STUDIES ON THE NATURE OF THE IODINE IN BLOOD*

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Iodine metabolism is known to be significant in the maintenance of normal body function and is involved in certain pathological conditions. The rôle of the blood iodine in iodine metabolism can be understood only through a knowledge of the type or types of compounds present. In this study an attempt has been made to determine the nature of the blood iodine by means of comparing it with inorganic iodides, simple organic iodine compounds (thyroxine and diiodotyrosine), and iodine-containing protein. Comparisons have been made with regard to solubility, ultrafiltrability, and precipitation with protein precipitants.

Both human and animal bloods have been used in this study. The human blood samples and those from dogs were obtained by venipuncture, and extreme precautions were taken against the possibility of iodine contamination from the needle, syringe, anticoagulant, or the glassware used. The various blood samples were not obtained during the fasting state, but care was exercised to eliminate all persons who used iodized salt or who had eaten foods reported to be high in iodine content. In no case was a sample taken if iodine in any form had been used by the individual during the preceding 2 weeks. Animal bloods were obtained at the abbatoir directly from the animal at the time of decapitation. The animals used had received a diet of grain, hay, and water and were known not to have received iodized salt for at least 10 days before the blood was taken.

A series of analyses of whole blood and plasma showed a dis-

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tribution of the total iodine in proportion to the water content of the plasma and cells. Although it is recognized that a qualitative difference may exist between the plasma and cell iodine, there has been no evidence that such is the case. Whole blood and plasma have been used interchangeably according to the ease of manipulation. Iodine analyses were carried out according to the method of Trevorrow and Fashena (1, 2).

EXPERIMENTAL

Ethyl Alcohol Extraction of Blood with and without Addition of Iodine Compounds—Ethyl alcohol was used to extract the blood iodine, as shown in Table I. The blood was precipitated with 4 volumes of purified 95 per cent ethyl alcohol and the precipitate so obtained washed three times with 1 volume of alcohol. These four portions of alcohol were combined for analysis. The precipitate was then transferred quantitatively to a Soxhlet apparatus and extracted for 4 hours with fresh alcohol according to the method of Lunde, Closs, and Pederson (3). This alcohol was analyzed separately. The extraction was then continued for 24 hours and a third alcohol fraction so obtained. The blood protein residue was then analyzed for alcohol-insoluble iodine.

As can be seen in Table I, the iodine of blood is completely extracted by this procedure, 30 to 50 per cent being removed in the first fraction, 30 to 45 per cent in the 4 hour continuous extraction, and the remainder in the next 24 hours. The recoveries in these experiments ranged from 90 to 108 per cent and the protein residue in no case contained detectable amounts of iodine. Dodds and coworkers (4) have reported a long continued extraction of blood with hot alcohol and state that some of the blood iodine remains in the insoluble protein fraction. We believe these results to be in error as a result of an inadequate method of iodine analysis.

In similar extraction experiments in which potassium iodide, thyroxine, and finely minced thyroid gland were added to the blood, it was found that both the iodide and thyroxine were removed slowly by alcohol but that the thyroid iodine remained insoluble. It would appear, therefore, that the iodine of blood is not in protein combination similar to that found in the thyroid gland. In the last two experiments cited in Table I, blood con-

taining 2.8 micrograms of iodine and a similar sample of the same blood to which 26.0 micrograms of thyroid iodine had been added were extracted simultaneously under entirely comparable conditions. The iodine removed in the three alcoholic fractions was identical in amount in the two cases (within the limits of error of the method).

Acetone Extraction of Blood with and without Addition of Iodine Compounds—Acetone was next used for the extraction of the

TABLE I

Extraction of Blood Iodine with C_2H_5OH

Material used	I_2 present	I_2 in C_2H_5OH from precipitation and three washings	I_2 in C_2H_5OH from Soxhlet extraction		I_2 in residue after extraction	Recovery
			First 4 hrs.	Next 24 hrs.		
	micrograms	*micrograms*	*micrograms*	*micrograms*	*micrograms*	*per cent*
50 cc. dog blood	2.5	1.4	0.8	0.5	<0.1	108
50 " human blood	2.8	1.1	0.8	0.6	<0.1	90
50 " " " + thyroxine	2.8 + 12.7	9.1	2.3	2.9	1.6	103
50 cc. human blood + KI	2.8 + 12.3	10.0	2.3	1.3	0.4	93
357.7 mg. thyroid	179.0		5.4	6.2	161.0	97
50 cc. beef blood + thyroid	2.8 + 26.0	0.8	1.3	0.6	*	
50 " " "	2.8	0.9	1.2	0.5	<0.1	93

* Not analyzed.

blood iodine, as shown in Table II. 1 volume of blood was added to 4 volumes of purified acetone and the mixture shaken vigorously in a mechanical shaking machine for from 1 to 12 hours as shown in Table II. The mixture was then centrifuged and the precipitate again shaken with 4 volumes of 80 per cent acetone. Four extractions were made in each case. The results are in accord with those of Eufinger and Schulte (5); namely, that all of the iodine is acetone-soluble. The time required for complete extraction was found to vary with the amount of iodine and the volume of blood used. 12 hour extraction periods served to extract completely 18 micrograms of iodine from 600 cc. of beef blood, whereas 3 hour extraction periods removed only 45 per

cent of the iodine from a similar 600 cc. sample. With smaller amounts of blood containing only 2 micrograms of iodine, 1 hour extraction periods were adequate.

Thyroxine and potassium iodide, when added to blood, were removed by acetone. In the 1 hour extraction periods used, only 72 and 86 per cent respectively of the iodine of these samples was dissolved by the acetone, but there can be no doubt that each of the added materials was being dissolved and would eventually have been completely removed from the precipitate.

The extracts obtained by this procedure were protein-free, as shown by the biuret test on a concentrated aqueous suspension of

TABLE II

Extraction of Blood Iodine with Acetone

Material used	Iodine present	Iodine in acetone		Iodine in residue		Time for each extraction*
	micrograms	*micrograms*	*per cent*	*micrograms*	*per cent*	*hrs.*
Beef blood	2.7	2.7	100	<0.2		1
" " + KI	1.3 + 3.0	3.7	86	0.6	14	1
" " + thyroxine	1.3 + 3.0	3.1	72	1.1	26	1
Human blood	2.4	2.4	100	<0.2		1
Beef blood	18.0	8.1	45	†		3
" "	18.0	17.2	96	†		12

* Four extractions were performed in each case.

† Not analyzed.

the extracted material from which the acetone had been removed. This finding supports the view that the blood iodine is not in protein combination, but no differentiation can be made between simple organic iodine (thyroxine) and inorganic iodine.

Ultrafiltration of Blood with and without Addition of Iodine Compounds—Attempts were made to secure a protein-free preparation of the blood iodine by means of ultrafiltration. Theoretically, this procedure should give a simple aqueous solution of the blood salts and all simple organic compounds such as thyroxine and other amino acids, urea, sugar, etc. The membranes used were made from a 5 per cent solution of nitrocellulose (parlodion) in glacial acetic acid, deposited on the filter cone of a Giemsa filtration apparatus. This concentration of nitrocellulose is adequate

for the retention of all protein material of plasma. Leaks in the membrane surfaces were avoided by the use of a double thickness of collodion on each tube, the second layer being deposited after the first had been hardened in water. Each membrane was washed with several hundred cc. of redistilled water until the acetic acid had been removed and a 50 cc. sample of the filtrate showed no trace of iodine. After being washed and found iodine-free, the membranes were dried as completely as possible by suction, and used immediately.

Aqueous solutions of potassium iodide and thyroxine were ultrafiltered and the filtrate (after the first portions were discarded because of unavoidable dilution by water in the membrane) was found to contain the same concentration of iodine as that of the unfiltered material. Preliminary experiments with plasma and serum showed that no appreciable amount of the iodine present passed through the filter. The technique was then altered in such a way that a maximum amount of filtrate would be obtained from a given sample of plasma. 25 to 100 cc. of plasma were filtered until the non-filtrable residue had been reduced to one-fifth the original volume. Water was then added to the residue and the volume again reduced. The process was repeated a third time and the combined filtrates so obtained were analyzed as a whole. By this means 90 to 99 per cent of the filtrable iodine should be removed from the protein residue. The protein residues were also analyzed, as shown in Table III. The total iodine recovered ranged from 83 to 105 per cent, the low values presumably being due to a loss of protein residue which was thick, tenacious, and difficult to remove from the membrane.

As can be seen in Table III, the filtrates of blood plasma, serum, and laked cells showed no detectable iodine except in one instance in which 17 per cent of the total serum iodine appeared to be filtrable. Inorganic iodine was recovered quantitatively in the filtrate. Thyroxine and diiodotyrosine, although filtrable in simple aqueous solution, were retained in the unfiltrable residue after addition of an aqueous solution of the compounds to serum or plasma. Thyroxine added to a solution of egg albumin was also rendered non-filtrable, indicating that the property is not specific for blood but probably represents a simple non-specific adsorption of thyroxine by protein.

Ultrafiltration of certain protein solutions can therefore be used to differentiate between inorganic iodine and the iodine of thyroxine or diiodotyrosine. The results of this study of blood lead

TABLE III

Behavior of Blood Iodine, Potassium Iodide, Thyroxine, and Diiodotyrosine toward Ultrafiltration

Material used	Iodine present	Filtrate iodine	Non-filtrable iodine
	micrograms per 100 cc. H_2O	*micrograms per 100 cc. H_2O*	
Aqueous KI	20	19	
" "	20	21	
" thyroxine	90	94	
" "	74	74	
" "	64	62	
" "	48	48	
Sheep serum + KI	8.0+105.0	100	
Beef plasma + KI	7.8+133.0	140	
" " + KIO_3	8.0+ 50.0	44	
	micrograms	*micrograms*	*micrograms*
Beef plasma	3.0	<0.2	3.0
" "	3.4	<0.2	3.0
" "	4.1	<0.2	3.4
" "	6.5	<0.2	6.2
" "	1.7	<0.1	1.6
" serum	6.8	<0.2	*
" "	13.6	2.4 (17%)	12.1
" plasma + 1 volume H_2O	1.7	<0.1	1.6
" " + 1 " "	2.1	<0.1	*
" cells + 1 volume H_2O	4.0	<0.2	3.5
" plasma + 1 volume thyroxine solution	3.8 + 46.5	<0.2	48.5
" " + thyroxine	1.0 + 19.5	0.6	20.2
" " + "	2.3 + 17.0	<0.2	*
Egg albumin + "	2.9 + 9.6	<0.2	*
Sheep serum + diiodotyrosine	3.6 + 44.0	0.4	47.2
Beef plasma + "	1.4 + 12.0	<0.2	13.2

* Not analyzed.

one to conclude that the inorganic iodine constituents do not exceed 20 per cent of the total iodine and are usually less than 10 per cent or completely absent in so far as the present method permits their determination.

Since the completion of this work, Leipert (6) has published similar ultrafiltration experiments in which the filtrable iodine is said to constitute 60 to 90 per cent of the total. No analyses are given for the iodine content of the non-filtrable residue, this fraction being determined by difference only. The membranes used by Leipert were prepared from 6.5 to 7 per cent collodion in glacial acetic acid to which 2 per cent potassium carbonate had been added. The writer has prepared membranes of this type but has been unable to confirm Leipert's results. Leipert's method of iodine determination has been found by the writer (1) to be subject to variable positive errors which would partially account for the discrepancies between his results and those of the present study. Beef blood has been used in both studies. The results shown in Table III were obtained with blood from animals which had not received iodized salt. Four samples of blood from animals which had been fed iodized salt showed the filtrable iodine to range from 30 to 50 per cent of the total. It is possible that the blood used by Leipert may have been from animals on an iodine-rich diet which served to increase greatly the proportion of inorganic iodine present in the blood.

Use of Zinc Sulfate-Sodium Hydroxide and of Heat and Acetic Acid to Precipitate Blood with and without Addition of Iodine Compounds—Confirmatory evidence that the iodine of blood does not normally exist in inorganic form is shown in the following experiments. The proteins of whole blood were precipitated by two commonly used precipitating agents and the filtrates analyzed for iodine. Table IV shows the results obtained with the use of heat and acetic acid and Table V, those with zinc sulfate and sodium hydroxide. In neither case did the filtrate contain more than 10 per cent of the total iodine, and in both cases added iodide could be recovered in the filtrate and added thyroxine was retained to a large extent on the precipitate. Precipitation by heat and acetic acid was carried out according to the method of Benedict, Newton, and Behre (7), except that the treatment with colloidal iron was omitted; the zinc sulfate-sodium hydroxide precipitation, by the method of Somogyi (8). All reagents were iodine-free.

The results obtained by the use of alcohol and acetone extraction of blood have shown that the blood iodine probably does not exist in protein combination. Ultrafiltration and the experiments

with heat-acetic acid or zinc sulfate-sodium hydroxide precipitation of blood give evidence that not more than 10 per cent of the blood iodine is in inorganic form. Throughout the work, the

TABLE IV

Behavior of Blood Iodine Alone, and of Potassium Iodide and Thyroxine Added to Blood, toward Precipitation of Proteins with Heat and Acetic Acid

Material used	Iodine represented by filtrate	Iodine present in filtrate
	micrograms	*micrograms*
Beef blood	0.8	Trace
" "	1.4	<0.1
" "	1.9	Trace
" "	1.5	0.6 ?
" "	1.7	Trace
" " + thyroxine	1.5 + 4.2	0.7
" " + "	1.7 + 4.0	0.5
" " + KI	1.5 + 4.2	4.6
" " + "	1.7 + 4.0	3.3

TABLE V

Behavior of Blood Iodine Alone, and of Potassium Iodide and Thyroxine When Added to Blood, toward Precipitation of Blood Proteins with $ZnSO_4$ and NaOH

Material used	Iodine represented by filtrate	Iodine present in filtrate
	micrograms	*micrograms*
Human blood	2.2	<0.2
" "	3.7	<0.2
" "	3.4	Trace
" "	6.7	0.5
" " + thyroxine	0.3 + 1.8	<0.2
" " + "	0.2 + 2.0	Trace
" " + "	0.4 + 5.7	<0.2
" " + KI	0.2 + 2.6	2.4
" " + "	0.3 + 2.4	2.6
" " + "	0.4 + 6.3	4.0

blood iodine has behaved exactly as have added thyroxine and diiodotyrosine. The next problem is to show whether either or both of these iodine-containing amino acids may be present.

Analysis of Blood Extracts for Thyroxine by a Modified Leland and Foster Technique—Thyroxine can be determined chemically by the method of Leland and Foster (9) which is based upon the distribution of thyroxine between butyl alcohol and aqueous sodium hydroxide as contrasted with the solubility of other known iodine compounds in these solvents. This method was originally described for use with thyroid tissue, in which the ratio of thyroxine to organic material is much greater than that found in blood and the thyroxine is in protein combination. In the original

TABLE VI

Extraction of Blood Iodine with Butyl Alcohol

Material used	Iodine present	Total iodine extracted		Distribution of total extracted iodine					
				Butyl alcohol			2 N NaOH		
	micrograms	*micrograms*	*per cent*	*micrograms*	*per cent of original*	*per cent of extracted*	*micrograms*	*per cent of original*	*per cent of extracted*
Human blood	18.0	20.0	111						
Beef blood	19.5	14.5	75						
" " + thyroxine	1.0 +9.4	10.2	98						
" plasma + thyroxine	3.8 +5.0	8.6	98	5.7			2.9		
" "	3.8	3.5	92	1.2	32	34	2.3	60	66
" "	71.0	31.8	45	19.2	27	60	12.6	18	40
" "	40.0	27.7	69	20.0	50	72	7.7	19	28
" "	35.0	27.4	79	18.0	51	65	9.6	27	35
" "	47.0	37.9	81	27.3	58	72	10.6	22	28

method a long period of alkaline hydrolysis was employed to liberate the thyroxine from its combination in the protein molecule. During this treatment with alkali, approximately 20 per cent of added free thyroxine was found to be destroyed. Since the thyroxine in blood, if present, could not be in protein combination, it was deemed advisable to remove the protein by some means other than hydrolysis and thus avoid the destruction by alkali. This has been done by using butyl alcohol to extract the original blood. Table VI shows the results of these experiments. 1 volume of blood or plasma was added dropwise to 3 volumes of butyl alcohol and the mixture stirred gently, but constantly, for 15

minutes. Too vigorous mixing resulted in the formation of a thick emulsion which required as long as 24 hours for complete separation. This tendency toward emulsion formation was more marked with whole blood than with plasma, a finding which led to the exclusive use of plasma when large amounts were required. In cases where more than 100 cc. of plasma were extracted, the extraction was made on a series of 100 cc. portions, since this allowed for a more complete mixing of the plasma and butyl alcohol. After each extraction of a given sample, the mixture was centrifuged and the clear, yellow butyl alcohol removed from the aqueous phase and protein residue by decantation. 3 volumes of fresh butyl alcohol were added and the process repeated until all of the water (from the blood) had been removed and only a

TABLE VII

Distribution of Thyroxine, Diiodotyrosine, and Potassium Iodide between Equal Volumes of Butyl Alcohol and 2 N Sodium Hydroxide

Material used	Total iodine	Iodine in butyl alcohol	Iodine in NaOH	Volume of each phase
	micrograms	*micrograms*	*micrograms*	*cc.*
Thyroxine	8.4	7.9	0.3	100
"	8.4	8.0	0.3	10
Diiodotyrosine	8.0	0.4	7.5	10
KI	8.0	0.6	7.2	10

finely granular residue remained. At this point the supernatant butyl alcohol was clear and colorless. From four to six extractions were made in each case.

Control experiments showed that all of the iodine of blood or plasma could be removed from small samples by this procedure and that added thyroxine could be recovered in the butyl alcohol. This again confirms the conclusion that the blood iodine is non-protein in nature. The butyl alcohol extract so obtained was concentrated under reduced pressure on a steam bath to a volume of 25 cc. for each 100 cc. of plasma used, and the concentrated butyl alcohol shaken with 1 volume of 2 N aqueous sodium hydroxide. Table VII shows the distribution of small amounts of pure thyroxine, diiodotyrosine, and potassium iodide between butyl alcohol and 2 N sodium hydroxide. The results are in agree-

ment with those reported by Leland and Foster (9) for larger amounts of material.

The mixtures obtained after shaking butyl alcohol extracts of blood with aqueous sodium hydroxide were allowed to separate completely and the two phases analyzed separately. As shown in Table VI, the butyl alcohol phase after this separation contained from 27 to 58 per cent of the total plasma iodine. Thyroxine when added to plasma was recovered in the butyl alcohol phase. In the last four experiments shown in Table VI, large amounts of plasma were used (500 to 1500 cc.), and the original removal of iodine from the protein was not complete. It is interesting to note that as the percentage of the total iodine removed increased in the different experiments, the increase was largely in the thyroxine-like fraction; the non-thyroxine iodine remained relatively constant at 20 to 30 per cent of the total plasma iodine. Too much emphasis should not be placed upon this finding, since in one experiment, in which 3.8 micrograms of iodine were completely removed from 50 cc. of plasma, 60 per cent was in a non-thyroxine form. It should be pointed out that this method, although added thyroxine can be recovered by it, may not be specific for this substance alone in the presence of iodine compounds other than diiodotyrosine and simple iodides. It is quite possible that the blood might contain some iodine compound other than thyroxine which has a high solubility in butyl alcohol as compared with its solubility in aqueous media.

The separation of the iodine into butyl alcohol- and water-soluble fractions is an actual separation of at least two different forms, as shown by the fact that the iodine of the butyl alcohol phase remains in the butyl alcohol with repeated extractions with 2 N sodium hydroxide and does not follow a percentage distribution of the order observed when the first separation was made. Were only a single compound present in the original extract, the aqueous sodium hydroxide would be expected to remove a constant percentage of the amount with each extraction.

Because of the non-specific nature of the above evidence for the presence of thyroxine, attempts were made to use biological tests on material obtained by the butyl alcohol and the acetone methods of extraction. This material, purified in various ways, has been used in the attempt to stimulate metamorphosis of tad-

poles. The results to date have been uniformly inconclusive because of the toxicity of the extracts. Further attempts to use methods of biological assay are planned for future work.

DISCUSSION

The results of this study are at variance with many of those reported in the literature, both in regard to factual material and the interpretation placed thereon. Part of this discrepancy may be due to positive errors in some of the methods of iodine analysis which have been used. As shown by us (1), Leipert's original method, in which arsenious oxide is used as the reducing agent, is subject to a variable positive error. We believe that all methods in which dry ashing is used to destroy organic material, alcohol extraction to remove the iodine from the ash, and final titration with sodium thiosulfate, are similarly subject to a positive error. This error results from the non-specificity of the Winkler titration in which any oxidizing agent capable of liberating iodine from potassium iodide or reacting with the thiosulfate will lead to erroneously high values. For example, in one of our experiments a portion of blood was extracted with ethyl alcohol until all of the original iodine had been removed and *could be accounted for in the extract.* The protein residue was analyzed by the Trevorrow and Fashena method and no iodine found. A second portion of the residue analyzed by the method of Turner (10) showed iodine to be present in an amount representing 4.0 micrograms per cent for the original blood. This type of error has therefore led to a general acceptance of the presence of "protein" iodine in the blood. Baumann and Metzger (11), in 1937, abandoned the dry ashing method previously published by them because of the variable positive errors involved.

That the method used in the present study is not subject to this error has been demonstrated in another way. 73 cc. of human blood were digested according to our analytical procedure and the iodine distilled. An aliquot of the distillate, representing 8.5 cc. of blood, was treated in the usual manner and iodine representing 5 micrograms per cent was found. The remainder of the distillate was treated with sodium bisulfite to reduce all of the iodine to iodide, evaporated to 2 cc., acidified, evaporated to 1 cc. (excess SO_2 boiled off), treated with palladium chloride, and the

color produced compared with that of standard iodide solutions. Iodine representing 5 micrograms per cent was found. Since the colorimetric procedure does not depend in any way upon oxidation or reduction reactions, the agreement between the results obtained by the colorimetric and titrimetric methods speaks well for the specificity of both procedures.

Besides the errors of analysis, we also believe that errors in interpretation have been made, especially with respect to the inorganic fraction of the blood iodine. Veil and Sturm (12), Lunde, Closs, and Pederson (3), and Eufinger and Schulte (5) have observed that a portion of the blood iodine can be extracted with solvents which remove inorganic iodides and have therefore concluded that this part of the blood iodine is inorganic in nature. These experiments have been inadequately controlled in that no consideration was given to the possible existence of simple organic iodine compounds. We have shown that the amino acids thyroxine and diiodotyrosine when added to blood can be extracted with these solvents.

By means of ultrafiltration and experiments with heat and acetic acid or zinc sulfate and sodium hydroxide precipitation, we have been able to differentiate between inorganic and simple organic iodine. The results of these studies lead to the conclusion that the blood samples studied cannot contain more than 20 per cent of their iodine in inorganic form.

SUMMARY

1. The iodine in the blood of animals on an iodine-poor diet is not in protein combination.

2. Not more than 20 per cent of this iodine is in inorganic form.

3. The greater part of the blood iodine possesses properties similar to those of thyroxine and diiodotyrosine, and a portion of this is not diiodotyrosine but is thyroxine-like in its solubility.

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