SUMMARY

1. The cellular components of human blood are shown to be a convenient system for studies of fatty acid and lipid synthesis and exchange.

2. After incubation of whole blood with [$Me-^{14}C$]acetate all the common saturated and unsaturated acids are found to be labelled, including the mono- and poly-unsaturated acids of a variety of chain lengths.

3. The 'essential' fatty acids arachidonic acid, and linoleic acid or linolenic acid or both, are also labelled.

4. The long-chain fatty acids are incorporated into triglycerides and some neutral lipids of undefined structure, as well as into phospholipids, but not into cholesterol esters.

5. The triglycerides and other neutral lipids are rapidly secreted into plasma, where they are incorporated into the plasma α -lipoproteins but not into β -lipoprotein.

6. The phospholipids are more readily incorporated into the plasma β -lipoproteins.

We should like to thank Mr H. Hadaway for a great deal of expert technical assistance.

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The Localization of ^{131}I in Biosynthetically Labelled [^{131}I]Thyroxine

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Work in this Laboratory on the biosynthesis and peripheral metabolism of thyroid hormones necessitated a method for the degradation of thyroxine whereby the proportions of radioiodine present on each of the two aromatic rings could be determined. The diazo-coupling reaction described in this paper was the best of several chemical reactions investigated with a view to displacing iodine specifically from the ring distal to the alanine side chain. Thyroxine, a phenolic coupling component, has no unsubstituted position *ortho* or *para* to the hydroxyl group. On coupling therefore iodine must be displaced from organic combination or the diphenyl-ether linkage is destroyed. The reaction has been studied by other workers, but never in conjunction with isotopic-tracer techniques. Komant (1950) coupled thyroxine with diazotized 2:4-dichloro-aniline. Barac & Morren (1953) studied the reaction

with sulphanilic acid and *p*-arsanilic acid. They isolated the *p*-arsanilic acid azo-compound in 60% yield and claimed that it was an azo-substituted tri-iodothyronine. It was then apparent that a large proportion of the diazo-coupling occurs by the elimination and replacement of the *ortho* substituent.

At best therefore in a reaction between equimolecular quantities of thyroxine and diazonium salt, where coupling is confined to the *ortho* position, a product of structure (I) is to be expected, where R is a substituted aryl nucleus. If the structural formula is correct, it follows that when [$3:5-^{131}I_2$]thyroxine is subjected to diazo-coupling, no radioiodine is displaced from organic combination. If the ^{131}I were present on both rings in the same amounts, 25% would be converted into inorganic iodide. On theoretical grounds therefore it is

likely that a linear relationship exists between the percentage of ^{131}I eliminated from the organic molecule, and the percentage of it which was situated in the 3':5' positions. This paper shows

that the distribution of ^{131}I within the molecule of labelled thyroxine may be determined by subjecting the compound to diazo-coupling and measuring the percentage of its radioiodine content which is eliminated from organic combination.

METHODS

Preparation of thyroxine samples. [3':5'- $^{131}\text{I}_2$]Thyroxine was prepared by the method of Gross & Leblond (1950). [3:5- $^{131}\text{I}_2$]Thyroxine was prepared by a micromodification of the method of Michel, Roche & Tata (1952). All the [^{131}I]thyroxine samples were purified before use by descending chromatography on Whatman no. 3MM paper in the solvent butan-1-ol-pentan-1-ol-aq. 2N-NH₃ soln. (1:1:2, by vol.). The thyroxine band was located by radioautography and eluted into ethanol-aq. 2N-NH₃ soln. (1:1, v/v).

Choice of amine. It was clearly desirable that the coupling reaction should occur as extensively as possible, and yet be very selective in its point of attack on the thyroxine molecule. The aim was a quantitative conversion of thyroxine into a homogeneous azotri-iodothyronine product. The work of Komant (1950) indicated that this might be almost obtainable with a 20% excess of 2:4-dichloroaniline. The yield was greatly superior to those claimed for sulphanilic acid and *p*-arsanilic acid (Barac & Morren, 1953).

In some preliminary trials, [3:5- $^{131}\text{I}_2$]thyroxine was coupled with 2:4-dichloroaniline and with sulphanilic acid, by the methods described by the above authors. The products were chromatographed on Whatman no. 3MM paper in the solvent systems butan-1-ol-2N-acetic acid (1:1) and butan-1-ol-dioxan-aq. 2N-NH₃ soln. (4:1:5, by vol.). The positions of the peaks of radioactivity were determined by use of an automatic scanning device. For the coupled product derived from 2:4-dichloroaniline, the greater part of the radioactivity ran as a single peak close to the solvent front in both solvents. Similar chromatographic trials with the sulphanilic acid derivative separated it into multiple components.

2:4-Dichloroaniline was therefore employed in most of the present work, since its coupled product with thyroxine is both obtainable in higher yield and appears to be more nearly homogeneous than that of sulphanilic acid. The term 'diazonium salt' is hereafter used without qualification to denote 2:4-dichlorobenzene-diazonium chloride.

Experimental procedure. Stable sodium L-thyroxine (20 mg.) was added to a solution of [^{131}I]thyroxine (0.5 μC) in ethanol-aq. 2N-NH₃ soln. (1:1, v/v), and the sample evaporated to dryness under vacuum. The residue was re-

dissolved in 10 ml. of 0.5N-NaOH, reprecipitated with 0.7 ml. of conc. HCl and the mixture was centrifuged. The supernatant was discarded. The thyroxine precipitate was dissolved in 2 ml. of water and 0.6 ml. of 2N-NaOH and the solution cooled in ice. The diazonium solution was prepared by adding, dropwise and with stirring, 1.7 ml. of 5% (w/v) NaNO₂ to a solution of 200 mg. of 2:4-dichloroaniline in 14 ml. of 2N-HCl, all at less than 5°; 2.77 ml. of water was then added. A portion (0.5 ml.) of the diazonium solution was added to the cooled thyroxine solution, drop by drop, with vigorous stirring. The pH was next varied by alternately adding 0.5 ml. volumes of 2N-HCl and of 2N-NaOH. Three complete oscillations of pH were carried out in this way, but the final addition of alkali was doubled in quantity. The sample was then stored at 0° for 16 hr., and the azo-compound was precipitated by addition of 2 ml. of 2N-HCl and centrifuged.

The extreme insolubility of the azo-compound in acid was thus exploited to separate it from inorganic iodide.

Separation. After centrifuging for 15-20 min. at 900 g the supernatant fluid was removed and its volume adjusted to 10 ml. The precipitate was then dissolved in 0.2N-NaOH-aq. 40% (v/v) ethanol-aq. 5.4% Teepol 530 (2:1:1, v/v) and the volume of this solution was also adjusted to 10 ml.

Assay of ^{131}I . This was carried out in a Geiger-Müller liquid counting tube (20th Century Electronics, type M6) having a sensitivity of 12 400 counts/min./ μC . Five-minute counts were made on both supernatant and precipitate. Although the supernatants and precipitates were dissolved in different solvents, this did not necessitate a correction factor. There was no detectable difference between the absorbance of ^{131}I β -radiation by the two solutions.

RESULTS

The reaction was first carried out on a series of thyroxine samples prepared by mixing together the two radioactively labelled varieties in different, known proportions. The results are shown in Fig. 1. The marked difference between the percentage precipitations obtained with the two kinds of labelled thyroxine confirms the view that iodine is more easily released from the 3':5' positions than from the 3:5 positions. However, the slope drawn from the experimental results is not nearly so steep as the theoretical graph.

Thus at this stage the method was effective but inefficient in distinguishing between [3:5- $^{131}\text{I}_2$] and [3':5'- $^{131}\text{I}_2$]thyroxine. If the standard curve (Fig. 1) ranges from 95% precipitation for [3:5- $^{131}\text{I}_2$]thyroxine to 75% for 3':5'-labelled thyroxine, an experimental result of 85% should indicate that the thyroxine sample is equally labelled on both rings. Since the measurements are subject to an experimental error of about 2%, however, the correct result for the percentage of ^{131}I in the 3':5' positions could lie anywhere between 40 and 60%.

It was accordingly necessary to study the mechanism and conditions of the reaction with a view to improving the slope of the standard curve. The unexpectedly high percentage precipitation

figure for [$3':5'-^{131}\text{I}_2$]thyroxine was found to be due partly to a reaction between the released inorganic iodide and the diazonium salt, in which the radioactivity was reincorporated into the precipitable fraction. When this reaction was studied by coupling stable thyroxine with diazonium salt in the presence of inorganic radioiodide, it was found that 36 % of the radioactivity was precipitated along with the reaction products. This effect could be diminished either by diluting the radioiodide with carrier NaI or by carrying out the reaction in the presence of sodium thiosulphate (Table 1).

When [^{131}I]thyroxine was coupled under these conditions the reaction was found to be more efficient in distinguishing between [$3:5-^{131}\text{I}_2$] and [$3':5'-^{131}\text{I}_2$]thyroxine (Table 2), but the precipitation figure for [$3':5'-^{131}\text{I}_2$]thyroxine, 65–70 %, still differs seriously from the theoretical figure of 50 %. Side reactions apparently consume much of the diazonium salt and render it unavailable for coupling. It was therefore necessary to employ a larger excess of the diazonium salt to compensate for this loss. Table 3 shows that, provided sufficient thiosulphate is added, more iodine can be displaced in this way from organic combination, thus improving the accuracy of the method.

Use of m-nitroaniline. Although 2:4-dichloroaniline couples well at pH values which render thyroxine readily soluble, it cannot be used profitably in quantities greater than 2 moles/mole of thyroxine, because the reaction is terminated prematurely by the precipitation of the coupled products. It was found possible to remedy this defect by replacing 2:4-dichloroaniline with an amine whose azo-compound was less hydrophobic. *m*-Nitroaniline, which proved to be the most

Fig. 1. Precipitation results obtained by coupling 2:4-dichlorobenzenediazonium chloride with the two kinds of [^{131}I]thyroxine, mixed in known proportions. Ordinate values show the percentage of total thyroxine ^{131}I not liberated on diazo-coupling.

Table 1. *Coupling of thyroxine with 2:4-dichlorobenzenediazonium chloride: effect of sodium iodide or thiosulphate on the binding of $^{131}\text{I}^-$ into the precipitable fraction of the reaction products*

Additions made to thyroxine solutions before coupling	Percentage of ^{131}I in ppt.
^{131}I only	36.3
$^{131}\text{I} + \text{NaI}$ (6.5 mg.)	25.2
$^{131}\text{I} + \text{NaI}$ (26.0 mg.)	12.1
$^{131}\text{I} + \text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ (1.2 mg.)	19.2
$^{131}\text{I} + \text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ (5.4 mg.)	5.5
$^{131}\text{I} + \text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ (10.8 mg.)	4.6

Table 2. *Coupling of [^{131}I]thyroxine with 2:4-dichlorobenzenediazonium chloride: effect of sodium iodide or thiosulphate on the release of thyroxine ^{131}I from the precipitable fraction of the reaction products*

Additions to thyroxine solutions before coupling	Precipitation (%)		
	[$3:5-^{131}\text{I}_2$]Thyroxine	[$3':5'-^{131}\text{I}_2$]Thyroxine	Difference
None	95.0	76.1	18.9
NaI (26.0 mg.)	91.9	67.2	24.7
$\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ (5.4 mg.)	95.6	69.6	26.0

Table 3. *Coupling of [$3':5'-^{131}\text{I}_2$]thyroxine with 2:4-dichlorobenzenediazonium chloride: effect of excess quantities of diazonium salt on the release of thyroxine ^{131}I from the precipitable fraction of the reaction products*

Diazonium salt (moles/mole of thyroxine)	Precipitation (%)		
	No addition	With 2.7 mg. of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$	With 10.8 mg. of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$
1:20	76.3	69.6	74.7
1:56	—	65.9	67.5
1:68	76.2	—	—
1:92	—	63.6	63.6
2:20	85.7	70.0	60.8

suitable, could be employed in 260 % excess, and the results were the most favourable yet obtained. [3:5-¹³¹I₂]Thyroxine gave 96 % precipitation and [3':5'-¹³¹I₂]thyroxine 50 %. Since the accuracy of the method had been thus improved by 130 % the use of *m*-nitroaniline was adopted as a routine procedure. Complete experimental detail for this modification of the reaction procedure is therefore given below.

Recommended experimental procedure. [¹³¹I]-Thyroxine (20 mg.), purified by reprecipitation as described above, is dissolved in 5.2 ml. of 0.463 *N*-NaOH; 0.1 ml. of 0.218 *M*-Na₂S₂O₃ is then added and the mixture cooled in ice to less than 5°. The diazonium solution is made by dissolving 215 mg. of *m*-nitroaniline HCl in 10.5 ml. of 2 *N*-HCl, cooling below 5° and mixing in 1.7 ml. of 5 % (w/v) NaNO₂ dropwise; 1.66 ml. of water is added. Coupling is carried out by addition of 1 ml. of the diazonium solution to the thyroxine solution, drop by drop, with vigorous stirring. The following additions are made to cause pH variations which result in a permanent deepening of the colour of the solution: 0.4 ml. of 2 *N*-HCl, 0.3 ml. of 2 *N*-NaOH, 0.3 ml. of 2 *N*-HCl, 0.3 ml. of 2 *N*-NaOH, 0.3 ml. of 2 *N*-HCl, 0.6 ml. of 2 *N*-NaOH. The mixture is stored overnight (16 hr.) at 0° and the reaction products are finally precipitated with 1 ml. of 2 *N*-HCl. The supernatant fluid is then separated from the precipitate by centrifuging, and this is followed by assay of ¹³¹I. The percentage of the ¹³¹I of the original thyroxine sample in the 3':5' position can then be read off from the standard slope. If percentage precipitation is 73.0 ± 2.0 then the percentage of ¹³¹I in the 3':5' positions is 50.0 ± 4.5.

DISCUSSION

The coupling reaction between thyroxine and diazonium salts was described by Komant (1950) as 'atypical', because a pre-existing substituent had to be eliminated from the molecule before coupling could occur. Thyroxine is a fairly complex molecule to employ as a coupling component, and this complexity multiplies the number of theoretically possible ways in which a diazo-compound could react with it. The side chain bears an α-amino group which is available for triazen formation. Furthermore, phenol ethers are known to behave in an exceptional way on coupling; the ether linkage is broken but the azo group does not take up the position which this linkage formerly occupied (Saunders, 1949). The existence of these possibilities, together with simple coupling in the *ortho* or *para* position, indicates that the possible structures for the reaction products in the so-called 'azotri-iodothyronine' precipitate are many and diverse. Although chromatography of the reaction products obtained from 2:4-dichloro-

aniline failed to separate them into different components, Professor C. Rimington (personal communication) has found that these reaction products can be separated into two different fractions, one of which is readily soluble, and the other only sparingly soluble, in sodium hydroxide solution.

In view of the multiplicity of possible side reactions it is not surprising that when thyroxine and diazonium salt are employed in equimolecular proportions, the displacement of iodine from the 3':5' positions is incomplete. Side reactions are apparently active in consuming the diazonium salt well before all the thyroxine has become involved in the reaction. Chromatography of the reaction products and subsequent staining of the chromatograms with ceric sulphate (Bowden, MacLagan & Wilkinson, 1955) showed that even after 1.44 moles of the diazonium salt had been added per mole of thyroxine, some unchanged thyroxine was still readily detectable.

Some interest attaches to the cause of the displacement of iodine from the 3:5 positions. It seems unlikely on theoretical grounds that diazo-coupling can occur in those positions while the diphenylether link is intact. If it were ruptured, however, coupling might well occur readily with the fragment, as it does with 3:5-di-iodotyrosine.

The solubility properties of thyroxine limit the range of amines with which it can be satisfactorily coupled. In order to dissolve the thyroxine, very alkaline conditions are required; this inhibits the coupling reaction by converting the diazohydroxide into an inactive *isodiazoate*. The investigator is therefore restricted to the use of amines whose diazohydroxides are stable at unusually high pH values.

Application of the method. In applying the diazo-coupling method to thyroxine from biological sources, it was necessary to take more stringent precautions for the purification of the samples. These were chromatographed in two or three different solvents before being finally eluted, mixed with carrier and reprecipitated. Not only was it necessary thus to separate the thyroxine from other radioactive metabolites, but much unwanted non-radioactive material was always present which had to be removed lest it should contain phenols or amines capable of coupling with the diazonium salt and thereby consuming it.

Provided that careful chromatographic purification was carried out, reproducible results were always obtainable.

SUMMARY

1. The coupling reaction between thyroxine and diazonium salts has been investigated with thyroxine labelled in either ring.

2. The results indicate that coupling occurs readily in the 3':5' positions, with the elimination

of iodine. Coupling in the 3:5 positions occurs only slightly or not at all.

3. This reaction has been utilized as the basis of a method for the localization of ¹³¹I in the thyroxine molecule.

4. The method is applicable to biosynthesized [¹³¹I]thyroxine.

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The Hydrolysis of Rabbit γ -Globulin and Antibodies with Crystalline Papain

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The molecular size of rabbit γ -globulin is such that any direct attempt to relate structure to biological activity is not feasible at present. An alternative approach is to degrade antibody molecules in such a way that activity will persist in smaller fragments and so to reduce the structural problems involved. In an earlier investigation of this type (Porter, 1950*b*) it was found that, if rabbit γ -globulin containing antiovalbumin was digested with crude papain, a fragment with a molecular weight of about 40 000 could be produced which retained the ability to combine specifically with ovalbumin though it would no longer form a precipitate. It seemed probable that this fragment contained an antibody-combining site.

In that work, the amounts of crude enzyme used relative to γ -globulin were large, making subsequent investigation of the products of digestion rather difficult. As crystalline papain can now be prepared easily (Kimmel & Smith, 1954) and fractionation techniques have improved, these earlier experiments have been repeated. It has been found that γ -globulin is split by papain into three large pieces with very little release of amino acids or small peptides. If the γ -globulin contains antibody against any of the several antigens investigated, then two of these pieces retain combining though not precipitating power. The third piece, which may be readily crystallized, has no antibody activity but it has most of the antigenic sites of the original molecule. The isolation and properties of these three fractions will be described.

A preliminary account of this work has been given (Porter, 1958*a*).

EXPERIMENTAL

Materials

Antisera. Rabbit antiovalbumin, antiovine serum albumin and antihuman serum albumin were prepared by intravenous injection of the alum-precipitated protein (Porter, 1955).

Rabbit antipneumococci polysaccharide type 3 was prepared by injection of a suspension of the formalin-killed bacteria in 0.9% NaCl solution.

Goat antirabbit γ -globulin was prepared by intravenous injection of alum precipitate and subcutaneous injection of γ -globulin with adjuvant (Freund & McDermott, 1942).

Rat antiserum was prepared according to the following schedule. The rats were given two intramuscular injections of protein and adjuvant into different thighs, with 1 week's interval between injection. Five weeks later they were given three injections, with 3-day intervals between injections, of alum-precipitated protein into the tail vein. They were bled by heart puncture 6 days after the last injection.

Rabbit γ -globulin. This was prepared either by chromatography, with diethylaminoethylcellulose (Sober, Gutter, Wyckoff & Peterson, 1956), or by Na₂SO₄ precipitation according to the method of Kekwick (1940).

Crystalline papain. This was prepared from crude enzyme powder purchased from Hopkin and Williams Ltd., London. The enzyme was crystallized once as the free enzyme and twice as the inactive Hg dimer (Kimmel & Smith, 1954). It was freeze-dried and stored as the dimer.

Methods

Quantitative estimation of precipitating antibody. This was carried out according to Kabat & Mayer (1948).

Estimations of inhibitory power. These were made by quantitative antibody assay in the presence of the inhibitor