5				
1st 4 ml		2637	23.4	77	1.1
2nd 4 ml		202	1.8	22	0.3
			90.0		5.1
5m Ammonium Hydroxide					
1st 4 ml		777	6.9	5749	84.6
2nd 4 ml		146	1.3	865	12.3
			8.2		96.9

place while the protein is on the resin. For the percentages to be reproducible by passing the protein over consecutive columns, the dissociation must be fast enough to allow the equilibrium distribution between the serum proteins to be reestablished before the serum is passed over the next cation column. In the preceding experiment the protein portion, serum, 2 ml of water and 2 ml of pH 8.0 solution, was applied to the top of the second column as soon as it had been completely eluted from the first column. Although it took about 20 minutes to pass the 6 ml of solution through the 5 cm resin column, any particular portion was actually in contact with the resin for a very short time, as the void volume of the resin bed was less than 0.1 ml.

The separation efficiency of two consecutive cation columns for the iodothyronines was studied using three different time delays between elution from one column and adsorption on the next. In case (i), the two columns were connected so that a volume of about 0.5 ml of solution existed between the two resin beds; in case (ii), the 6 ml sample was collected from the first column and then applied to the second column; in case (iii) the sample was allowed to stand for 15 minutes after being completely eluted from the first column before being applied to the second column. In all cases the wash solution, pH 9.5, was passed through immediately after the serum. The results obtained are shown

in Table 13. From these results it can be seen that even the time delay due to an 0.5 ml column head on the second column is sufficient to reestablish the equilibria. Thus the columns may be connected directly, one below the other.

Optimum Resin Crosslinkage for the Separation of Iodothyronines

The resin used to separate the iodothyronines was Bio-Rad AG 50W ×2 mesh 200-400. An attempt was made to modify the separation of the iodothyronines and a cross-linkage of 4 used. However, it was found to be very difficult to elute the iodothyronines from a resin of this cross-linkage. A 5 cm column was eluted with 40 ml of an ammonium hydroxide solution 0.5M pH 11.0, and only 54% of the thyroxide and 40% of the triiodothyronine was recovered. In this elution there was no peak just a general smear of radioactivity. More dilute solutions, i.e. 0.2M ammonium hydroxide, gave similar results. If the pH was lowered to 9.5, no triiodothyronine was eluted, but the thyroxine eluted only very slowly. Thus, the resin of crosslinkage 4 was not used for the separation of iodothyronines. The explanation of this phenomenon may lie in the fact that the higher percentage of divinylbenzene in the resin polymer gives a more closely interlocked polymer network into which the iodothyronine molecule may diffuse. However, because of its elongated shape, it may become "trapped", thus causing the iodothyronines to be

Table 13

The effect on the separation of Iodothyronines of the time delay between the two consecutive cation columns

	Thyroxine		Triiodothyronine	
	Radioactivity c/m	% of Total Radioactivity	Radioactivity c/m	% of Total Radioactivity
Case (i)				
Radioactivity added	9297		9777	
Eluted volume Initial 6 ml	6432	69.3	670	6.9
Ammonium Hydroxide pH 9.5				
1st 4 ml	1840	19.8	369	3.8
2nd 4 ml	337	3.6	208	2.1
		92.7		12.8
Case (ii)				
Radioactivity added	11284		7034	
Eluted volume Initial 6 ml	8969	77.0	624	8.9
Ammonium Hydroxide pH 9.5				
1st 4 ml	1693	15.0	158	2.2
2nd 4 ml	164	1.4	74	1.1
		93.4		12.2
Case (iii)				
Radioactivity added	15767		14470	
Eluted volume Initial 6 ml	9943	63.1	1221	8.4
Ammonium Hydroxide pH 9.5				
1st 4 ml	3651	23.3	396	2.7
2nd 4 ml	830	5.3	27	0.2
		91.7		11.3

eluted in very diffuse bands.

Optimum Mesh of the Resin for the Separation of the Iodothyronines

For the separation of the iodothyronines, three resin bead sizes were investigated, meshes 50-100, 100-200 and 200-400. Preliminary experiments showed that to obtain the optimum separation from each bead size a solution of different pH was required. For mesh 50-100 pH 8.7, mesh 100-200 pH 8.9 and mesh 200-400 pH 9.5. From the results recorded in Table 14 it is clear that only the fine mesh resin gives a good recovery of thyroxine under conditions which allow the elution of only about 35% of the triiodothyronine. For the larger mesh resins it is necessary to use an eluting solution of a higher pH to obtain better recoveries of the thyroxine, resulting in a greater percentage of the triiodothyronine being eluted. Thus, instead of using two consecutive columns, it would be necessary to use three or possibly four in the case of the 50-100 mesh resin.

2.4 Separation Procedure Used for Analysis of Blood Serum

From the preceding discussion the following conditions for the separation of the iodoamino acids in human serum have been selected.

Separation Technique

A 2 ml serum sample was passed through a mixed

Table 14

The effect of the mesh of the resin on the separation of the Iodothyronines on cation columns

	Thyroxine		Triiodotyronine	
	Radioactivity c/m	% of Total Radioactivity	Radioactivity c/m	% of Total Radioactivity
(i) 50-100 Mesh				
Radioactivity added	63634		31606	
Eluted volume Initial 6 ml	32310	49.3	8817	27.9
Ammonium Hydroxide pH 8.7				
1st 4 ml	12503	19.7	3629	11.4
2nd 4 ml	6054	9.2	1216	3.8
		78.2		43.1
(ii) 100-200 Mesh				
Radioactivity added	47067		54050	
Eluted volume Initial 6 ml	16098	34.3	8545	15.8
Ammonium Hydroxide pH 8.9				
1st 4 ml	6763	14.4	4575	8.5
2nd 4 ml	12692	27.1	6770	13.5
		75.8		36.8
(iii) 200-400 Mesh				
Radioactivity added	14194		11367	
Eluted volume Initial 6 ml	11456	80.6	2439	21.4
Ammonium Hydroxide pH 9.5				
1st 4 ml	1874	13.2	909	8.0
2nd 4 ml	245	1.7	799	7.1
		95.5		36.5

resin column and the column eluted with 2 ml of distilled water, removing the serum proteins. The column was then eluted with 18 ml of 0.2M ammonium acetate adjusted to pH 6.7 with ammonium hydroxide. This was followed by a further elution with 18 ml of molar acetic acid adjusted to pH 4.3 with ammonium acetate. The 4 ml from the original sample and distilled water wash was diluted with 2 ml of 0.2M ammonium acetate adjusted to pH 8.0 with ammonium hydroxide. This sample was then passed through two consecutive cation columns, followed by an 8 ml wash of 0.2M ammonium hydroxide adjusted to pH 9.5 with ammonium acetate and 8 ml of 5M ammonium hydroxide. The effluent from the cation columns was collected in three fractions: (i) the initial 6 ml ; (ii) the pH 9.5 fraction; and (iii) the 5M ammonium hydroxide fraction.

Recovery of Iodoamino Acids

In the development of the separation procedure I^{131} labelled compounds were used as tracers. Some typical results obtained are recorded in Tables 15 and 16. Table 15 indicates that the thyroxine and the majority of the triiodothyronine remain with the serum proteins when they are passed through the mixed resin column. The monoiodotyrosine was eluted at pH 6.7, while the diiodotyrosine was recovered at pH 4.3. The diiodotyrosine fraction may contain as much as 5% of the

Table 15

The separation of Iodoamino Acids on mixed resin columns

	I		II	
(i) Monoiodotyrosine	Radioactivity c/m	% of Total Radioactivity	Radioactivity c/m	% of Total Radioactivity
Radioactivity added	12572		14309	
Eluted volume Sample + 2 ml water	366	2.9	558	3.9
Ammonium Acetate pH 6.7				
1st 6 ml	6302	50.1	9118	64.0
2nd 6 ml	4382	34.8	2540	17.8
3rd 6 ml	387	3.1	438	3.4
		88.0		85.2
Acetic Acid pH 4.3				
1st 6 ml	400	3.2	595	4.2
2nd 6 ml	165	1.3	79	0.5
3rd 6 ml	47	0.3	34	0.2
		4.8		4.9
Total Radioactivity Eluted		95.7		94.0
(ii) Diiodotyrosine				
Radioactivity added	25204		15008	
Eluted volume Sample + 2 ml water	202	0.8	212	1.4
Ammonium Acetate pH 6.7				
1st 6 ml	353	1.4	391	2.6
2nd 6 ml	731	2.9	345	2.3
3rd 6 ml	1032	4.1	603	4.0
		8.4		8.9
Acetic Acid pH 4.3				
1st 6 ml	12897	51.2	7423	49.5
2nd 6 ml	5202	20.6	3085	20.6
3rd 6 ml	1273	5.0	817	5.4
		76.8		75.5
Total Radioactivity Eluted		86.0		85.8

	Radioactivity c/m	% of Total Radioactivity	II Radioactivity c/m	% of Total Radioactivity
(iii) Triiodothyronine				
Radioactivity added	8784		13817	
Eluted volume Sample + 2 ml water	7034	84.3	11367	82.2
N.B.	No Radioactivity was eluted at PH 6.7 or PH 4.3			
(iv) Thyroxine				
Radioactivity added	12066		15300	
Eluted volume Sample + 2 ml water	11284	93.5	14194	93.0
N.B.	No Radioactivity was eluted at PH 6.7 or PH 4.3			

Table 16

The separation of Iodothyronines on cation exchange columns

	I		II	
	Radioactivity c/m	% of Total Radioactivity	Radioactivity c/m	% of Total Radioactivity
(i) Triiodothyronine				
Radioactivity added 1st column	7034		11367	
Eluted volume Initial 6 ml	1498	21.3	2439	21.4
Ammonium Hydroxide pH 9.5				
1st 4 ml	660	9.4	909	8.0
2nd 4 ml	485	6.9	799	7.1
		37.6		36.5
5M Ammonium Hydroxide				
1st 4 ml	4224	60.1	7221	63.5
2nd 4 ml	41	0.6	110	1.0
		60.7		64.5
2nd column				
Eluted volume Initial 6 ml	624	8.9	956	8.4
Ammonium Hydroxide pH 9.5				
1st 4 ml	158	2.2	212	1.9
2nd 4 ml	74	1.1	209	1.8
		12.2		12.1
5M Ammonium Hydroxide				
1st 4 ml	5756	82.0	8827	77.6
2nd 4 ml	564	8.2	1431	12.6
		90.2		90.2

	Radioactivity c/m	% of Total Radioactivity	II Radioactivity c/m	% of Total Radioactivity
(ii) Throxine				
Radioactivity added 1st column	11284		14194	
Eluted volume Initial 6 ml	10334	91.5	11456	80.6
Ammonium Hydroxide pH 9.5				
1st 4 ml	314	2.8	1874	13.2
2nd 4 ml	138	1.2	245	1.7
		95.5		95.5
5M Ammonium Hydroxide				
1st 4 ml	256	2.3	577	4.1
2nd 4 ml	100	0.9	40	0.2
		3.2		4.3
2nd column				
Eluted volume Initial 6 ml	8969	77.0	8981	63.2
Ammonium Hydroxide pH 9.5				
1st 4 ml	1693	15.0	3352	23.6
2nd 4 ml	164	1.4	420	3.0
		93.4		89.8
5M Ammonium Hydroxide				
1st 4 ml	426	3.8	838	5.9
2nd 4 ml	121	1.1	37	0.2
		4.9		6.1

monoiodotyrosine from tailing of the monoiodotyrosine bond, while up to 9% of the diiodotyrosine appears to be eluted in the monoiodotyrosine fraction. Part of this 9% of the diiodotyrosine radioactivity may be monoiodotyrosine from radiation-induced decomposition[103] but it would appear that complete resolution had not been achieved. The small percentage of radioactivity,from the iodotyrosines which were eluted with the serum proteins, was found to remain with them if passed over a second mixed resin column and is therefore probably in the form of iodothyronines formed by isotopic exchange.

The 16-18% of the triiodothyronine left on the mixed resin is composed of two distinct portions:

(i) Inorganic iodide from radiation induced decomposition of the triiodothyronine[102]

(ii) A small percentage of the triiodothyronine adsorbed by the resin.

This was shown by taking the effluent from the mixed resin column and passing it through an identical column, in which case approximately 93% of the radioactivity was recovered, indicating that, of the approximately 17% retained by the first mixed resin column, about 10% was present as an impurity - probably iodide - and about 7% of the triiodothyronine was adsorbed by the resin in competition with the

serum proteins. The radioactivity retained by the mixed resin column was not eluted at pH 6.7, nor at pH 4.3. Thus, the iodotyrosines are not contaminated by the triiodothyronine.

The radioactivity from the labelled thyroxine adsorbed on the mixed resin column appears to be mainly iodide from the radiation-induced decomposition, since when the effluent was passed over a second mixed resin column more than 98% of the radioactivity was recovered. It was also noted that the radioactivity absorbed on the mixed resin column was not eluted at pH 6.7 or pH 4.3, so that no contamination of the iodotyrosines occurred.

Table 16 shows that triiodothyronine was separated from the serum proteins and the thyroxine by retention on cation columns from which it was removed with 5M ammonium hydroxide. The thyroxine was only partially retained by the cation columns. Thus the protein fraction contained some of the thyroxine, and the remainder was eluted from the cation columns with 8 ml of pH 9.5 solution. The 12% of the triiodothyronine which was recovered in the thyroxine fraction would be reduced to 5% by using a third cation column. However, as triiodothyronine is normally present in concentrations of about one sixth of the concentration of thyroxine, this seems unnecessary. The 5% of the thyroxine

radioactivity which was found to be eluted with the 5M ammonium hydroxide was also found to migrate with the triiodothyronine fraction if it was neutralized, added to a fresh serum sample, and fractionated again. This would be expected if the approximately 6% radioactivity retained by the mixed resin column was iodide from radiation-induced decomposition; the other decomposition product was triiodothyronine[102]. The iodide would be washed straight off the cation column, although it would be retained by the mixed resin. However, the other product or products may contribute to the radioactivity which is found in the triiodothyronine fraction, even if they are not triiodothyronine. This problem is not carried over into the analysis of serum as it is peculiar to radioactively labelled compounds which are not employed in the analytical procedure.

Recovery of Iodoamino Acids from a Serum Sample Containing all Four Labelled Iodoamino Acids.

The results recorded in Tables 15 and 16 indicate that when using these labelled iodoamino acids one may expect the recovery distribution shown in Table 17. As discussed above, these percentage recoveries represent the amount of radioactivity found in each fraction, and not necessarily the percentages of the particular iodoamino acid. Since exchange and radiation induced decomposition introduce errors which have not been accurately evaluated.

To test the separation procedure one drop of each

of the four I^{131} labelled iodoamino acids was added to 6 ml of fresh serum, the radioactivity being measured after each addition. The serum was allowed to equilibrate for half an hour at 10°C. Then two 2 ml portions, having radioactivities of 6726 c/m and 7036 c/m, were run through the separation procedure. The composition of the test sample is shown in Table 18. Table 19 shows the radioactivity of the various fractions obtained from the separation procedure together with the expected radioactivities calculated from Tables 17 and 18. From these results one sees that there is good agreement between experiments isolating I^{131} labelled compounds singly and experiments where a mixture of all four labelled compounds were separated from each other.

Table 17

Recoveries of I^{131} labelled Iodoamino Acide

Labelled Iodoamino Acid	"MIT" Fraction	"DIT" Fraction	"T_3" Fraction	"T_4" Fraction	Suspected Iodide
MIT	85-90%	~5%			
DIT	~ 9%	~75%			
T_3			~76%	~ 10%	~10%
T_4			~ 5%	83-86%	~7%

Table 18

The radioactive composition of "doped" serum

Iodoamino Acid	Radioactivity (counts/min)
Monoiodotyrosine	6733
Diiodotyrosine	3313
Triiodothyronine	7883
Thyroxine	2612
Total Radioacitivy	24641

Table 19

The radioactivity of separated fractions

		"MIT" Fraction	"DIT" Fraction	"T_3" Fraction	"T_4" Fraction
(i)	Sample No. 1				
	Initial Radioactivity 6726 c/m				
	Radioactivity obtained	1700	740	1767	1694
	Radioactivity expected	1650-1740	770	1730	1766-1816
(ii)	Sample No. 2				
	Initial radioactivity 7036 c/m				
	Radioactivity expected	1829	782	1882	1789
	Radioactivity obtained	1715-1805	800	1800	1834-1894

ANALYTICAL PROCEDURE

3.1 Neutron Activation Analysis

If N atoms of a target isotope having a neutron absorption cross section σ are irradiated for a time t_1 in a neutron flux ϕ, the induced radioactivity A in disintegrations per sec. is given by

$$A = \sigma \phi N (1-e^{-\lambda t_1}) e^{-\lambda t_2} \qquad (1)$$

where λ is the decay constant of the isotope produced and t_2 is the time from the end of the irradiation until A is determined. If therefore,one could determine A after the irradiation of a material for a known time in a known reactor flux, one could determine the number of atoms of any particular isotope, providing its half life and neutron absorption cross section are known. In practice,the observed count rate, C, in a given detector is related to the quantity A by the relationship

$$C = G.\ E.\ S.\ I.\ A. \qquad (2)$$

where G is a geometric factor, E is the detector efficiency for the radioactive decay concerned, S is the sample self absorption factor and I is the fraction of the isotopic disintegration giving rise to the observed gamma ray. To overcome the difficulty of determining all these quantities in

order to evaluate N accurately, an internal standard system was used. In this method a constant known amount of an element which is not present in the sample is added immediately prior to irradiation. In the resultant gamma ray spectrum, only the photopeaks arising from the element being determined and the internal standard are of interest. The ratio of the observed disintegration rates of the two isotopes obtained from these two photopeaks allows the evaluation of the quantity of the desired element with the aid of a calibration curve. This calibration curve is prepared by plotting the ratio of the observed disintegration rates against the concentration of the element to be determined in irradiated standard samples containing varying known amounts of the element to be determined along with the same constant amount of the internal standard. If the observed count rate for the photopeak of the isotope being determined is given by

$$C = G.\ E.\ S.\ I.\ \sigma\ \phi\ N\ (1-e^{-\lambda t_1})\ e^{-\lambda t_2} \qquad (3)$$

and the observed countrate for the photopeak of the internal standard is given by

$$C_s = G.\ E_s.\ S_s.\ I_s.\ \sigma_s\ \phi_s\ N_s\ (i-e^{-\lambda_s t_1})\ e^{-\lambda_s t_2} \qquad (4)$$

then the ratio of the observed radioactivities will be

$$\frac{C}{C_s} = \frac{E\ S\ I\ \sigma\ \phi\ N\ (1-e^{-\lambda t_1})\ e^{-\lambda t_2}}{E_s\ S_s\ I_s\ \sigma_s\ \phi_s\ N_s\ (1-e^{-\lambda_s t_1})\ e^{-\lambda_s t_2}}. \qquad (5)$$

If in all determinations, both calibrations and analyses, t_1 and t_2 are kept constant, then equation (5) may be rewritten

$$\frac{C}{C_s} = N\ K \qquad (6)$$

where

$$K = \frac{E\ S\ I\ \sigma\ \phi\ (1-e^{-\lambda t_1}\ e^{-\lambda t_2}}{N_s\ E_s\ S_s\ I_s\ \sigma_s\ \phi_s\ (1-e^{-\lambda_s t_1})\ e^{-\lambda_s t_2}}$$

and is a constant dependent only on the reactor neutron energy distribution provided that N_s, the number of atoms of internal standard added, is kept constant and that the size of the sample does not vary, causing a change in the self absorption. K may be evaluated from the calibration curve. Thus, in order to obtain the number of atoms of unknown in a given sample it is only necessary to measure the ratio of the photopeaks of the gamma rays from the sample and the standard. It is necessary to keep t_1 and t_2 constant unless the decay constants of the two isotopes of interest are identical, i.e. $\lambda = \lambda_s$. In the choice of the internal standard it is desirable that the energy of its photopeak be slightly lower than that of the element being determined so that any Compton from the internal standard does not contribute to the background of the photopeak of the element being determined. A sufficient quantity of internal standard should be added so that the counting statistics of the internal standard photopeak do

not significantly affect the uncertainty in the photopeak ratio.

In this work the method used was the same as that used by Tang and Tomlinson[8]. Naturally occurring iodine is monoisotopic and forms I^{128} by neutron capture. The decay scheme of this isotope, which has a half life of 25 minutes, includes a single gamma ray with an energy of 442 keV. Natural strontium is 0.56% Sr^{84}, 9.8% Sr^{86}, 7% Sr^{87}, 82.5% Sr^{88}. The only gamma-emitting isotopes produced by the thermal neutron irradiation are Sr^{85} and Sr^{87m}. The highest energy gamma ray emitted is the 388 keV gamma ray of Sr^{87m} which has a 2.8 hour half life. This gamma ray energy is conveniently close to, but slightly below, the energy of the gamma ray from I^{128}. 1 ml of a solution of strontium nitrate containing approximately 100 µg/ml was added to each sample immediately prior to irradiation.

3.2 Contamination

Preliminary neutron activation experiments showed that all fractions, except the thyroxine which remained with the serum proteins, were contaminated with sodium ions. The iodotyrosine fractions were contaminated with chloride as well as sodium ions. Other contaminants may have been present but did not constitute analytical problems as the samples were only irradiated for ten minutes and then allowed to "cool" for twenty minutes before being "counted". Under

such conditions the induced radioactivity of other isotopes was found to be insignificant. Before the gamma ray spectrum could be used to determine the quantity of iodine in each fraction the sodium ions and chloride contamination had to be removed.

Removal of Chloride Contamination

It was decided to use an ion exchange system to remove the chloride contamination from the iodotyrosines. Two different approaches to such a system are available: either an anionic column to retain the chloride anion, or a cationic column to retain the cationic iodoamino acid. In either system it is first necessary to acidify the sample so that the iodoamino acid is in the cationic form. A few trial runs showed that the anion exchange resin did not give a sufficiently high chloride decontamination factor for the neutron activation analysis system, as it is very sensitive to the presence of chloride. On the other hand, it was found that I^{131} labelled iodotyrosines, after preliminary removal of any radioactive iodide, were quantitatively absorbed from acid solutions (pH below 1) by a 5 × 0.5 cm column of Bio-Rad 50W × 2, mesh 100-200 cationic resin, and couldbe quantitatively recovered in a 5 ml wash of molar ammonium hydroxide. Thus the chloride contamination was removed by acidifying the 18 ml monoiodotyrosine and

and diiodotyrosine samples with 1 ml of concentrated nitric acid to lower their pH below 1, and then percolating them through a 5 × 0.5 cm column of Bio-Rad 50W ×2 mesh 100-200. The columns were then washed with 1 ml of molar nitric acid to remove any final traces of chloride, and then the iodoamino acids were quantitatively recovered with 5 ml of molar ammonium hydroxide. This method has the advantage of concentrating the original 18 ml of sample to 5 ml which is a convenient volume for irradiation in the pneumatic rapid transfer (rabbit) system of the McMaster Nuclear Reactor.

Removal of Sodium Ions

The sodium ions are removed after irradiation using a technique developed by Girardi and Sabboni[109] in which the radio-sodium is removed by passing the irradiated material, in an acidic medium, over a column of hydrated antimony pentoxide. The hydrated antimony pentoxide was obtained from Carlo Erba, Milan, Italy. The details of the preparation of this compound are not given by the suppliers. Each irradiated fraction, except that of the serum proteins which did not contain any sodium ions, was acidified with 1.2 ml of concentrated nitric acid and passed over the hydrated antimony pentoxide column, 5 × 0.5 cm, followed by a 5 ml wash of 50% nitric acid. The recovery efficiencies of the iodoamino acids passed over the hydrated antimony

pentoxide was investigated using I^{131} labelled iodoamino acids. One drop of the labelled iodotyrosines were added to 5 ml of molar ammonium hydroxide, and the solution was then acidified. The labelled triiodothyronine and thyroxine were added to 8 ml of 5M ammonium hydroxide and 8 ml of pH 9.5 solution respectively. These were then evaporated to approximately 5 ml under a heat lamp before the acid was added. In all cases, it was found that 1.2 ml of nitric acid was sufficient to lower the pH below 1. The acidified samples were passed over hydrated antimony pentoxide columns and the columns washed with 5 ml of 50% nitric acid. Quantitative yields of the iodoamino acids were obtained, as may be seen from Table 20. This is not conclusive proof for the quantitative recovery of the iodine originally present as iodoamino acids, from the hydrated antimony pentoxide columns as some of the iodoamino acids will be destroyed during irradiation, (Szilard-Chalmers reaction). However a comparison of the analysis values obtained and the protein bound iodine values, together with the normal ranges of the thyroxine concentration obtained in this work compared to those obtained by other workers indicate that no significant losses occur, (see later).

Although Girardi and Sabboni[109] claimed that under these conditions the hydrated antimony pentoxide is highly selective for sodium ions, it was desirable to ascertain that

Table 20

The recovery of Iodoamino Acids from hydrated hydrated antimony pentoxide

	Radioactivity (counts/min)
(i) Monoiodotyrosine	
Radioactivity added	22065
Eluted volumne	
Sample	17401
50% Nitric Acid	
1st 1 ml	2651
2nd 1 ml	1536
3rd 1 ml	421
Total Radioactivity Eluted	22009
(ii) Diiodotyrosine	
Radioactivity added	15193
Eluted volume	
Sample	11510
50% Nitric Acid	
1st 1 ml	2306
2nd 1 ml	888
3rd 1 ml	412
Total Radioactivity Eluted	15116

	Radioactivity (counts/min)
(iii) Triiodothyronine	
Radioactivity added	15496
Eluted volume	
Sample	10943
50% Nitric Acid	
1st 1 ml	2103
2nd 1 ml	1275
3rd 1 ml	881
Total Radioactivity Eluted	15202
(iv) Thyroxine	
Radioactivity added	11551
Eluted volume	
Sample	8213
50% Nitric Acid	
1st 1 ml	1971
2nd 1 ml	892
3rd 1 ml	341
Total Radioactivity Eluted	11417

the internal standard, strontium, was not retained by the hydrated antimony pentoxide. This was shown by taking 1 ml of the strontium nitrate solution in 5 ml of molar ammonium hydroxide, and irradiating, counting and acidifying it with 1 ml of nitric acid before passing it through a hydrated antimony pentoxide column, followed by a 5 ml 50% nitric acid wash. The resultant eluant was counted in two 6 ml fractions using identical counting geometry to that used for the original sample. The radioactivity of these two eluted fractions, after correction for radioactive decay, indicated that the strontium radioactivity was quantitatively recovered from the hydrated antimony pentoxide column.

3.3 Preparation of the Calibration Curve

The calibration curve was obtained by using a series of standard solutions containing known amounts of iodine varying between 1.74×10^{-2} and and 3.466×10^{-1} µg in the form of potassium iodate. To each of these had been added 1 ml of strontium nitrate solution. These samples were each sealed in a polyethylene vial and frozen in liquid nitrogen before being irradiated for ten minutes in the Reactor. They were allowed to "cool" for twenty minutes, during which time each was transferred to a fresh polyethylene vial to reduce the background radioactivity, before being counted for twenty minutes. It is essential in using

this internal standard method that the time intervals be kept constant for both the calibration and the serum samples. The ratio of the two photopeaks, I^{128} 442 keV and Sr^{87m} 388 keV, in the resultant gamma ray spectrum was obtained by summing the top five channels on each and subtracting the background values obtained by averaging 10 channels, 5 from each side of the peak. This ratio was plotted against 50 times the amount of iodine in the calibration samples. In this way the results obtained from a 2 ml serum sample could be read off the calibration curve in μg of iodine per 100 ml of serum, which is the conventional way of reporting protein bound iodine analyses. The values obtained for such a calibration are shown in Table 21. Figure 2 shows the straight line obtained by applying a least squares fit to these experimental results. "Blanks" were also irradiated, containing strontium nitrate solution and various concentrations of ammonium hydroxide and acetic acid. In no case was any iodine photopeak detectable in the resultant γ spectrum.

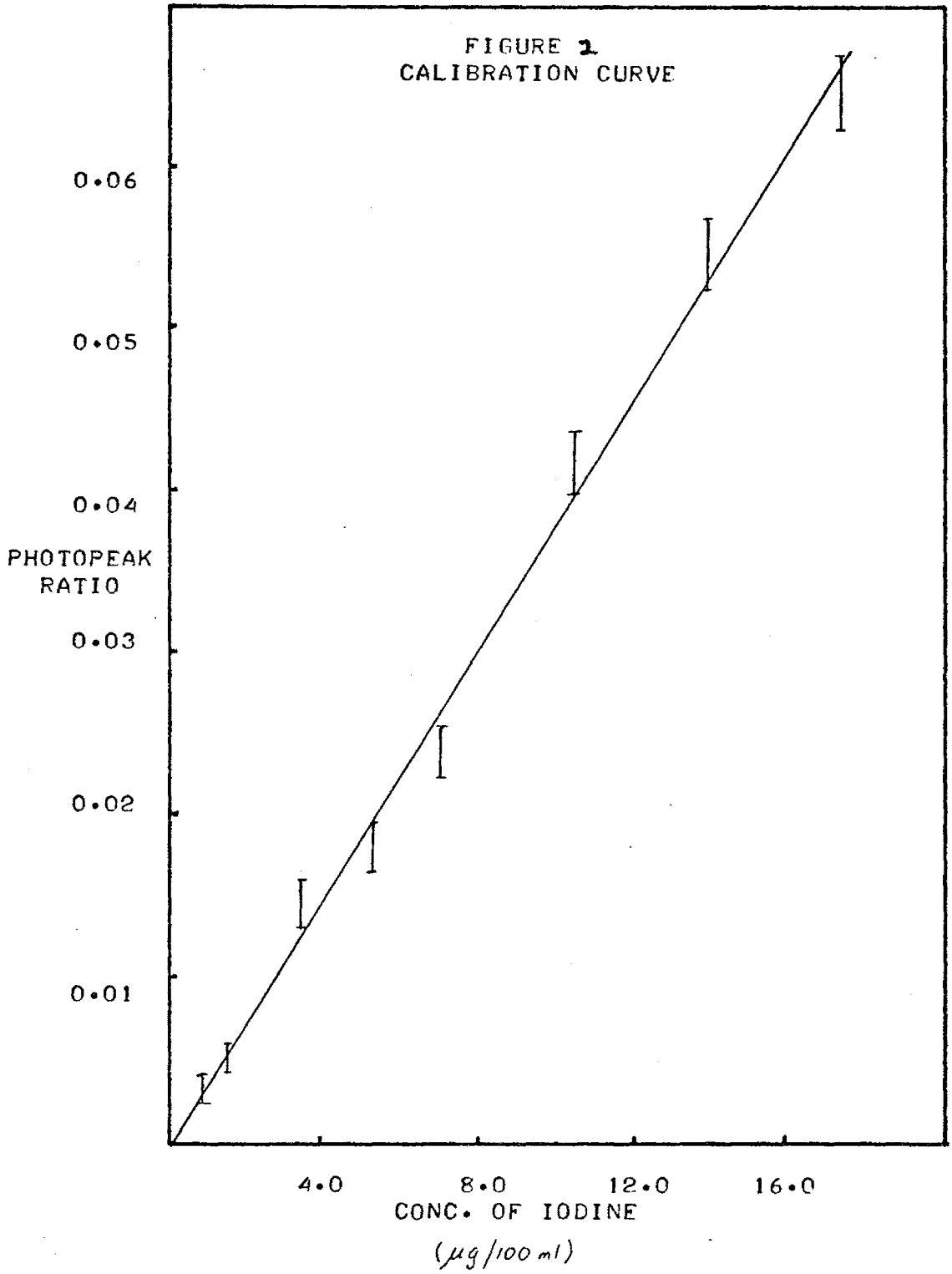
Comar[120] observed that when an aqueous solution of ammoniom iodide (0.05 μg/ml) was irradiated in a polyethylene tube for 20 min. about half the iodide was adsorbed onto the polyethylene tube. The fact that the calibration curve is a straight line passing through the origin indicates that this is not the case with the type of polyethylene used here.

Table 21

Iodine to strontium photopeak ratios for the calibration curve

Quantity of Iodine μg	Ratio of Photopeak Areas	σ_{stat} of Ratio
0.0174	0.0030	0.0010
0.0346	0.0050	0.0010
0.0693	0.0143	0.0014
0.1040	0.0181	0.0015
0.1386	0.0240	0.0016
0.2080	0.0416	0.0019
0.2772	0.0545	0.0021
0.3466	0.0646	0.0023

FIGURE 2.
CALIBRATION CURVE



A further test involving the irradiation of an ammonium hydroxide solution containing 0.1 μg of iodide, as potassium iodide, indicated that no adsorption of the iodide was taking place.

3.4 Irradiation of Serum Samples

The fractions obtained from the serum samples were treated in exactly the same way as the potassium iodate calibration samples. To each was added 1 ml of strontium nitrate solution. They were sealed in polyethylene vials, frozen in liquid nitrogen and irradiated for ten minutes. During the "cooling" time of twenty minutes all fractions, except that of the serum proteins, were treated for the removal of sodium as described previously. The samples were then counted for twenty minutes using the live-time clock on the analyser. The ratio of the 442 keV I^{128} peak to the 388 keV Sr^{87m} peak was calculated as described above and the concentration of iodine read off the calibration graph.

3.5 Analytical Procedure Used for Analysis of Blood Serum

The monoiodotyrosine and diiodotyrosine fractions were acidified with 1 ml of conc. nitric acid and passed over a 5 × 0.5 cm Bio-Rad 50W × 2 mesh 100-200 column, followed by a 1 ml molar nitric acid wash. The iodotyrosines were quantitatively recovered in 5 mls of molar

ammonium hydroxide. The triiodothyronine and that portion of the thyroxine eluted with the pH 9.5 solution were evaporated to approximately 5 ml. One ml of strontium nitrate was then added to each fraction before they were sealed in polyethylene vials, frozen in liquid nitrogen and irradiated for ten minutes. During the "cooling" time of twenty minutes, each fraction was transferred to a fresh polyethylene vial for counting. All samples, except that of the serum proteins, were acidified with 1.2 ml of concentrated nitric acid and passed over a 5 × 0.5 cm column of hydrated antimony pentoxide, followed by a 5 ml wash of 50% nitric acid. Each fraction was counted for twenty minutes. The ratio of the radioactivities of the 442 keV and 388 keV photopeaks in the resultant gamma ray spectrum, Figure 3, was calculated and the concentration of iodine determined from the calibration curve.

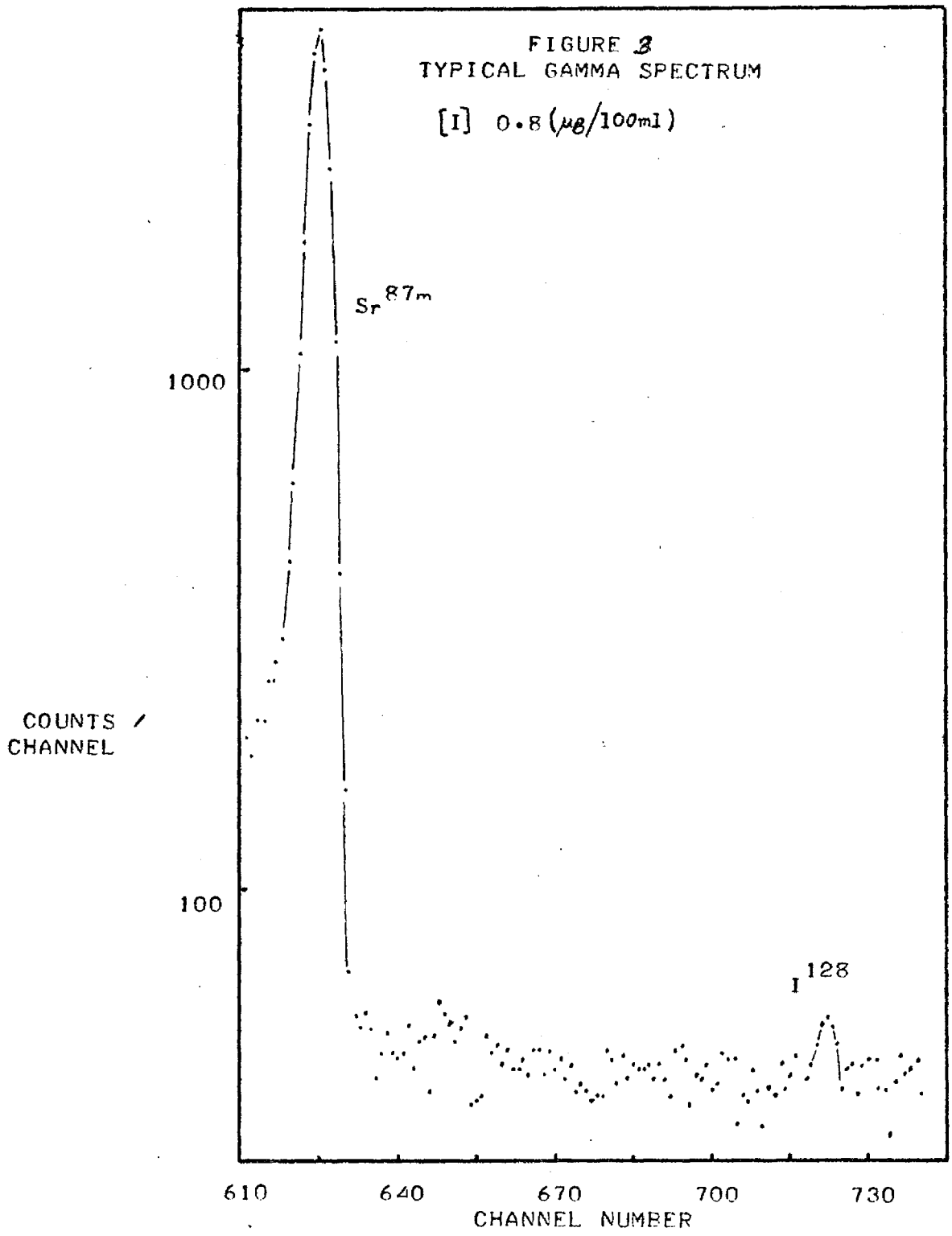


FIGURE 3
TYPICAL GAMMA SPECTRUM
[I] 0.8 (μg/100ml)
Sr 87m
I 128
1000
100
COUNTS / CHANNEL
610
640
670
700
730
CHANNEL NUMBER

RESULTS AND DISCUSSION

4.1 Reproducibility of Analytical Results

The reproducibility of analyses made using the analytical system described in Sections 2.4 and 3.5 was investigated by doing a series of determinations on aliquots taken from the same blood sample. The results obtained are shown in Table 22.

σ_{Stat} is the standard deviation associated with the counting statistics. The quantity of strontium nitrate added to each sample is such that the counts in the Sr^{87m} photopeak are more than 20 times greater than those in the I^{128} photopeak, so that the standard deviation of the ratio of the counts in the photopeaks is essentially that associated with the I^{128} photopeak. For T_4 total, σ_{Stat} is given by

$$\sigma_{Stat} = \pm \left[\sigma_{T_{4A}}{}^2 + \sigma_{T_{4B}}{}^2 \right]^{\frac{1}{2}}$$

where T_{4A} = the thyroxine remaining with the serum proteins

T_{4B} = the thyronine eluted from the cation column at pH 9.5

The standard deviation is calculated from the spread of the experimental values using the formula

Table 22

Multiple analysis of one serum sample
as a test of reproducibility

Iodoamino Acid	µg I/100 ml	σ Stat	av [I]	Std. Dev.
MIT	1.3	0.3		
	1.0	0.3		
	0.8	0.3		
	1.1	0.3	1.0	0.2
	0.7	0.2		
	1.0	0.3		
	1.1	0.3		
DIT	1.0	0.3		
	0.6	0.2		
	0.5	0.3		
	0.8	0.3	0.7	0.2
	1.0	0.3		
	0.5	0.2		
	0.6	0.3		
T_3	1.3	0.3		
	1.0	0.3		
	0.9	0.3	0.9	0.4
	1.4	0.3		
	0.8	0.3		
	0.6	0.3		
	0.6	0.3		
T_{4B}	1.2	0.4		
	1.2	0.4		
	2.0	0.3		
	2.8	0.4		
	1.1	0.3		
	1.0	0.3		
	0.4	0.4		

Iodoamino Acid	µg I/100 ml	σ Stat	av [I]	Std. Dev
T_{4A}	3.6	0.6		
	3.9	0.6		
	2.6	0.7		
	2.8	0.6		
	4.9	0.7		
	5.2	0.6		
	5.3	0.6		
T_4 total	4.8	0.7		
	5.1	0.7		
	4.6	0.8		
	5.6	0.7	5.4	0.6
	6.0	0.8		
	6.2	0.7		
	5.7	0.7		

$$\text{Std. dev.} = \frac{\Sigma|\bar{x}-x|^2}{n-1}$$

where $\bar{x}$ = average value

x = the individual value

n = number of values used.

If the experimental variations are insignificantly small compared to the counting statistics, the σ_{Stat} should be equal to the standard deviation. The results in Table 22 show that the standard deviation is less than or equal to σ_{stat} indicating that the reproducibility is controlled by the counting statistics.

4.2 Comparison of Analyses of Serum with Protein Bound Iodine Determinations

Serum samples on which protein bound iodine determinations had been carried out were analyzed using this method. The results are recorded in Table 23. It will be seen that on the average, the value obtained for the thyroxine concentration alone is 1.2 times the protein bound iodine value reported by the hospital laboratory, and the total hormonal fraction, triiodothyronine and thyroxine, is 1.4 times the hospital protein bound iodine. It is interesting to compare these values with those reported by Smith *et al*[7] who used neutron activation to measure the protein bound iodine and obtained values which were systematically higher than those obtained by chemical

Table 23

Comparison of activation analysis with
P. B. I. determinations on serum samples

	MIT	DIT	T_3	T_4	PBI	$\frac{T_4}{PBI}$	$\frac{T_4+T_3}{PBI}$	T_4+T_3-PBI
1	1.0	0.7	0.3	4.9	3.7	1.3	1.4	1.5
2	4.8	0.9	1.0	4.3	3.6	1.2	1.5	1.7
3	0.8	0.1	0.7	2.1	1.4	1.5	2.0	1.4
4	0.7	1.6	0.8	3.8	3.8	1.0	1.2	0.8
5	1.1	0.3	0.3	3.5	0.9	3.9	4.2	2.9
6	0.4	0.8	0.8	2.8	1.9	1.5	1.9	1.7
7	0.2	0.6	1.3	10.8	9.7	1.1	1.2	2.4
8	0.7	3.0	0.8	6.8	4.3	1.6	1.8	3.3
9	0.7	0.2	0.7	6.3	6.6	0.95	1.1	0.4
10	0.0	0.3	0.3	10.2	6.8	1.5	1.5	3.7
11	1.3	1.0	0.4	5.8	5.0	1.2	1.2	1.2
12	1.2	0.5	0.8	6.1	5.5	1.1	1.3	1.4
13	0.4	0.6	0.7	6.5	5.9	1.1	1.2	1.3
			Average neglecting No. 5			1.3	1.4	1.7

methods. On the average, the values they obtained were systematically higher by 1.8 μgm/100 ml, while on the average values obtained in this work for thyroxine plus triiodothyronine are systematically higher than the protein bound iodine values by 1.7 μgm/100 ml. Smith et al used ion exchange resins to remove the inorganic iodide from the plasma. If their resin columns removed the iodotyrosines, then there is good agreement between their results and those obtained by the method used in this work. Cuaron[69], who measured only thyroxine, found values systematically higher than the chemical analysis of the protein bound iodine by about the same amount.

A protein bound iodine determination employing neutron activation analysis and giving good agreement with the chemical analysis of the protein bound iodine values has been reported by Tang and Tomlinson[8]. In this work, however, the isolation of the iodine compounds for analysis was essentially the same as that used by the hospital, and the comparison was therefore between the actual analyses of iodine.

4.3 Determination of the Ranges of Normal Concentration of the Various Iodoamino Acids

Before it is possible to relate abnormal thyroid function to the analysis of the iodine bound in the various organic forms, it is necessary to establish their normal

levels. To ascertain the normal concentration ranges of these four iodoamino acids, blood samples were taken from twelve clinically euthyroid people. The serum from these blood samples was separated and analyzed for the four iodoamino acids as described previously. The results obtained are recorded in Table 24. The average concentration of each of the iodoamino acids was calculated and the standard deviation of the distribution of each was evaluated using the formula

$$\sigma = \sqrt{\frac{\Sigma |\bar{x}-x|^2}{n-1}} .$$

The fact that the standard deviations of the concentrations of iodine in the forms of monoiodotyrosine, diiodothyrosine, triiodothyronine and thyroxine are the same as the statistical standard deviations, arising from the statistical factors of radioactive counting, indicates that these levels form a narrower distribution than can be seen within the high σ of radioactive analyses. The results obtained by this analysis system will be a close approximation to the Gaussian Distribution of the radioactive analysis. Thus, if we use the 90% probability range, i.e. a 2σ width, we obtain the normal ranges shown in Table 25. This range for thyroxine compares remarkably well with that obtained by Cuaron[69], 6.6 - 12.7 µg of thyroxine/100 ml, using a "radioimmuno-assay-like" technique.

Table 24

Distribution of organic iodine in euthyroid sera

SERUM	MIT	DIT	T_3	T_4	$T_3 + T_4$	Total [I]
1	0.6	0.5	1.4	5.0	6.4	7.5
2	0.4	1.1	1.2	5.1	6.3	7.8
3	0.4	0.7	1.1	5.2	6.3	7.4
4	0.3	0.8	1.4	5.2	6.6	7.7
5	0.6	1.2	1.2	5.8	7.0	8.8
6	0.0	0.9	0.7	6.1	6.8	7.7
7	0.4	1.1	1.4	6.8	8.2	9.7
8	0.4	0.7	1.1	6.9	8.0	9.1
9	0.4	0.6	1.4	7.0	8.4	9.4
10	0.8	0.8	1.2	7.1	8.3	9.9
11	0.5	0.9	1.4	7.4	8.8	10.2
12	0.6	0.6	1.6	8.0	9.6	10.8
Average	0.4	0.8	1.3	6.30	7.6	8.8
σ	0.2	0.2	0.2	1.03	1.1	1.2

N.B. These values are given in µg of Iodine /100 ml.

Table 25

Range of normal concentrations of
Iodoamino Acids in human sera

	µg I/100 ml	µg/100 ml
Monoiodotyrosine	0.05 - 0.85	0.12 - 2.06
Diiodotyrosine	0.40 - 1.24	0.68 - 2.11
Triiodothyronine	0.78 - 1.74	1.33 - 2.98
Thyroxine	4.24 - 8.36	6.48 - 12.80

Murphy[68] also used a radioimmuno-assay-like technique, and reported a normal range of 4.00-11.00 µg/100 ml before correaction for a 77% recovery, thus giving a true normal range of 5.2-14.3 µg/100 ml which, although a considerably wider distribution, has the same mean value of 9.75 µg/100 ml. The normal concentration of triiodothyronine of 0.33 ± 0.07 µg of triiodothyronine/100 ml obtained by Nauman et al[110] is only one seventh of the value obtained here. These workers used paper chromatography to isolate the triiodothyronine. The paper was dried, and the triiodothyronine spot cut out and extracted. The triiodothyronine was then determined using a radioimmuno-assay-like technique. Such systems have been criticized on the grounds that, under these drying conditions, triiodothyronine and thyroxine are subject to rapid deiodination[31]. It is also observed that Werner[13], who analyzed the eight euthyroid sera found triiodothyronine contributed from 2 to 26%, with an average of 10%, of the serum iodine. Our results show an average of 13.5% of the organic iodine. As discussed in section 1.3, values of the concentrations of the iodotyrosines vary so greatly that comparison with the values o tained in this work is of no real value. The average values show monoiodotyrosine accounting for approximately 5% and diiodotyrosine about 10% of the organically bound iodine. Thus a total iodotyrosine fraction of 15% is found which is in the general region ob-

tained by workers using chemical analysis[22,25], although I^{131} tracer techniques have found little or no iodotyrosines [13,18].

4.4 Determination of the Concentrations of Iodoamino Acids in the Serum of Patients with Thyroid Disorders

Serum samples were obtained from patients suspected of having thyroid disorders and analyzed by the method developed in this work. In all cases at least two of the following four laboratory tests of thyroid function were also carried out; triiodothyronine-thyroxine-resin uptake, protein bound iodine assay, and neck uptake of I^{131} 24 hours after administration.

Analysis of Hyperthyroid Sera

Sera from patients who were hyperthyroid by the laboratory tests and who were judged to be hyperthyroid in the opinion of the physician, were analyzed. The results obtained, together with those of the laboratory tests are recorded in Table 26. If these results are examined with respect to the normal concentration ranges of each of the iodoamino acids, the following general statements may be made about these analyses.

(i) In all but one case the thyroxine concentration is high.

(ii) In only one case is the triiodothyronine concen-

Table 26

Iodoamino Acid concentrations in the serum of hyperthyroid patients

Patient	MIT µg/100 ml	DIT µg/100 ml	T_3 µg/100 ml	T_4 µg/100 ml	T_3 Uptake %	T_4 Uptake %	P.B.I µgI/100 ml	I^{131} Neck Uptake %
1	2.90	0.85	5.81	13.16	40	12.6		
2	2.18	1.19	4.28	24.62	56	19.0	14.0	100
3	2.90	1.36	3.42	14.53	44	19.5	16.0	
4	2.66	1.87	3.42	22.65	43	16.4	8.9	85
5	2.18	2.04	6.33	20.65	53	>30	13.2	79
6	2.18	0.68	5.30	13.61	47	19		80
7	1.94	1.36	4.10	13.16	43	11.2	12.3	85
8	0.73	1.53	8.72	13.30	56	16.3		95
9	0.73	1.19	9.92	15.90	41	19.0	14.1	
10	1.45	1.53	9.06	20.02	55	29.0	16.7	
11	1.45	1.36	4.45	16.21	52	17.5	14.1	58
12	1.21	1.36	4.28	26.47	45	13.1	11.9	52
13	1.21	1.36	4.10	29.21	59		15.7	100

Patient	MIT µg/100 ml	DIT µg/100 ml	T_3 µg/100 ml	T_4 µg/100 ml	T_3 Uptake %	T_4 Uptake %	P.B.I. µgI/100 ml	I^{131} Neck Uptake %
14	0.97	1.02	5.99	20.97	62	>30	19.4	100
15	1.45	2.04	3.76	22.35	45	20.0	14.8	97
16	1.21	0.51	7.35	19.28	40	18.4	16.0	55
17	2.42	1.53	2.74	13.91	53	19.5		86
18	3.39	0.00	1.54	17.29	36	14.0	8.8	
19	1.94	1.53	2.91	20.48	39	14.4	10.6	45
20	0.73	1.19	2.56	13.16	43	15.9	11.1	21
21	0.97	1.53	2.74	16.36	20	14.0	7.6	35
22	1.45	1.36	1.20	13.16	36	14.0	9.2	
23	1.94	0.68	3.08	15.76		12.0	8.2	
24	1.45	0.68	2.56	15.76	40	14.8		
25	0.48	0.0	4.28	11.93	42	12.0		25
26	0.0	0.68	0.68	12.85		16.8	9.2	

tration low, and this one has a high thyroxine.

(iii) The diiodotyrosine concentration is never high and only in eight cases does it exceed the average of the normal range (0.8 μg I/100 ml).

(iv) The monoiodotyrosine concentrations do not appear to be restricted as both normal and above normal values are observed.

These results for hyperthyroid patients may be put into the broad groupings shown in Table 27. It can be seen that at this stage, it is not possible to say which feature is of the greatest clinical importance. Is it the ratio of triiodothyronine to thyroxine, the level of monoiodotyrosine, the level of diiodotyrosine or none of these that determines whether or not a patient will respond to chemical treatment? Clearly it will be necessary to follow the changes of the concentrations of these iodoamino acids in the serum of patients under treatment. It may then be possible to predict whether or not a specific treatment will be successful for persons having a particular iodoamino acid distribution.

Analysis of Hypothyroid Sera

Sera from seventeen patients who were judged to be hypothyroid, by the same standards as those used for hyperthyroid patients, were analyzed and results obtained, together with the results of their laboratory tests recorded in Table 28.

Table 27

Classes of Iodoamino Acid distribution in hyperthroid sera

CLASS	MIT	DIT	T_3	T_4	No. of Cases	No. of cases with a high $\frac{T_3}{T_4}$ ratio
1	High	Normal	High	High	6	2
2	Normal	Normal	High	High	10	3
3	Normal	Normal	Normal	High	5	0
4	High	Low	Normal	High	1	0
5	Normal	Low	High	High	1	1
6	High	Normal	Normal	High	1	0
7	Normal	Low	High	Normal	1	1
8	Normal	Normal	Low	High	1	0

Table 28

Iodoamino Acid concentrations in the serum of hypothyroid patients

Patient	MIT µg/100 ml	DIT µg/100 ml	T_3 µg/100 ml	T_4 µg/100 ml	T_3 Uptake %	T_4 Uptake %	P.B.I. µgI/100 ml	I^{131} Neck Uptake %
1	3.39	0.68	0.85	3.67	27	1.5	0.9	7
2	2.90	0.85	0.68	2.76	20	2.3	2.1	
3	2.66	1.02	1.20	4.74		1.2	2.6	
4	1.21	0.51	1.54	5.96			2.7	
5	1.94	1.87	1.71	5.50	21		2.8	
6	1.94	1.70	1.03	6.27		1.0	2.8	2.2
7	2.18	1.36	1.03	2.90	24	0.1	1.6	
8	0.0	1.70	0.85	4.28	25	0.0	1.6	5
9	0.73	1.53	0.68	4.89	25	0.3	2.8	
10	0.73	1.19	2.22	4.89	22	0.0	0.8	4
11	3.39	1.36	1.20	7.50	21	0.0	1.6	
12	2.90	1.53	1.20	11.48	16	3.8	3.1	17
13	0.97	1.19	0.0	7.95	24	1.2	3.2	
14	1.45	0.68	0.68	6.58	25	0.0	1.3	
15	2.18	0.68	0.85	7.65	19	2.0	2.9	
16	2.18	0.51	0.68	9.48	22	0.0		15
17	2.90	0.51	0.85	7.34	25	0.0	2.9	

Again, comparing these analyses with the normal concentration ranges, it will be noted that the most dominant characteristics are:

(i) In ten out of seventeen cases, the thyroxine concentration is low and in no case is it high.

(ii) In twelve cases the triiodothyronine concentration is low, and in no case is it high.

(iii) In all cases either the triiodothyronine or the thyroxine is below normal concentration.

(iv) In half the cases the monoidotyrosine has an abnormally high concentration.

(v) The diiodotyrosine concentration is normal in all but three cases where it is low.

These results may be put into the broad groupings shown in Table 29.

Another eight cases who were suspected of being hypothyroid, were examined but at least one of the laboratory tests carried out on them indicated that they were within the normal range for that test. The analysis results for these eight are recorded in Table 30. It will be seen that of this eight, one belongs to class 1, 1 to class 3 and 1 to class 5. Four previously unobserved classes appear: one characterized by a high triiodothyronine and low thyroxine

Table 29

Classes of Iodoamino Acid distribution in hypothyroid sera

CLASS	MIT	DIT	T_3	T_4	No. of Cases
1	Normal	Normal	Normal	Low	2
2	Normal	Normal	Low	Low	3
3	Normal	Normal	Low	Normal	12
4	High	Normal	Low	Low	4
5	High	Normal	Low	Normal	3
6	High	Low	Low	Normal	2
7	Normal	Low	Normal	Low	1

Table 30

Iodoamino Acid concentrations in the sera of possibly hypothyroid patients

	MIT µg/100 ml	DIT µg/100 ml	T_3 µg/100 ml	T_4 µg/100 ml	T_3 Uptake %	T_4 Uptake %	P.B.I. µgI/100 ml	I^{131} Neck Uptake %
Patient								
1	0.0	3.91	2.91	8.57	25	0.0	4.2	
2	0.0	1.36	1.54	10.26	32	2.5	3.3	
3	2.66	0.85	3.08	8.57	34	2.9	2.9	
4	2.18	1.53	2.56	6.58	31	3.7	3.2	
5	1.69	1.36	0.0	9.94	30	2.1		
6	2.18	2.04	1.03	7.65	28	0.0	2.6	
7	1.45	0.51	3.94	5.66	25	0.0	2.6	19
8	1.69	1.19	1.37	5.66	26	2.5	3.1	

concentration, one by a high monoiodotyrosine level, one with a high diiodotyrosine level, and the third by high monoiodotyrosine and triiodothyronine. In the eighth case no abnormality was observed, in this case the T_3 uptake was well within the normal range (32%) and the protein bound iodine close to the normal (3.3 µg/100 ml). This could be a case of abnormal protein distribution, i.e. an increased concentration of thyroxine binding prealbumin.

Considering both these groups of hypothyroid patients, it is seen that we have a total of twelve different groups, on the basis of normal concentration levels, in 25 cases. Obviously, it is impossible to rule out any further variations, or to make any statements about the major type of hypothyroidism. Further study before, during and after treatment might lead to a greater understanding of the thyroid function and to some guidelines as to the successful treatment for particular iodoamino acid distribution.

4.5 Discussion of Analytical Procedure

The results recorded for the analysis of the serum samples were obtained directly from the gamma ray spectrum with the aid of a calibration curve. No corrections have been made for the recoveries from the separation procedure, although it was shown in Section 3.3, that when I^{131} labelled

compounds were used that approximately 85% of monoiodotyrosine, 75% of diiodotyrosine, 75% of triiodothyronine and 85% of thyroxine were recovered. Corrections were not made on the basis of these recoveries as it was believed that much of the losses indicated were due to radioactive impurities in the labelled compounds used, as discussed in Sections 1.6 and 3.3. It is difficult to devise experiments which could evaluate the recovery with sufficient precision to warrant the use of recovery factors. For example, it would seem that an accurate evaluation of the recoveries might be obtained by first analyzing the iodoamino acids in one batch of serum and then repeating the analysis after the addition of an accurately known quantity of unlabelled iodoamino acid to another aliquot of the same serum. However, the added iodoamino acid must not upset its equilibrium with the proteins. This restricts the quantity of iodoamino acid which can be used to "dope" the serum. The counting statistics of the I^{128} photopeak, obtained in the subsequent neutron activation analysis, make it difficult to obtain precise values for the separation recoveries. No method of determining the efficiency of recovery with sufficient accuracy to warrant its usage in the analytical procedure used has been devised.

As shown in Section 4.1, the reproducibility of the analysis is controlled by the counting statistics. The

statistical standard deviations due to the radioactive counting are approximately ± 40% for monoiodotyrosine, ± 25% for diiodotyrosine, ± 15% for triiodothyronine and ± 12% for thyroxine in the normal ranges. There is no other procedure which gives comparable analyses of blood sera. Furthermore, both hypothyroid and hyperthyroid subjects show concentration variations from the normal by more than twice the standard deviation. This analytical procedure therefore represents a promising clinical technique. However, it is obvious that it is desirable to improve the counting statistics if possible. This might be achieved by:

(i) increasing the volume of the original serum sample;

(ii) increasing the time of the irradiation, so that a greater proportion of the theoretical maximum iodine radioactivity is obtained;

(iii) decreasing the "cooling" time;

(iv) increasing the time for which the sample is counted.

It is not desirable for medical reasons to increase the quantity of serum used in each analysis for clinical use. Increasing the size of the serum sample would also make it necessary to carry out further concentrations of the samples prior to irradiation, thereby increasing the possibility of loss of iodine. (The volumes being irradiated in the above procedure are the maximum possible in the pneumatic rabbit

system of the McMaster Nuclear Reactor.)

An irradiation of 25 minutes would yield twice the radioactivity in the same sample as that obtained using 10 minutes irradiation. However, problems arise with the heating of the sample while it is in the reactor. In a 10 minute irradiation a 6 ml sample, frozen in liquid nitrogen immediately prior to the irradiation, melts . Longer irradiations cause the temperature to rise further and the pressure within the polyethylene vial builds up, so that in a 20 minute irradiation a significant proportion of the sample vials split and the sample is lost. Besides the loss of the sample, this also causes undesirable contamination of the rabbit container. A further problem with a long irradiation is that the long lived radioactivities are also produced in greater amounts, and the significance of the increased I^{128} radioactivity is not fully realized as the background radioactivity is proportionally even greater. It is not desirable to reduce the cooling time significantly as the interferences from short lived contamination, e.g. N^{13}, become significant. To get twice the number of counts in the peak by increasing the counting time would necessitate counting for 60 minutes instead of 20. The background, as it is partially electronic and partially due to longer lived isotopes, would increase by a factor of nearly three, so

that no improvement in the percentage standard deviation would be gained by counting for a longer time. Thus it is apparent that a significant improvement in the counting statistics is not feasible using the existing equipment. Improvements may be possible with such refinements as anti-coincidence counting to reduce the background, on a larger detector with a greater detection efficiency. Another possible way to improve the counting statistics would be to use a higher neutron flux in the irradiation, i.e. irradiate the sample in a reactor with a neutron flux in the region of 10^{14} neutron cm^{-2} sec^{-1}. It is also worth noting that at a flux of about 10^{12} neutron cm^{-2} sec^{-1} it would be impossible to obtain analyses such as reported in this work without significantly better counting systems than are currently in existence.

4.6 Concentrations of Iodoamino Acids in the Thyroid Gland

Robbins and Rall[71] published the degradation rates of the iodoamino acids in the serum of a euthyroid person. If these values are multiplied by the concentrations of the corresponding iodoamino acids in the serum, reported in Table 24 then one should obtain the rates of secretion of the iodoamino acids from the thyroid gland, neglecting any secondary sources of iodoamino acids and assuming equal distribution volumes. The results of this approach are shown in Table 31. If it is then proposed that there is no

preferential elution, the ratio of the secretion rates should then be the same as the ratio of the concentrations of the iodoamino acids in the thyroid gland. Unfortunately, the concentrations of the iodoamino acids in euthyroid people do not appear to be available. However, Pitt-Rivers and Cavaliri[70] have compiled a table of the ratios of the iodine distribution in the thyroid based on assumed isotopic equilibrium of I^{131}. The values obtained are as follows: monoiodotyrosine 19-35%, diiodotyrosine 38-56%, triiodothyronine 1-2%, thyroxine 7-19% and some inorganic iodide. The agreement is good for the iodotyrosines but the iodothyronines are different by factors of more than 2 in opposite directions. The significance of these values is open to question since Stolc[113], using rats and guinea pigs, showed that there were discrepancies between the concentration ratios of the iodoamino acids as studied with I^{131} tracer compared to the results obtained by chemical analysis of the stable iodine. For rates, Stolc reported that the organic iodine in the thyroid was 35% monoiodotyrosine, 48% diiodotyrosine, 5.5% triiodothyronine and 11% thyroxine using chemical analysis. The ratio using I^{131} depended to some extent on the time between the injection of the I^{131} and the taking of the blood sample; but, after 34 hours it was 32% monoiodotyrosine, 57% diiodotyrosine, 1.35% triiodothyronine and 10.7% thyroxine. For guinea pigs

Table 31

Secretion of Iodoamino Acids from the thyroid

Iodoamino Acid	Conc. in Serum µg/100 ml	Degradation rate (71)	Secretion Rate µg/hr	Proposed Thyroid Ratio
MIT	0.97	0.41/hr	0.40	38%
DIT	1.36	0.41/hr	0.56	53%
T_3	2.05	0.0093/hr	0.047	4.5%
T_4	9.80	0.0017/hr	0.041	3.9%

he reported analyses for stable iodine giving 35% monoiodotyrosines, 36% diiodotyrosine, 8% triiodothyronine and 21% thyroxine. The corresponding I^{131} results 48 hours after I^{131} injection were: 38.5% monoiodotyrosine, 47.7% diiodotyrosine, 1.77% triiodothyronine and 12.1% thyroxine.

Although direct analysis of the iodotyrosines in the serum has been uncertain it was fairly clear that the values were very much lower than that of the thyroxine. This is entirely different from the distribution in the thyroid itself. The two compartment model of the thyroid[114] has been proposed to account for these observations. In the two compartment model, the first compartment involves the iodination of the tyrosine and the coupling of the iodotyrosine residues to form the iodothyronines while the second compartment is concerned with the deiodination of the iodotyrosines. We see that the distribution of the iodoamino acids in the thyroid, calculated from the serum concentrations and the degradation rates in Table 25 agrees in general with the analysis of the thyroid and therefore the two compartment model is not necessary. The large differences in the concentrations between the thyroid and the serum may be explained in terms of their relative degradation rates.

It should be noted that the degradation rates on which this approach to the relative thyroid concentrations are based

are derived from *in vivo* I^{131} labelled iodoamino amino acid experiments. The interpretation of such results is based on the assumption that no exchange occurs between the labelled iodoamino acid and a different unlabelled iodoamino acid or the inorganic iodide. Such an assumption may or may not be valid. Of special concern in this direction are the triiodothyronine results as the triiodothyronine's 3' iodine atom appears to be the most labile iodine atom in any of these iodoamino acids. If such exchange occurred, then the true degradation rate would be slower than that indicated, and the corresponding concentration necessary in the thyroid gland would also be lower.

4.7 Interpretation of I^{131} Data

The experiments described in this work present analyses for iodotyrosines in serum, although there are many papers in the literature which indicate that these do not exist in the serum. In this section, an attempt is made to understand this difference.

Most of the evidence against the presence of iodotyrosines in serum has been obtained from the inability of research workers to find any radioactivity in the iodotyrosines on chromatograms of serum after the *in vivo* administration of radioactive iodine[13,14,15]. In these experiments the key observations are that after administration of radioactive inorganic iodide it is possible to observe

the presence of the labelled iodothyronines within a few hours, but that no labelled iodotyrosines are found in these chromatographic studies. There are two types of mechanisms whereby radioiodine may be incorporated in the iodoamino acids. One method is through synthesis and the other through isotopic exchange. Since the degradation rate of thyroxine is as slow as 10%/day, a considerable period of time must be required to obtain a steady state distribution of the radioiodine in the iodoamino acids by synthetic routes. Thus, any deductions concerning the distribution of iodoamino acids based on the distribution of radioiodine would require a continuous intake of radioiodine and a period of the order of 20 days in order to obtain a steady state distribution.

Exchange of the radioiodine with the iodine in the various iodoamino acids is much more rapid, the common preparation for the labelled iodoamino acids being through this mechanism (see section 1.6).

Stolc[113] studied rat thyroid and Feuer[115] studied the rat serum after injection of I^{131}. Feuer observed a rapid rise in the radioactivity of the serum thyroxine with the maximum I^{131} incorporation 8 hours after injection, while after only 1 hour about 80% of this maximum radioactivity was obtained. Stolc, in studying the thyroid gland, observed a much slower rise in the radioactivity incorporated into the thyroxine and only after 34

hours did this incorporation reach a maximum. Thus it would appear that the rapid incorporation of the radioactivity by the thyroxine in the blood is due to isotopic exchange of iodine atoms. The combined effects of the greater lability of the 3' and 5' positions of the iodothyronines and their longer biological half-lives, as compared to the iodotyrosines, ensure that the radioactivity incorporated in the iodothyronines completely dominates that of the iodotyrosines. It will be observed from the results reported by Stolc that the incorporation of the radioactivity into the iodotyrosines in the thyroid occurs rapidly, probably by synthesis, since the iodine entering the thyroid becomes organically bound very rapidly. The question may then be asked, "If the iodotyrosines incorporate radioactivity in the thyroid and all four iodoamino acids are secreted from the thyroid at rates proportional to their concentrations within the thyroid, then why are the iodotyrosines not observed in the blood serum?" The reason for this probably lies in the dilution factor involved in the thyroid. It is reported[116] that the normal thyroid contains about 8,900 µg iodine. Using the average euthyroid values obtained from the analyses reported in this thesis, 0.4, 0.8, 1.3 and 6.3 µg I/100 ml for monoiodotyrosine, diiodotyrosine, triiodothyronine and thyroxine respectively, and multiplying

the sum of these, 8.8 µg I/100 ml, by the average volume of serum (2.6 litres) a serum iodine content of 220 µg is obtained. These figures show that the thyroid contains 40.5 times as much iodine as the serum. In the normal administration of radioiodine by either injection or ingestion the radioiodine is first in contact with the serum. Although there would be little synthesis of the iodoamino acids in the serum, exchange would occur. This exchange with the iodotyrosines may be slower than with the iodothyronines and could lead to their lower specific activities, but the more important factor is the rates of their degradation. The iodotyrosines degrade 100 times faster than thyroxine and 20 times faster than triiodothyronine (Table 31). The iodoamino acids are replaced in the blood serum from the thyroid and the specific activity of the iodoamino acids coming from this source must be lower by a factor of about 40 as shown above. Even if all the iodine entering the thyroid went initially into the monoiodotyrosine the specific activity would be reduced by a factor in excess of 10.

At early times after administration of the radioiodine it is probable that the iodothyronines coming from the thyroid will have lower specific activities than the iodotyrosines. Because the specific activity of the iodothyronines in the serum was determined by the initial exchange

and their degradation rates are slow, the relative specific activities of the iodothyronines remain greater than those of the iodotyrosines for a considerable period of time after administration.

As indicated at the beginning of this section, it is necessary to wait more than twenty days before one can be sure that the specific activities of the various iodine compounds are the same. For this work, the short-lived iodine isotope, I^{131}, is unsuitable as it has a half-life of only 8 days and large administrations can influence the thyroid function. However, Globel[17] used a single 25 μCi does of I^{131} and followed the relative radioactivities in the iodinated compounds in the serum for 26 days. He was unable to observe any radioactivity in the iodotyrosines for the first 8 days while after 26 days 10% of the radioactivity was in the iodotyrosines, this is in good agreement with the approximately 14% reported here. The only really satisfactory method for investigating the relative concentrations of the iodoamino acids in the serum would be maintenance of the subject on a radioactive iodine diet, with the specific radioactivity adjusted to the biological half-life of the iodine isotope, for a period of several weeks. This is undesirable with human subjects owing to the radiation doses involved. Pitt-Rivers and Rall[18] fed rats on a I^{131} diet for 14-35 days, and obtained

a thyroid distribution of approximately 25% monoidotyrosine, 52% diiodotyrosine, 3% triiodothyronine, 18% thyroxine and a serum distribution of approximately 93% thyroxine, 4% triiodothyronine and 2% iodotyrosines. However, these values cannot be compared with the values calculated in Section 4.7 as the degradation rates are different for the rat, i.e. the degradation rate of thyroxine is 92%/day and of triiodothyronine is 170%/day in the rat.

CONCLUSIONS

In this thesis it has been shown that, contrary to most of the published reports using radioactive iodine as a tracer, but in agreement with most of the published chemical analyses, there are significant levels of iodine in the blood in the form of iodotyrosines. In euthyroid people the organically bound iodine in the blood serum is about 4.5% monoiodotyrosine, 9% diiodotyrosine, 13.5% triiodothyronine and 73% thyroxine.

It has been shown that these results are consistent with a kinetic mechanism and the relatively large amounts of the iodotyrosines with the iodothyronines found in thyroid. The apparent disagreement in regard to the iodotyrosine analyses of the present work compared to that in other laboratories using tracer studies is reconciled in terms of the rates of iodine exchange.

The analyses of hypothyroid and hyperthyroid sera clearly indicate that there are a number of iodoamino acid distributions or subdivisions within each of these thyroid disorders. It would seem logical that each of these different abnormalities of iodoamino acid distributions are due to a different cause, or combination of causes, and would therefore probably be best treated by different methods.

The procedures developed in this thesis offer a method of analysis for such studies.

BIBLIOGRAPHY

1. T. WHARTON

   Adenographia (1656)

2. E. BAUMANN

   Z. Physion Chem. $\overline{21}$ 319 (1896)

3. E. BAUMANN

   Munch Med, Wochsohr. $\overline{43}$ 309 (1896)

4. E. C. KENDALL

   J. Amer. Med. Assoc. $\overline{64}$ 2042 (1915)

5. C. R. HARINGTON, G, BARGER

   Biochem. J. $\overline{21}$ 169 (1927)

6. J. GROSS, R. PITT-RIVERS

   Lancet $\overline{1}$ 439 (1952)

7. E. M. SMITH, J. M. MOZLEY, H. N. WAGNER, Jr.

   J. Nucl. Med. $\overline{5}$ 828 (1964)

8. C. W. TANG, R. H. TOMLINSON

   Nuclear Activation Techniques in the Life Sciences

   Vienna, 1967. I.A.E.A. pp. 427

9. C. KELLERSHOHN, D. COMAR, C. LEPOEC

   Int. J. Applied Radiat. Isotop. $\overline{12}$ 87 (1961)

10. E. FERMI

    Nature $\overline{133}$ 757 (1934)

11. S. HERTZ, A. ROBERTS, R. D. EVANS

Proc. Soc. Exp . Biol. (N.Y.) 38 510 (1938)

12. J. G. HAMILTON, M. H. SOLEY

Amer. J. Physiol. 127 557 (1939)

13. S. C. WERNER

Nature 183 406 (1959)

14. R. J. BLOCK,S. C. WERNER, R.H. MANDL, V. V. ROW, I. RADICHEVICH

Archives Biochem Biophys. 88 98 (1960)

15. S. LISSITZKI, J. BISMUTH , C. SIMON

Nature 199 1002 (1963)

16. S. C. WERNER, I. RADICHEVICH

Nature 197 877 (1963)

17. B. GLÖBEL

Radionuklide in Kreislaufforschung und Kreislaufdiagnostik Wien 1967 p. 499.

18. R. PITT-RIVERS, J. RALL

Endocrinology 68 309 (1961).

19. A. TAUROG, J. C. PORTER, D. T. THIO

Endocrinology 74 902 (1964)

20. K. MATSUDA, M. A. GREER

Endocrinology 76 1012 (1965)

21. J. WYNN

Archives Biochem. Biophys. 87 120 (1960)

22. R. H. MANDL, R. J. BLOCK

Archives Biochem. Biophys. 81 25 (1959)

23. T. KONO, L. A. MIDDLESWORTH, E. B. ASTWOOD

Endocrinology 66 845 (1960)

24. M. L. WELLBY, B. S. HETZEL

Nature 193 752 (1962)

25. A. DIMITRIADOU, R. FRASER, P.C.R. TURNER

Nature 201 575 (1964)

26. E. T. BACKER,Th. J. POSTMES, J. D. WIENER

Clin. Chem. Acta 15 77 (1967)

27. A. TAUROG, I. CHAIKOFF

J. Biol. Chem. 176 639 (1948)

28. V. A. GALTON, R. PITT-RIVERS

Biochem. J. 72 310 (1959)

29. R. PITT-RIVERS, B. I. SACKS

Biochem. J. 82 111 (1962)

30. R. J. BLOCK, R. H. MANDL

Biochem. J. 81 37P (1961)

31. F. BJORKSTEN, R. GRÄSBECK, B. A. LAMBERG

Acta Chem. Scand. 15 1165 (1961)

32. B. L. BROWN, W. S. REITH, W. TAMPION

Biochem. J. 97 30P (1965)

33. E. H. MOUGEY, J. W. MASON

Anal. Biochem. 6 223 (1963)

34. G. HOCMAN

J. Chromatography 21 413 (1966)

35. E. MAKOWETZ, K. MÜLLER, H. SPITZY

Microchem. J. 10 194 (1966)

36. F. BLASI, R. V. DeMASI

J. Chromatography 28 33 (1967)

37. B. M. DOBYNS, S. R. BARRY

J. Biol. Chem. 204 517 (1953)

38. J. ROCHE, R. MICHEL

Methods of Biochemical Analysis 12 143 (1964)

39. B. A. RHODES, H. N. WAGNER , JUN.

Nature 210 647 (1966)

40. M. W. O'HALLORAN, M. L. WELLBY

Nature 211 1195 (1966)

41. B. MALAMOS, C. J. MIRAS, J. N. KARLI SAMOUIBDOU, D. A. KOUTRAS

J. Endocrin 35 223 (1966)

42. S. B. BARKER, M. J. HUMPHREY

J. Clin. Endocrinol. 10 1136 (1950)

43. K. G. SCOTT, J. C. REAVIS, W. W. SAUNDERS, W. E. WHITE

Proc. Soc. Exp. Biol. Med. 76 592 (1951)

44. J. J. MORAN

Anal. Chem. 24 378 (1952)

45. A. L. CHANEY

Ind. Eng. Chem., Anal. Ed. 12 179 (1940)

46. B. ZAK, H. H. WILLARD, G. B. MYERS, A. J. BOYLE
Anal. Chem. 24 1345 (1952)

47. V. STOLC, J. KNAPP
Mikrochim. Acta. 941 (1963)

48. C. H. BOWDEN, N. F. MACLAGAN, J. H. WILKINSON
Biochem. J. 59 93 (1955)

49. V. J. PILEGGI, G. KESSLER
Clin. Chem. 14 339 (1968)

50. E. T. BACKER, J. D. WIENER
Clin. Chem. Acta. 20 155 (1968)

51. J. E. JONES, J. S. SCHULTZ
J. Clin. Endocrin. Metab. 27 877 (1967)

52. R. GMELIN, A.I. VIRTANEN
Acta. Chem. Scand. 13 1469 (1959)

53. P. F. MULVEY, J. A. CARDANELLI, M. ZOUKIS, R.D. COOPER, B. A. BURROWS, J. Nucl. Med. 7 603 (1966)

54. M. H. FELDMAN, J. McNAMARA, R. C. REBA, W. WEBSTER
J. Nucl. Med. 8 123 (1967)

55. M. W. HAMOLSKY, M. STEIN, A. S. FREEDBERG
J. Clin. Endocrin. Metab. 17 33(1957)

56. W. N. TAUXE, M. Y. YAMAGUCHI
Amer. J. Clin. Path. 36 1 (1961)

57. V. HERBERT, C. W. GATTLIEB, K.S. LAU, P. GILBERT, S. SILVER
J. Lab. Clin. Med. 66 814 (1965)

58. W. J. IRVINE, R. M. STANDEVEN

J. Endocrin. 41 31 (1968)

59. G. B. McADAMS, R. F. REINFRANK

J. Nucl. Med. 5 112 (1964)

60. D. L. TABERN, J. KEARNEY, A. DOLBOW

J. Nucl. Med. 8 740 (1967)

61. J. F. SCHOLER

J. Nucl. Med. 3 41 (1962)

62. F. H. FOECKLER, Jr., N. M. BERING, J. SIRAGUSA, O. B. HUNTER, Jr.

J. Nucl. Med. 4 239 (1963)

63. W. N. TAUXE, T. KUSAKABE

Radioisotopes in Medicine - In vitro studies.

U.S.A.E.C. 273 (1968)

64. L. OLIVER, J. C. HARRIS

J. Nucl. Med. 5 218 (1964)

65. B. SHAPIRO, J. L. RABINOWITZ

J. Nucl. Med. 3 417 (1962)

66. I. S. JENKINSON, J. D. YOUNG

J. Nucl. Med. 7 281 (1966)

67. B. E. P. MURPHY

J. Lab. Clin. Med. 66 161 (1965)

68. B. E. P. MURPHY

Radioisotopes in Medicine - In vitro studies.

U.S.A.E.C. 43 (1968)

69. A. CUARON

J. Nucl. Med. 10 532 (1969).

70. R. PITT-RIVERS, W. R. TROTTER in The Thyroid Gland, Vol. 1. p. 87, Butterworths, London (1964)

71. C. H. GRAY, A. L. BACHARACH, Hormones in the Blood, Vol. 1 p. 383, Academic Press, New York 1967.

72. F. BLASI

Biochim. Biophys. Acta 121 204 (1966).

73. A. NISHINGA, H. J. CAHNMANN, H. KON, T. MATSUURA

Biochemistry 7 388 (1968)

74. J. B. STANBURY

Ann. N. Y. Acad. Sci. 86 417 (1960).

75. J. ROBBINS

Archives Biochem. Biophys. 63 461 (1956)

76. S. C. WERNER, ARchives Biochem. Biophys. 75 508 (1958).

77. K. MIYIA, F. K. ITOH, H. ABE, Y. KUMAHARA

Clin. Chem. Acta. 22 341 (1968)

78. W. DEISS, E. ALBRIGHT, F. LARSON

Proc. Soc. Exp. Biol. Med. 84 513 (1953)

79. F. LARSON, E. ALBRIGHT

Endocrinology 56 737 (1955)

80. S. TANAKA, P. STARR

J. Clin. Endocrin. Metab. 19 485 (1959)

81. J. ROBBINS, J. RALL

J. Clin. Invest. 34 1331 (1955)

82. J. ROBBINS, J. RALL

J. Clin. Invest. 34 1324 (1955)

83. J. ROBBINS, J. RALL

Recent Progress in Hormone Research 13 161 (1957)

84. L. E. BRAVERMAN, S. H. INGBAR

Endocrinology 76 547 (1965)

85. K. STERLING, M. TABACHNICK

J. Biol. Chem. 236 2241 (1961)

86. G. L. TRITSCH

Archives Biochem. Biophys. 127 384 (1968)

87. C. OSARIO

Proceedings Intern. Symp. on Salicylates.

Lond. 82 (1962)

88. P. K. BONDY, M. A. HAGEWOOD

Proc. Soc. Exp. Biol. Med. 81 328 (1952)

89. B. S. HETZEL, W. W. SCHOTTSTAEDT, W. J. GRACE, H. G. WOLFF

J. Psychosom. Res. I 177 (1956)

90. T. YAMADA, A. LEWIS

Endocrinology 82 91 (1968)

91. R. HENINGER, E. ALBRIGHT

Endocrinology 79 309 (1966)

92. R, M. LEMMON, W. TARPEY, K G. SCOTT

J. Amer. Chem. Soc. 72 758 (1950)

93. W. H. MILLER, G. W. ANDERSON, R. K. MADISON, D. J. SOLLEY
Science 100 340 (1944)

94. E. FRIEDEN, M. B. LIPSETT, R. J. WINZLER
Science 107 353 (1948)

95. A. HOYE
Acta. Chem. Scand. 22 695 (1968)

96. P. SÜE
Proc. Intern. Congr. Pure and Applied Chem. 11 279

97. A. CRITCHLOW, M. GOLDFINCH
Proc. Ind. Radioisotopes Conf. Oxford. Medical and Physiol. Applications 271 (1954)

98. M. ANDREOLI, J. ROBBINS, J. RALL, M. BERMAN
Proc. 5th Intern. Thyroid Conf. 635 (1964)

99. G. O. F. LIPPERT,J. A. MEINTJES
South African J. Lab. Clin. Med. 3 57 (1957)

100. R. J. BAYLY, E. A. EVANS
J. Label. Compounds 2 1 (1966)

101. E..M. VOLPERT, M. MORTINEZ, J. H. OPENHEIMER
J. Clin. Endocrin. Metab. 27 421 (1967)

102. J. R. TATA
Clin. Chem Acta. 4 427 (1959)

103. J. LIEBSTER, J. KOPOLDOVA
Advances in Radiation Biology 1 157 (1964)

104. W. T. BARNES
J. Clin. Endocrin. Metab. 23 222 (1963)

105. TETRASORB

Radio Pharmaceuticals: Abbott Laboratories

106. J. B. DALTON, P. L. KIRK, C. L. A. SCHMIDT

J. Biol. Chem. 88 589 (1930)

107. P. S. WINNEK, C. L. A. SCHMIDT

J. Gen. Physiol. 18 889 (1935)

108. J. R. TATA

Biochem. J. 72 214 (1959)

109. F. GIRARDI, E. SABBONI

J. Radioanal. Chem. T. 169 (1968)

110. J. A. NAUMAN, A. NAUMAN, S. C. WERNER

J. Clin. Invest. 46 1346 (1967)

111. V. A. GALTON, R. PITT-RIVERS

Endocrinology 64 835 (1959)

112. R. PITT-RIVERS

Biochem. J., 82 108 (1962)

113. V. STOLC

Endocrinology 71 564 (1962)

114. C. PINCUS, K. V. THIMANN, E. B. ASTWOOD

The Hormones, Vol. 5, pp. 159.

Academic Press. New York. 1964

115. G. FEUER

Biochem. J. 73 349 (1959)

116. A. B. GUTMAN, E. M. BENEDICT, B. BAXTER, W. W. PALMER

J. Biol. Chem. 97 303 (1932)

117. W. O. THOMPSON, L. L. McLELLAN, P. K. THOMPSON, L. F. N. DICKIE

J. Clin. Invest. 12 235 (1933)

118. E. T. BACKER, V. J. PILEGGI

J. Chromatography 36 351 (1968)

119. P. I. JAAKANMAKI, J. E. STOUFFER

J. Gas Chromatography 5 303 (1967)

120. D. COMAR

Advances in Activation Analysis, Vol. 1, p. 170 (Acad. Press 1969).