THE NATURE OF THE CIRCULATING THYROID HORMONE*

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Iodine exists in the thyroid gland in at least two well defined chemical entities, namely diiodotyrosine and thyroxine, which, according to Harington, account for practically all of the organic iodine in the gland ((2) p. 91). These two compounds are not present in a free form but are combined with other amino acids to form the characteristic thyroid protein, thyroglobulin.

Iodine is also a component of plasma, but its concentration there is so minute (about 5 γ per 100 cc.) that the chemical form in which it exists has eluded discovery. It was formerly believed that plasma iodine is also present as thyroglobulin (3, 4), but this view has been abandoned, mainly as a result of the studies of Trevorrow (5) and of Lerman (6). Despite the fact that crystalline thyroxine, when administered, produces in the mammalian organism all the known effects of thyroid tissue, investigators have nevertheless hesitated to assert that the circulating form of the thyroid hormone is thyroxine *per se*. This rejection is based on the following observations: (1) the failure of some investigators to account completely for the biological activity of thyroglobulin by its thyroxine content ((7, 8), ((9) p. 114), (2) the delayed response of animals to injected thyroxine ((2) p. 123, (10)), and (3) the failure of thyroxine to act *in vitro* (11, 12). These observations led Harington in 1935 (8, 13) to postulate that the circulating hormone is a peptide containing both thyroxine and diiodotyrosine. Recently, however, reevaluating this older evidence, Harington (14) has arrived at the conclusion that the peptide concept is an unnecessary complication and that thyroxine itself is probably the circulating hormone.

Two powerful tools, namely a refined method for determining small quantities of iodine (15) and the radioactive isotope of iodine (I^{131}), made possible a new attack on the problem of the nature of plasma iodine. The evidence provided by these means is presented here.

EXPERIMENTAL

The experiments presented here may be grouped conveniently as follows: (1) those dealing with the extractability of plasma iodine with butyl al-

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cohol; (2) those dealing with the butyl alcohol extractability of thyroxine added to plasma; (3) the demonstration that the thyroid hormone of plasma labeled by means of radioactive iodine behaves exactly the same as added thyroxine carrier, as judged by the latter's recrystallization to constant specific activity and by its distribution between two immiscible solvents; (4) those dealing with the combination of thyroxine with plasma protein.

Extraction of Plasma Iodine with Butyl Alcohol

The organic solvent *n*-butyl alcohol has proved helpful in the determination of the thyroxine content of thyroid tissue. It was first used for this purpose in 1932 by Leland and Foster (16), who showed that it extracted all of the thyroxine from thyroid protein that had been subjected to strong hydrolysis with 2 N NaOH. Later several investigators (5, 17–19) used butyl alcohol for the fractionation of blood iodine, but their efforts led to no agreement as to the nature of the circulating thyroid hormone. This is not surprising in view of the difficulties encountered in measuring the small quantities of iodine involved in such experiments.

A sensitive and reliable method for the determination of plasma iodine, previously reported from this laboratory (15), enabled us to carry out butyl alcohol fractionation on smaller quantities of plasma than was previously possible and thus to achieve complete extraction with convenient volumes of solvent. The details of this procedure are described below.

3 cc. of heparinized plasma were added, with shaking, to 15 cc. of normal butyl alcohol (reagent grade) in a 50 cc. narrow necked centrifuge tube. The tube was stoppered (the rubber stopper had previously been treated with alkali and acid and then soaked in butyl alcohol) and shaken thoroughly. The butyl alcohol layer obtained by 10 minutes of centrifugation was quite clear and was transferred to a 125 cc. separatory funnel. The residue was reextracted twice, each time with 15 cc. of butyl alcohol, and the mixture centrifuged after each addition. The three clear butyl alcohol extracts were combined in the separatory funnel and shaken with 50 cc. of a reagent consisting of 4 N NaOH and 5 per cent Na_2CO_3. The latter reagent, introduced by Blau (20), extracts inorganic iodide and diiodotyrosine, but not thyroxine, from butyl alcohol. After the mixture was allowed to stand for a few hours, the lower aqueous layer was run out and the butyl alcohol fraction extracted a second time with 30 cc. of 4 N NaOH-5 per cent Na_2CO_3. This time the separation of the two layers was allowed to proceed for 15 hours, at the end of which time the butyl alcohol layer showed only a slight turbidity. The butyl alcohol was next transferred to the two-neck digestion flask used in the iodine determination and carefully concentrated to dryness under reduced pressure at 100°. The iodine deter-

mination was carried out on the residue as previously described by us for plasma iodine (15) except for the following modifications: (1) Hydrogen peroxide (1 cc. of a 1:20 dilution of Merck's superoxol) was added after the addition of the phosphorous acid (21). The slight color in the distillate previously encountered when the peroxide was added (15) did not appear in the present investigation. We attribute this to the more careful construction of the iodine still used in this later work. (2) It seemed desirable to add some non-iodine-containing organic material to the butyl alcohol residue in order to make it more comparable to the original plasma. For this purpose we used dried defatted muscle containing a minimum amount of iodine. Such muscle was obtained from rats that had been fed a low iodine diet containing 0.15 per cent propylthiouracil. In some of the samples wheat was added as organic carrier, since it was found in control runs that it gave exactly the same results as the dried muscle. Two samples of butyl alcohol were shaken with the alkali reagent and concentrated to dryness as described above; these served as reagent blanks.

The results obtained by this butyl alcohol fractionation are shown in Table I. Blood was obtained from normal human subjects; a sufficient quantity was removed from each to permit carrying out triplicate or quadruplicate analyses on each plasma sample. The total and protein-bound iodine content of plasma was also determined for each subject. The protein-bound fraction was determined on a zinc hydroxide precipitate which had been washed twice with redistilled water.

It is clear from Table I that 90 per cent or more of the iodine in plasma is extractable with butyl alcohol at room temperature. This may be taken to mean that the iodine in plasma is not stably bound to protein. These results should be contrasted with those in Table II, which shows the extent to which the iodine of fresh thyroid tissue can be extracted with butyl alcohol; only a small per cent was found soluble. Only after strong hydrolysis does thyroid iodine become markedly soluble in butyl alcohol. There can be little doubt therefore that the chemical form of the iodine in plasma is different from that in the thyroid gland and that some degradation of the thyroid protein molecule takes place before the hormone is secreted into plasma.

As is shown in Table I, some iodine can be reextracted from the butyl alcohol by shaking the latter with the 4 N NaOH-5 per cent Na_2CO_3 reagent. This iodine, amounting to about 10 to 15 per cent of that present in the butyl alcohol, should represent the combined inorganic iodide and diiodotyrosine contents of the extract. Thus the diiodotyrosine content of plasma is at most only about 10 per cent of total plasma iodine. In the gland, however, diiodotyrosine iodine represents at least 60 per cent of the

total iodine (22). It is apparent therefore that there is a preferential release of thyroxine by the gland into the circulation; only a small fraction of the gland's iodine leaves the gland as diiodotyrosine.

TABLE I

Extractability of Plasma Iodine with Butyl Alcohol

Plasma source	Iodine in 3 cc. plasma					
	Total	Protein-bound	Butyl alcohol-extractable		Butyl alcohol-extractable, not reextractable with 4 N NaOH-5 per cent Na_2CO_3	
	γ	γ	γ	*per cent of total*	γ	*per cent of total*
Human female	0.188	0.183	0.174	93	0.150	80
" male	0.165	0.165	0.162	98	0.135	82
" "	0.168	0.174	0.147	88	0.156	93
" "	0.195	0.198			0.158	81
" "	0.162	0.156	0.153	94	0.126	78
" "	0.153	0.150			0.123	82
" "	0.168	0.156	0.162	96	0.123	73
Rat, pooled	0.094	0.096	0.093	99	0.081	84

TABLE II

Extractability of Rat Thyroid Iodine with Butyl Alcohol

Thyroids of ten large rats were pooled, minced with scissors, and duplicate portions taken for the following treatment.

Total I in gland	Trichloroacetic acid-soluble I*	Butyl alcohol-extractable I†	Butyl alcohol-extractable, not reextractable with 4 N NaOH-5 per cent Na_2CO_3
mg. per cent	*per cent of total*	*per cent of total*	*per cent of total*
17.3	6.9	3.4	2.4

* Thyroid tissue was homogenized with 1 cc. of 10 per cent trichloroacetic acid in a small glass tube.

† Thyroid tissue was homogenized with 1 cc. of butyl alcohol in a small glass tube. The residue was then extracted three times, each time with 3 cc. of butyl alcohol.

Behavior of Thyroxine Added to Plasma

The finding that approximately 80 per cent of the iodine in plasma can be extracted with butyl alcohol from which it cannot be reextracted with the 4 N NaOH-5 per cent Na_2CO_3 reagent suggested that this iodine is thyroxine. It therefore became of interest to compare the properties of this iodine fraction of plasma with those of crystalline thyroxine.

Butyl Alcohol Extraction—Crystalline thyroxine was added to human

plasma in an amount comparable to that already present, and this treated plasma was subjected to the butyl alcohol extraction procedure described above. Approximately 80 per cent of the added thyroxine was recovered in the final butyl alcohol extract (Table III). This compares favorably with the solubility properties of the iodine originally present.

Protein Precipitation—From the results in Table I it is clear that practically all of the iodine in plasma precipitates with proteins when zinc hydroxide is used as the precipitating agent. When crystalline thyroxine is added to plasma, it too precipitates quantitatively with proteins, as shown in Table IV. These results are in accord with those reported previously by Trevorrow (5) and by Bruger and Member (23).

TABLE III

Butyl Alcohol Extractability of Crystalline Thyroxine Added to Human Plasma

Procedure	Iodine determined in 3 cc. plasma		Thyroxine I added per 3 cc. plasma*	Thyroxine I recovered	
	Initial value	After adding thyroxine			
	γ	γ	γ	γ	*per cent of that added*
Total iodine	0.168	0.336	0.168		
Extractable with butyl alcohol but not reextractable with 4 N NaOH-5% Na_2CO_3	0.123	0.252		0.129	77
Total iodine	0.174	0.393	0.219		
Extractable with butyl alcohol but not reextractable with 4 N NaOH-5% Na_2CO_3	0.129	0.300		0.171	78

* The difference between the third and second columns.

Dialysis—The iodine in plasma is not dialyzable, as is shown in Table V and as reported previously by Silver and Tyson (24). A small amount of thyroxine when added to plasma is not dialyzable, although in simple aqueous solution thyroxine will dialyze readily.

Extraction of Diiodotyrosine from Plasma to Which It Had Been Added—Crystalline diiodotyrosine was added to plasma and the mixture extracted with butyl alcohol as described above. The amount of iodine added as diiodotyrosine was approximately equal to the total iodine initially present in the plasma sample. Table VI shows that the addition of 5 to 6 γ per cent of diiodotyrosine iodine to plasma did not affect the value obtained for the iodine in the thyroxine fraction. The method described here, therefore, should prove valuable in distinguishing between thyroxine iodine and diiodotyrosine iodine in plasma.

Unfortunately, the presence in plasma of 17 γ per cent of the gallbladder

dye, tetraiodophenolphthalein, did affect the thyroxine iodine value appreciably. The method described here is therefore not suitable for the determination of hormonal iodine in patients in which visualization tests of the gallbladder were recently carried out with this organic iodine compound.

It may be concluded from the experiments described in this section that crystalline thyroxine added to plasma behaves almost exactly like the

TABLE IV

Precipitation of Added Thyroxine Iodine with Plasma Proteins When Zinc Hydroxide Was Used As Precipitating Agent

Plasma source	Iodine initially present in 3 cc. plasma		Thyroxine I added	Iodine present in protein ppt.	Recovery of added thyroxine in protein ppt.
	Total	Protein-bound			
	γ	γ	γ	γ	*per cent*
Human	0.141	0.138	0.234	0.363	96
"	0.168	0.156	0.168	0.336	107
Dog	0.14		2.08	2.16	97

TABLE V

Dialysis of Thyroxine Added to Human Plasma

Iodine initially present in 3 cc. plasma		Thyroxine I added	Non-dialyzable I	Recovery of non-dialyzable thyroxine I	
Total	Protein-bound				
γ	γ	γ	γ	γ	*per cent of added thyroxine*
	0.147	0	0.156		
0.168	0.156	0	0.168		
0.141	0.138	0.234	0.367	0.229	98
0.168	0.156	0.168	0.327	0.171	102

naturally occurring iodine of plasma. Such experiments suggest very strongly that the iodine in plasma is mainly in the form of thyroxine loosely attached to plasma protein. More evidence on this point was obtained with the aid of radioactive iodine, to be described in the following section.

Experiments with Radioactive Iodine

It has been shown in this laboratory that, after the injection of a carrier-free dose of radioiodine into rats that have been fed a low iodine diet, the isotope is rapidly taken up by the thyroid gland, converted there to organic iodine, and then released into the plasma (25). Within 24 hours, about 90 per cent of the radioactive iodine in plasma is protein-bound and nearly

all of this percentage is presumably the thyroid hormone. This procedure thus permits one to obtain *labeled* thyroid hormone in its normal physiological state.

Butyl Alchol Extraction of Radioactive Iodine from Rat Plasma—Twelve large rats were injected with 80 microcuries of I^{131} and their blood removed 24 hours later and pooled. The plasma was separated, and 3 cc. portions were taken for determination of total iodine, protein-bound iodine, and

TABLE VI

Effect of Presence of Diiodotyrosine and Tetraiodophenolphthalein on Determination of Thyroxine Iodine Content of Human Plasma

Substance added	Iodine added per 3 cc. plasma	Iodine in butyl alcohol fraction prepared from 3 cc. of plasma	
		Original plasma	Plasma with added I
	γ	γ	γ
Diiodotyrosine	0.16	0.129	0.123
"	0.17	0.123	0.123
Tetraiodophenolphthalein*	0.51	0.123	0.345

* Trade name, Iodeikon.

TABLE VII

Extraction of Chemical and Radioactive Iodine from Plasma of Rats Injected 24 Hours Previously with Radioactive Iodine (I^{131})

	Iodine in 3 cc. plasma		Per cent of total I	
	Chemical I	Radioactive I	Chemical I	Radioactive I
	γ	*counts per sec.*		
Total I	0.096	348		
Protein-bound I	0.096	321	100	92
Butyl alcohol-extractable	0.093	250	97	72
" " but not reextractable with 4 N NaOH-5% Na_2CO_3	0.081	230	84	66

butyl alcohol-extractable iodine (Table VII). Both chemical and radioactive measurements were made on all plasma samples. Since the chemical method involved the distillation of the iodine into an alkaline medium, a portion of this alkaline distillate was used for the radioactive measurement by simply evaporating a suitable aliquot in a porcelain milk-ashing dish. Radioactivity was determined by means of a thin mica window Geiger-Müller tube.

As is shown in Table VII, the butyl alcohol extractability of the iodine in

rat plasma is quantitatively similar to that of human plasma. Almost all of the iodine passed into butyl alcohol, and about 84 per cent resisted further extraction with the 4 N NaOH-5 per cent Na_2CO_3 reagent. The results with the radioactive iodine were somewhat different. Only 72 per cent passed into the butyl alcohol, and 66 per cent remained in the butanol after the latter was subjected to extraction with alkali.

In seeking an explanation for the apparent discrepancy between the chemical and the radioactive data the following factors must be considered. (1) Not all of the iodine in the rat plasma had the same specific activity. If, for example, a small amount of diiodotyrosine-like iodine leaked into the plasma from the gland, this iodine would have a higher specific activity than the iodine of the thyroxine fraction (22). The injected iodide that was still present in the plasma would, of course, also have a much higher specific activity than the thyroxine iodine. (2) Inorganic iodide and diiodotyrosine, the compounds with the highest specific activity, are extractable from plasma with butyl alcohol to a lesser degree than thyroxine.

In the light of these considerations it would be expected that the chemical iodine in the plasma of the injected rats would be extracted with butyl alcohol to a greater extent than the radioactive iodine. Whether this alone accounts entirely for the discrepancy is difficult to say.

The possibility of some error (5 to 10 per cent) in the chemical method used here must also be considered in seeking to explain the discrepancy between the chemical and the radioactive data. Admittedly, the determination of 0.1 to 0.2 γ of iodine in the butyl alcohol extracts was not a simple matter, but it must be added that such analyses were always done in triplicate or quadruplicate. It seems unlikely to us that the average value of 80 per cent for the butyl alcohol-soluble, alkali-insoluble fraction of plasma iodine suffered from an error of more than 5 per cent.

Recrystallization of Thyroxine Carrier Added to Butanol Extracts—Butyl alcohol extracts, reextracted with the alkali reagent, were prepared from the plasma of rats injected 24 hours previously with 75 to 80 microcuries of I^{131}. 25 mg. of recrystallized thyroxine were added as carrier, and the butyl alcohol extract was concentrated to dryness on a boiling water bath under reduced pressure. The thyroxine in the residue was dissolved in hot 0.1 N K_2CO_3 and separated from a small amount of undissolved material by centrifugation. The thyroxine was then repeatedly recrystallized as follows: (1) The first crystallization was effected by the addition of glacial acetic acid to the hot K_2CO_3 solution of the residue. (2) The crystals obtained in this way were washed with water, dissolved in a minimum quantity of hot 0.1 N K_2CO_3, and then precipitated by cooling to 0°. (3) The crystals obtained in (2) were dissolved in an alkaline 70 per cent ethyl alcohol solution and precipitated by the addition of glacial acetic

acid. (4) The fourth recrystallization was made from a dilute NaOH solution by the addition of glacial acetic acid.

The specific activity of the iodine of each batch of crystals (counts per second per microgram of I^{127}) was determined. The results are recorded in Table VIII. The constancy in the values indicates that the I^{131} in the butyl alcohol extract of rat plasma is in the same form as the material which underwent recrystallization, namely thyroxine. Such an experiment is not absolutely conclusive, however, since a compound very similar to thyroxine in its structure might conceivably continue to precipitate with thyroxine.

Solvent Distribution Experiment—To provide further evidence as to whether the radioactive iodine in the butyl alcohol extract of plasma was

TABLE VIII

Specific Activity of Thyroxine Carrier after Successive Recrystallizations

See the text for details.

Experiment No.	Per cent of total I^{127} in BuOH fraction	Per cent of total I^{131} in BuOH fraction	Specific activity, counts per min. per γ I				
			Initial	After 1st recrystallization	After 2nd recrystallization	After 3rd recrystallization	After 4th recrystallization
1	84	66	2.64	2.58	2.58	2.47	2.53
2	84	66	2.57	2.25	2.20	2.28	2.28
3			2.90	2.69	2.81	2.74	2.70
4			2.89	2.69	2.71	2.66	2.63

thyroxine, its distribution between two immiscible solvents was compared with that of added thyroxine carrier. The carrier was added to the butyl alcohol extract containing the radioactive iodine and the mixture concentrated to dryness on a boiling water bath under reduced pressure. The residue was dissolved in 15 cc. of 0.1 N NaOH and shaken with an equal volume of either butyl alcohol or isoamyl alcohol. The distribution of chemical and radioactive iodine between the organic and the aqueous phases is recorded in Table IX. It was found that the distribution of the I^{131} always paralleled that of the added thyroxine.

This parallelism is all the more striking, inasmuch as the distribution ratios varied from experiment to experiment. The variation suggests that a variable breakdown or transformation of the thyroxine occurs during the process of concentrating the alkaline butyl alcohol extracts to dryness. Despite these variations, however, the distribution ratios for radioactive and chemical iodine in any given experiment were about equal. Such results would be expected only when the radioactive iodine was of the same molecular species as the added carrier.

Comparison of Behavior of Thyroxine and Thyroxine Peptide As Judged by Solvent Distribution Experiments—The evidence outlined above is best explained by the assumption that the iodine in plasma is mainly in the form of thyroxine loosely attached to protein. The question arises, however, whether a thyroxine-containing peptide is ruled out by the evidence at hand. To provide information on this point a thyroxine peptide was prepared by the procedure of Harington and Salter (26). Desiccated thyroid powder obtained from the Viobin Corporation was first hydrolyzed with pepsin (Merck) and then with pancreatin (Merck). A product was finally isolated which closely resembled that obtained by Harington and Salter. It contained 49.4 per cent I, 3.4 per cent N, 1.3 per cent amino N, and gave a

TABLE IX

Distribution of Crystalline Thyroxine Carrier and Radioactive Plasma Iodine between Two Immiscible Solvents

See the text for discussion.

Experiment No.	Amount of thyroxine carrier added	Concentration of I in organic solvent / Concentration of I in aqueous solution	
		Chemical I	Radioactive I
	mg.		
1*	10	1.67	1.78
2*	10	1.73	1.73
3*	1.5	1.43	1.35
4*	0.9	0.81	0.84
5†	10	0.069	0.072

* Solvent pair, 0.1 N NaOH-*n*-butyl alcohol.

† Solvent pair, 0.1 N NaOH-isoamyl alcohol.

strong nitrous acid color test for thyroxine. But it also gave a positive ninhydrin test, which indicates the presence of some free amino acid. If this free amino acid is assumed to have a molecular weight of 120 (average for ordinary amino acids), then it represents an impurity of about 6 per cent. If it is assumed to be free thyroxine, however, it would have amounted to about 40 per cent contamination because of the large molecular weight of thyroxine (777). The small amount of material available did not permit us to carry out all the analyses necessary to establish the true identity of this product. But it is safe to assume that it was composed mainly of thyroxine peptides, the ratio of total N to amino N (2.6) leading to the conclusion that it was a mixture of di- and tripeptides.

A solvent distribution experiment was carried out with this preparation in the manner described above for thyroxine, except that the butyl alcohol extract, after the addition of the carrier, was not concentrated to

dryness but shaken directly with 0.1 N NaOH. This avoided the possibility of chemical changes that might occur during the concentration. The results for both peptide carrier and thyroxine carrier are presented in Table X. It is evident that the radioactivity distributed itself very much like the thyroxine carrier but quite differently from the peptide carrier. *This demonstrates the sensitivity of this procedure in distinguishing between thyroxine and a closely related compound and lends further support to the view that the circulating thyroid hormone is actually thyroxine.*

It will be noted that the distribution ratios of thyroxine recorded in Table X are quite different from those in Table IX. In the former are recorded the results of the experiments in which the butyl alcohol extracts containing the thyroxine were not concentrated to dryness; the values found for the ratio of the concentration of I in butyl alcohol to the concentration

TABLE X

Comparison of Crystalline Thyroxine and Thyroxine Peptide Preparation in Solvent Distribution Experiment

Solvent pair, 0.1 N NaOH-*n*-butyl alcohol.

Sample No.	Carrier added	Amount of carrier added	Concentration of I in organic solvent / Concentration of I in aqueous solution	
			Chemical I	Radioactive I
		mg.		
1	Thyroxine	0.6	3.2	3.0
2	"	0.6	3.1	3.0
3	"	0.6	3.0	3.0
4	Peptide	1	1.5	3.1
5	"	1	1.5	3.2

of I in 0.1 N NaOH were much higher in these experiments than in those of Table IX. As already pointed out, the lower values observed in the experiments of Table IX may be attributed to some transformation of thyroxine which occurs while the butyl alcohol extract is being concentrated to dryness and which renders the iodine less soluble in butyl alcohol.

Combination of Thyroxine with Plasma Proteins

The data so far presented lead to the conclusion that thyroxine in a loose combination with plasma protein is the circulating thyroid hormone. It seems of interest, therefore, to determine the particular fraction of plasma proteins with which thyroxine is combined.

Iodine Content of Plasma Protein Fractions—Salter and his coworkers (18, 27) have carried out iodine analyses on the various plasma protein fractions prepared by Cohn (28). The two albumin fractions, Nos. V and

VI, were found to contain 75 per cent of the protein-bound iodine. The concentration of iodine appeared to be greatest, however, in the α-globulin fraction.

These results have been confirmed by us (Table XI). The following plasma fractions were analyzed: crystalline bovine albumin, human albumin (Fraction V), human α-globulin (Fraction IV-1), human β-globulin (Fraction IV-3,4), and human γ-globulin (Fraction II). The α-globulin fraction had the highest concentration of iodine (1.9×10^{-4} per cent), the γ-globulin the lowest ($< 1 \times 10^{-5}$ per cent). The iodine content of the albumin fraction was, unfortunately, too small for accurate analysis, and the value recorded is probably too low. Nevertheless the albumin fraction is the largest carrier of iodine because it constitutes the largest fraction of

TABLE XI

Iodine Content of Plasma Protein Fractions

See the text for discussion.

Fraction No.	Principal components	Iodine per 100 gm. protein	Approximate protein per 100 cc. plasma	Estimated I per 100 cc. plasma
		γ	*gm.*	γ
IV-1	α-Globulin β-Globulin	189	0.5	1
IV-3,4	β-Globulin α-Globulin	56	0.8	0.5
II	γ-Globulin	<10	0.8	0
V	Albumin	35	4	1.5
Crystalline bovine albumin	"	30		
I	Fibrinogen	<10	0.4	0

the plasma proteins. The values given in Table XI should, however, be regarded as preliminary observations; more complete results must await further refinements in the iodine method as well as a more abundant supply of pure α- and β-globulins.

Thyroxine-Protein Combination—Many reports have dealt with the combination of various molecules with plasma proteins. Thus Roberts and Szego (29) reported that circulating estrogen is attached to plasma protein, probably the β-globulin fraction, whereas the combination of many organic anions with serum albumin has been studied by Klotz *et al.* (30), Teresi and Luck (31), and others. Davis (32) has discussed the physiological significance of the binding of molecules by plasma proteins.

In Table XII are presented our results on the binding of thyroxine by various plasma protein fractions. 100 mg. of each protein were dissolved or suspended in 10 cc. of a phosphate-saline (0.01 M phosphate-0.15 M

NaCl) buffer at pH 7.3. To 3 cc. of a protein solution in a small dialysis bag (Visking casing) was added 1 cc. of a standard thyroxine solution. Dialysis was carried out in a cold room (6–7°) against 100 cc. of phosphate buffer or distilled water. After 4 hours the external solution was replaced with a fresh 100 cc. portion and the dialysis continued for another 12 hours. The bag was rotated by motor during the dialysis. It is clear from the data in Table XII that albumin, α-globulin, and β-globulin are all capable

TABLE XII

Combination of Thyroxine with Plasma Proteins

Thyroxine was added to a 1 per cent protein solution in a phosphate-saline buffer at pH 7.3 and dialyzed. See the text for details.

Protein fraction	Thyroxine added	Dialyzed against	Per cent of added thyroxine remaining in dialysis bag
	mg.		
None	26.2	Distilled water	3.2
Albumin, Fraction V	21.4	" "	98
" " "	21.4	Phosphate-saline buffer, pH 7.3	82
α-Globulin, Fraction IV-1	21.4	Distilled water	86
" " "	21.4	Phosphate-saline buffer, pH 7.3	81
β-Globulin, " IV-3,4	25.2	Distilled water	91
" " "	24.5	Phosphate-saline buffer, pH 7.3	70
γ-Globulin, " II	25.2	Distilled water	58
" " "	24.5	Phosphate-saline buffer, pH 7.3	22

of binding thyroxine to a high degree. γ-Globulin is much less potent as a thyroxine binder.

It may be concluded that plasma iodine is not confined to a single plasma protein fraction. Albumin, α-globulin, and, probably to a lesser extent, β-globulin share in binding the so called "protein-bound iodine" of plasma. γ-Globulin seems to play no rôle in this respect. It does seem, however, as pointed out by Salter (27), that the iodine in plasma is attached to the smaller protein molecules.

DISCUSSION

The concentration of "protein-bound" iodine in plasma is now widely used for the diagnosis of thyroid diseases (33–37).[1] This iodine is the frac-

[1] Chaney, A. L., private communication (1944).

tion that is precipitated from plasma along with the proteins by such agents as zinc hydroxide, tungstic acid, or acetic acid plus heat, the inorganic iodine being freed from the protein precipitate by simple washing. Protein binding, however, is such a non-specific reaction that the finding of iodine in the plasma protein precipitate does not contribute much to an understanding of the chemical nature of this iodine.

Alcohol and acetone have been widely employed to fractionate the iodine of plasma ((9) p. 72, (38–40)). But such solvents do not achieve a clear cut separation of organic from inorganic iodine of plasma. Their inadequacy has been pointed out by Trevorrow (5), Salter ((9) p. 72), and others (23, 24, 38). Butyl alcohol is much more satisfactory for this purpose, since it has been successfully applied to the thyroid gland and since inorganic and non-thyroxine organic iodine can be reextracted from butyl alcohol by shaking this solvent with appropriate reagents.

The first to apply Leland and Foster's butyl alcohol fractionation procedure to blood iodine were Elmer *et al.* (17). They reported that after strong alkali hydrolysis (which destroyed part of the thyroxine present) 40 to 60 per cent of the organic iodine in the blood was thyroxine-like as judged by solubility properties. However, their values for normal human blood are high enough to cast suspicion upon their method for the determination of iodine.

Some time later, Trevorrow (5) also applied Leland and Foster's method to beef plasma and whole blood. She concluded that all of the iodine in plasma or whole blood could be directly extracted with butyl alcohol at room temperature and without previous hydrolysis. After reextraction of the butyl alcohol with 2 N NaOH, which served to remove inorganic and diiodotyrosine iodine, a good part of the iodine remained in the butyl alcohol. Her data did not permit quantitative conclusions regarding the percentage of total iodine in the thyroxine fraction.

Bassett, Coons, and Salter (18) also applied a butyl alcohol fractionation to plasma that had been subjected to pepsin hydrolysis. They reported that the thyroxine-like fraction in normal human plasma amounted to 73 per cent of the protein-bound iodine, but the range of values and number of patients studied were not given. Interestingly enough, approximately this same percentage was found in the thyroxine fraction in three cases of hyperthyroidism.

Somewhat different results were reported by Wilmanns in an extensive study of the iodine in normal, hyperthyroid, and hypothyroid blood (19). He treated whole blood with hot butyl alcohol and reported that only 65 per cent of the iodine could be removed from normal blood with this solvent, and of this only 28 per cent was not reextractable with 1 N NaOH. He concluded that there are at least two organic iodine fractions in whole

blood: (1) free thyroxine (average 28 per cent) and (2) a stable protein-bound iodine fraction (average 34 per cent).

The results obtained in the present investigation confirm Trevorrow's observation that almost all of the iodine in plasma can be extracted with butyl alcohol at room temperature. This finding leads us to conclude that the iodine of plasma is not stably bound to protein and that its state thus differs from that in which iodine exists in the thyroid gland. The iodine of the gland becomes butyl alcohol-soluble only after strong hydrolysis. Not only is most of the iodine in plasma extractable with butyl alcohol, but, as Table I shows, only 10 to 15 per cent of it can be removed from this solvent with the reagent 4 N NaOH-5 per cent Na_2CO_3, which extracts inorganic iodine and diiodotyrosine, but not thyroxine, from butyl alcohol. In seven human subjects and in one sample of pooled rat plasma 73 to 93 per cent of the total plasma iodine (average 81 per cent) remained in the butyl alcohol extract after it had been treated with the alkali mixture. When crystalline thyroxine was added to plasma in physiological amounts and the mixture subjected to the butyl alcohol extraction procedure, 77 to 78 per cent of the thyroxine appeared in the final butyl alcohol extract. Such findings demonstrate that a large fraction (at least 80 per cent) of plasma iodine behaves like thyroxine in its solubility properties. The additional findings that thyroxine added to plasma precipitates quantitatively with the proteins (Table IV) and does not dialyze (Table V) also lend support to the view that the iodine in plasma is mainly in the form of thyroxine loosely attached to protein.

Further evidence that thyroxine exists in plasma is provided by the experiments with radioactive iodine (Tables VIII to X). Crystalline thyroxine carrier when added to the butyl alcohol extract of the plasma of rats injected with I^{131} showed a constant specific activity upon repeated recrystallization. Even more convincing, perhaps, was the finding that the radioactive iodine in the butyl alcohol extract distributes itself between two immiscible solvents in almost exactly the same manner as does added thyroxine carrier (Table IX), but quite differently from a thyroxine peptide carrier (Table X).

The results obtained here agree quite well with those of Bassett, Coons, and Salter (18), although these workers hydrolyzed the plasma protein with pepsin before extraction with butyl alcohol. Apparently, preliminary hydrolysis of the proteins does not appreciably affect the free thyroxine content of plasma.

Although Trevorrow reported that practically all of the iodine in small samples of blood or plasma could be extracted with butyl alcohol, she found that in the case of beef plasma a variable but large portion (42 to 73 per cent) of the butyl alcohol-soluble iodine could be reextracted with 2

N NaOH. Most of the values, however, were based on experiments in which large volumes of plasma were used and extraction with butyl alcohol was admittedly incomplete. Nevertheless, in view of this discrepancy between our results and Trevorrow's, the possibility of species variation must be considered.

In a recent paper dealing with the effects of thyroxine, thyroglobulin, and thyrotropin on tissue respiration (41), Williams and Whittenberger were led to the conclusion that the active form of the thyroid hormone is probably a thyroxine peptide. Their main evidence is that thyroxine had no calorigenic effect on liver tissue slices, whereas thyroglobulin[2] did. They also reported that the serum of myxedematous patients given thyroxine intravenously gradually acquired the capacity to raise the QO_2 of guinea pig liver slices incubated therein, the maximum effect being reached in 6 hours. The latter experiment was taken as evidence that injected thyroxine must undergo some process of activation before it can increase oxygen consumption. Peptide formation was suggested as a possible mode of activation. Salter also appears to favor the peptide hypothesis (27).

The results obtained by us favor thyroxine rather than thyroxine peptides as the chemical form of plasma iodine. If any activation of thyroxine is required before it can act on tissues, it seems unlikely that peptide formation is involved. More likely the solubility of thyroxine and its rate of penetration to the site of reaction are important.

Harington has reported immunological experiments which support the view that thyroxine itself circulates in the plasma (14). He found that antisera against artificial thyroxine-protein complexes protected rats against the characteristic response to a dose of thyroxine. He has also reviewed the evidence *against* the view that thyroxine itself is the normal circulating hormone and no longer finds it convincing. Nevertheless the whole question of the relation between the biological activity of thyroid preparations and their thyroxine content deserves accurate reinvestigation with proper attention to the effects of route of administration, solubility, relative activities of D- and L-thyroxine, accurate chemical thyroxine determinations, and method of biological assay.

The finding that the form in which thyroxine exists in plasma differs from that in the gland raises the question by what mechanism the hormone is released into the circulation. De Robertis and Nowinski (43) have reported the presence of a proteolytic enzyme in the gland which hydrolyzes thyroglobulin into smaller fragments. The concentration of this enzyme supposedly is increased in hyperthyroidism. Confirmation of these interest-

[2] The claim that the addition of thyroglobulin increases the respiration of liver slices has not been confirmed (42).

ing findings would be welcomed as a further step toward an understanding of the workings of the thyroid gland.

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SUMMARY

The following evidence is presented in support of the view that the circulating thyroid hormone in the normal animal consists of thyroxine loosely attached to plasma protein.

1. The iodine of normal plasma is almost completely extractable with butyl alcohol at room temperature. Most of this iodine (73 to 93 per cent) remains in the butyl alcohol even after the latter is shaken with 4 N NaOH-5 per cent Na_2CO_3, a reagent which extracts diiodotyrosine and inorganic iodine, but not thyroxine, from butyl alcohol.

2. When crystalline thyroxine is added to plasma, it behaves like naturally occurring protein-bound iodine of plasma in the following respects: (*a*) Approximately 80 per cent of it remains in the butyl alcohol extract after treatment with the 4 N NaOH-5 per cent Na_2CO_3 reagent. (*b*) It precpitates quantitatively with plasma proteins when $Zn(OH)_2$ is used as the precipitating agent. (*c*) It does not dialyze.

3. Protein-bound iodine of rat plasma labeled with I^{131} follows thyroxine carrier quantitatively when the latter is repeatedly crystallized or when it is distributed between two immiscible solvents.

BIBLIOGRAPHY

1. Taurog, A., and Chaikoff, I. L., *J. Biol. Chem.*, **171,** 439 (1947).
2. Harington, C. R., The thyroid gland, London (1933).
3. Hektoen, L., Carlson, A. J., and Schulhof, K., *J. Am. Med. Assn.*, **81,** 86 (1923).
4. Barnes, B. O., and Jones, M., *Am. J. Physiol.*, **105,** 556 (1933).
5. Trevorrow, V., *J. Biol. Chem.*, **127,** 737 (1939).
6. Lerman, J., *J. Clin. Invest.*, **19,** 555 (1940).
7. Means, J. H., Lerman, J., and Salter, W. T., *J. Clin. Invest.*, **12,** 685 (1933).
8. Harington, C. R., *Ergeb. Physiol. u. Exp. Pharmakol.*, **37,** 210 (1935).
9. Salter, W. T., The endocrine function of iodine, Cambridge (1940).
10. Thompson, W. O., Thompson, P. K., Brailey, A. G., and Cohen, A. C., *J. Clin. Invest.*, **7,** 437 (1929).
11. Gerard, R. W., and McIntyre, M., *Am. J. Physiol.*, **103,** 225 (1933).
12. Gordon, E. S., and Heming, A. E., *Endocrinology*, **34,** 353 (1944).
13. Harington, C. R., *Lancet*, **1,** 1261 (1935).

14. Harington, C. R., *Proc. Roy. Soc. London, Series B*, **132,** 223 (1944).
15. Taurog, A., and Chaikoff, I. L., *J. Biol. Chem.*, **163,** 313 (1946).
16. Leland, J. P., and Foster, G. L., *J. Biol. Chem.*, **95,** 165 (1932).
17. Elmer, A. W., Luczynski, Z., and Scheps, M., *Compt. rend. Soc. biol.*, **115,** 1714 (1934).
18. Bassett, A. M., Coons, A. H., and Salter, W. T., *Am. J. Med. Sc.*, **202,** 516 (1941).
19. Wilmanns, H., *Z. ges.-exp. Med.*, **112,** 1 (1943).
20. Blau, N. F., *J. Biol. Chem.*, **102,** 269 (1933).
21. Chaney, A. L., *Ind. and Eng. Chem., Anal. Ed.*, **12,** 179 (1940).
22. Taurog, A., and Chaikoff, I. L., *J. Biol. Chem.*, **169,** 49 (1947).
23. Bruger, M., and Member, S., *J. Biol. Chem.*, **148,** 77 (1943).
24. Silver, S., and Tyson, M. C., *J. Mt. Sinai Hosp.*, **12,** 701 (1945).
25. Chaikoff, I. L., Taurog, A., and Reinhardt, W. O., *Endocrinology*, **40,** 47 (1947).
26. Harington, C. R., and Salter, W. T., *Biochem. J.*, **24,** 456 (1930).
27. Salter, W. T., *Western J. Surg., Obs. and Gynec.*, **55,** 15 (1947).
28. Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, **68,** 459 (1946).
29. Roberts, S., and Szego, C. M., *Endocrinology*, **39,** 183 (1946).
30. Klotz, I. M., Walker, F. M., and Pivan, R. B., *J. Am. Chem. Soc.*, **68,** 1486 (1946).
31. Teresi, J. D., and Luck, J. M., *J. Biol. Chem.*, **174,** 653 (1948).
32. Davis, B. D., *Am. Scientist*, **34,** 611 (1946).
33. Salter, W. T., Bassett, A. M., and Sappington, T. S., *Am. J. Med. Sc.*, **202,** 527 (1941).
34. Man, E. B., Smirnow, A. E., Gildea, E. F., and Peters, J. P., *J. Clin. Invest.*, **21,** 773 (1942).
35. Lowenstein, B. E., Bruger, M., and Hinton, J. W., *J. Clin. Endocrinol.*, **4,** 268 (1944).
36. Talbot, N. B., Butler, A. M., Saltzman, A. H., and Rodriguez, P. M., *J. Biol. Chem.*, **153,** 479 (1944).
37. Soley, M. H., and Miller, E. R., *Med. Clin. North America*, 1 (1948).
38. Boyd, E. M., and Clarke, E. L., *J. Biol. Chem.*, **142,** 619 (1942).
39. McClendon, J. F., and Foster, W. C., *J. Biol. Chem.*, **154,** 619 (1944).
40. Davison, R. A., Zollinger, R. W., and Curtis, G. M., *J. Lab. and Clin. Med.*, **27,** 643 (1942).
41. Williams, R. H., and Whittenberger, J. L., *Am. J. Med. Sc.*, **214,** 193 (1947).
42. Williams-Ashman, H. G., *J. Endocrinol.*, **5,** p. xc (1948).
43. De Robertis, E., and Nowinski, W. W., *J. Clin. Endocrinol.*, **6,** 235 (1946).

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