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Protein-bound Iodine in Extrathyroidal Tissues

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It has been shown that the concentration of protein-bound iodine in serum usually reflects the concentration of the thyroid hormone, increasing in hyperthyroidism and decreasing in hypothyroidism. Several reasonably satisfactory analytical methods have been developed in the past decade and give results which are in essential agreement. There is evidence that normally most of the protein-bound

iodine which is separable from inorganic iodide in serum by zinc precipitation is present as thyroxine (Taurog & Chaikoff, 1948; Man, Kydd & Peters, 1951).

The desirability of determining the concentration of a hormone not merely in the manufacturing gland or in the blood stream, but also in the peripheral tissues, has led to many attempts, beginning with

Justus (1904), to extend the scope of iodine determinations from serum to tissues. Early work consisted of determinations of total, rather than protein-bound iodine, and was based on methods which were unreliable even for analysis of serum. Even the more recent reports give insufficient data regarding the reliability of the methods used. In view of recent progress in analytical methods, a new attempt to solve this problem seemed justified. Little would be gained, however, by adding to the conflicting values already in the literature without giving adequate data on the reliability of the method. We have therefore devoted considerable attention to this point. In this paper we shall describe the method used by us for serum analysis and indicate its limits of error, describe the modifications of this method which make it suitable for tissue analysis, indicate its limits of error when so used, and report the results of tissue analyses.

METHODS

Protein-bound iodine of serum

This is a modification of the method described by Barker (1948) and, like it, follows the general outline of Chaney's (1940) method in that the sample is digested in hot chromic acid and distilled over into a trap containing an alkaline reducing solution, the concentration of I_2 in the distillate being finally determined by its ability to catalyse the reduction of cerium.

Reagents

These are as described by Barker, with the following exceptions:

Double-distilled water. Once-distilled water is redistilled from alkali.

Sodium arsenite solution. 3.75 g. As_2O_3 are dissolved in 100 ml. 20% (w/v) NaOH and made up to 500 ml. This is used in the trap during distillation. No Na_2SO_3 or $n-H_2SO_4$ are used. This substitution has been found satisfactory by Barker.

Arsenious acid solution. 2 g. NaOH are dissolved in 200 ml. of water and 3.0 g. As_2O_3 and 87.5 g. NaCl are added. To the mixture is then added a solution which has been made by cautiously adding 764 ml. conc. H_2SO_4 (sp. gr. 1.84) to 1 l. of water. The acid solution should be cooled before mixing. After mixing, the solution is again cooled and made up to 2 l.

Standard iodide solutions. Each standard solution is prepared by diluting 40 ml. sodium arsenite solution and the required quantity of a standard solution of KI to 1 l. Thus prepared, 10 ml. of each standard solution contains the same quantity of trap solution as a 10 ml. sample of unknown distillate. These standard solutions are stable indefinitely. We have found that solutions containing 0.00, 0.01, 0.02, 0.06, 0.10 and 0.12 $\mu g.$ of iodide/10 ml. are sufficient to establish the standard curve.

Haemoglobin solution. Approximately 500 ml. fresh oxalated or defibrinated dog blood are centrifuged and the plasma removed. The erythrocytes are then washed five times

by suspension in 0.85% (w/v) NaCl and centrifugation. The volume of NaCl solution used in each wash should be at least equal to the volume of erythrocytes. The erythrocytes are finally added to water and the solution made up to a volume twice that of the original volume of whole blood.

Special apparatus

300 ml. digestion flasks are employed. In the end-point determination an Evelyn colorimeter with a no. 420 filter is used, and the test tubes are the 'selected absorption tubes' designed for this colorimeter. Each stirrer consists of a glass rod, about 25 cm. long, to the lower end of which is sealed a small, slightly concave glass disk, about 1.5 cm. diameter. The disk is sealed at right angles to the stirring rod, with the concavity directed upward, so that the stirrer resembles a shallow spoon with an elongated handle bent to form a right angle with the bowl of the spoon.

Procedure

This follows Barker's method with the following exceptions.

Digestion. The flasks are agitated during initial heating until boiling is smooth and vigorous.

Distillation. Sodium arsenite solution (1 ml.) is placed in the trap at the beginning of distillation. At the conclusion of distillation the trap is drained into a 25 ml. volumetric flask before the flame is turned off, in order to prevent suck-back. After the flame is turned off, the still is washed four times with water and the distillate plus washings is made up to 25 ml.

End point. A 10 ml. sample from each 25 ml. of distillate plus washings is placed in a colorimeter tube. Standard iodide solution (10 ml.) is placed in each of twelve test tubes to provide six pairs of standards containing from 0.00 to 0.12 $\mu g.$ of iodide. Arsenious acid solution (2 ml.) is added to each tube, which is then placed, after thorough shaking, in the previously warmed water bath, the standards being scattered at random among the unknowns. A stirrer is placed in each tube. Ceric ammonium sulphate solution is placed in a separate reservoir tube which is also placed in the bath. After 30 min. equilibration, 0.5 ml. ceric ammonium sulphate solution is withdrawn from the reservoir and added to each tube according to a definite schedule as described by Barker, except that 60 sec. are allowed between tubes. Immediately after addition of ceric ammonium sulphate solution the contents of the tube are vigorously stirred for 15 sec. by repeatedly drawing the stirrer up and down through the solution while the thumb and forefinger rotate the stirrer. A reading is taken 24 min. after addition of the ceric ammonium sulphate solution, the stirrer being removed just before the tube is placed in the colorimeter.

Blanks and duplicates. Each reagent blank contains 2 ml. of haemoglobin solution as 'carrier' protein, instead of the dialysed dog serum recommended by Barker. A pair of blanks is included with each set of unknowns and each unknown is analysed in duplicate. However, after distillation of all blanks and unknowns in a set, only one of each pair of blanks and unknowns is run in the end-point determination. The other member of each pair is set aside and analysed on the subsequent day. Thus, each day's end-point determination includes half of that day's set and half of the previous day's set. The distillates are stable at room temperature for at least 6 days.

Limits of error

The method was tested in three ways: (a) amounts of iodide ranging from 0.02 to 0.60 μg . were added to blanks containing haemoglobin solution before digestion, and the recovery and precision determined; (b) precision was tested by repeated analyses of the same serum; (c) the recovery of 0.1 μg . of iodide added before digestion to a serum of known iodine content was repeatedly determined. The results of these experiments are shown in Tables 1 and 2.

Analyses of sera from euthyroid humans by the present method have shown the normal range of values to be similar to that previously established with the Riggs-Man method (Riggs, 1947).

Protein-bound iodine of tissues

The method just described is used with the following further modifications.

Precipitating reagents

The NaOH solution is not modified, but the concentrations of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and H_2SO_4 in the acid zinc sulphate solution are three times those described by Barker (1948).

Procedure

Sampling of tissues. The dog tissues were taken from carcasses directly after exsanguination under light ether anaesthesia ('bleeder' dogs) or at the conclusion of heart-lung preparations ('HLP' dogs). All tissues were blotted free of gross blood, trimmed, and stored frozen at -7° . Only the ventricular muscle of the heart was used. As far as possible, renal cortex was separated from the medulla and only the cortex analysed. Intestine was stripped of the tunica serosa and tunica muscularis, and the contents of the lumen were washed out with tap water. The tunica albuginea was stripped from the testis.

Grinding and precipitation. A 2 g. (± 0.1 g.) sample of the thawed tissue is placed in a mortar with a small amount of alundum (grit no. 36B). As the tissue is ground it is moistened with small amounts of acid zinc sulphate solution. The total amount of this solution used in grinding and precipitation is 16 ml. The thoroughly ground tissue is transferred to a 50 ml. centrifuge tube with the aid of about 10 ml. of water and precipitated by adding the rest of the acid zinc sulphate solution and 6 ml. of 0.75N-NaOH. Washing is then carried out as described by Barker.

Digestion. 1 l. flasks are used. 75 ml. of 70% (w/v) H_2SO_4 and 9 ml. of 60% (w/v) CrO_3 are used. Haemoglobin solution (6 ml.) is added to each blank. After addition of the reagents and sample the flasks are stoppered and left overnight at room temperature ('cold' digestion) before digestion with heat is carried out.

Distillation. 20 ml. of 50% (w/v) H_3PO_3 are used.

Limits of error

The tissue method has been subjected to the same three types of test as the serum method. The results are listed in Tables 1 and 2. Except when otherwise noted, 2 g. samples of tissue were used, and the results were calculated in terms of wet tissue.

It should be noted that the amounts of iodine added to blanks and tissues in recovery experiments were of the same order of magnitude as those actually found in 2 g. samples of normal dog tissue. In calculating the percentage recovery of the added iodine, the variation between replicate analyses is necessarily exaggerated somewhat since all of the analytical error is ascribed to the added iodine. The standard deviation for the recovery of iodine added to tissue is therefore misleadingly high. The lower the ratio of added iodine to iodine already present, the higher this error will be. If the number of determinations of recovery is relatively small, such an increase in standard deviation may cause serious error in estimating the mean recovery. Table 2 shows that the standard deviation expressed as per cent recovery tended to vary inversely with the amount of added iodine. However, the standard deviation expressed as absolute amount of iodine was fairly constant from tissue to tissue.

Comments and precautions

Storage of tissues. The best conditions for storage of tissues before precipitation have not been precisely determined. However, the protein-bound iodine in a large sample of normal human serum stored at -7° has been determined repeatedly during a period of 88 weeks and has remained constant within the error of the method. A limited number of repeat analyses have indicated that storage of tissues for as long as a month at this temperature is probably also safe.

Grinding and precipitation. Comparison of a series of analyses of dog heart in which 0.7 g. samples were used, with a series in which 2 g. samples were used showed that the latter gave appreciable improvement in precision. In order to produce a precipitate which can be well washed, the tissue must be finely ground before precipitation. The Waring Blender and the Potter homogenizer gave unsatisfactory results. Grinding by hand with alundum for several minutes reduces even the tougher tissues to a satisfactory pulp. This step increases the total time consumed in the analysis, but prevents falsely high values caused by inefficient washing.

Digestion. In two experiments radioactive iodine (^{131}I) was added to the reagents as used for serum, and all vapours arising during digestion were condensed and collected in fractions. The principal loss of iodine occurred over a 3-4 min. period directly after the onset of boiling in the first digestion, and amounted to only about 2%.

Recoveries were poor when an excess of protein was added and digestion was therefore incomplete. Recoveries were also unreliable when too little protein was added and the resulting excess of CrO_3 was not completely reduced by the H_3PO_3 . When no protein was added, but care was taken to reduce all the excess CrO_3 by addition of excess H_3PO_3 , recoveries were still unreliable. The necessity for 'carrier' protein has been noted by Taurog & Chaikoff (1946), and by Barker (1948). A solution of dog haemoglobin can be simply prepared and furnishes 'carrier' protein of extremely low iodine content. Human haemoglobin has also been found satisfactory in the thyroid clinic of the Massachusetts General Hospital (Pratt, 1950). If the use of a sample weighing considerably less than 2 g. is unavoidable, enough haemoglobin should be added to bring the total amount of protein to about 400 mg. For tissues it was found necessary to triple the amounts of both digestion reagents since the use of 9 ml. of CrO_3 with 25 ml. of H_2SO_4 led to very poor recoveries.

Table 1. *Precision of protein-bound iodine method for human serum and dog tissues*

Material analysed	Dog	Preliminary 'cold' digestion	No. of pairs* of analyses	Protein-bound iodine	
				Mean $\pm$ S.D. (μ g./100 g.)	Coefficient of variation 100 S.D. M
Normal human serum	—	No	11	6.1 $\pm$ 0.19	3.1
Heart	D	No	3	1.7 $\pm$ 0.07	4
Heart	E	No	3	2.2 $\pm$ 0.31	14
Skeletal muscle	K	No	6	1.2 $\pm$ 0.37	31
Skeletal muscle	M	Yes	5	1.1 $\pm$ 0.31	28
Liver	J	No	6	6.1 $\pm$ 0.68	11
Liver	K	Yes	5	5.4 $\pm$ 0.98	18
Spleen	K	No	6	1.9 $\pm$ 0.24	13

* As explained in the text, one member of any given pair was usually digested and distilled on a different day from its mate and the two were never run in the same end-point determination. Furthermore, in all experiments listed in Tables 1 and 2, except those involving heart, the individual analyses contributing to any statistic were spread over four or more end-point determinations.

Table 2. *Recovery of iodine added to reagents, serum and tissues*

(2 g. samples of tissue used except where otherwise stated.)

Dog*	Material to which iodine was added	Iodine added (μ g.)	Form in which iodine added and time of addition†	No. of pairs of analyses	Percentage recovery. Mean $\pm$ S.D.
	Blank + haemoglobin (no precipitation)	0.02	KI, before digestion	8	97 $\pm$ 19
		0.10		20	98 $\pm$ 4.8
		0.60		6	87 $\pm$ 1.2
	Normal dog serum (no precipitation)	0.10	KI, before digestion	8	92 $\pm$ 2.8
	Blank + haemoglobin (no precipitation)	0.02	KI, before digestion	8	87 $\pm$ 9.6
		0.04		12	85 $\pm$ 7.4
		0.06		10	88 $\pm$ 7.9
		0.10		13	105 $\pm$ 7.2
		0.60		6	89 $\pm$ 1.1
D	Heart	0.06	KI, after pptn. and washing, before digestion	3	97 $\pm$ 3.2
E	Heart, 1.5–2.0 g.	0.061	1 ml. of normal human serum, before grinding	4	91 $\pm$ 12
K	Skeletal muscle	0.05	KI, after pptn. and washing, before digestion	8	36 $\pm$ 29
M	Skeletal muscle	0.05	KI, after cold digestion before hot digestion	6	95 $\pm$ 3.7
M	Skeletal muscle	0.05	KI, after pptn. and washing, before cold digestion	5	81 $\pm$ 13
J	Liver	0.08	KI, after pptn. and washing, before digestion	8	84 $\pm$ 4.6
K	Liver, 1.3 g.	0.134	2 ml. normal human serum after pptn. and washing, before cold digestion	6	81 $\pm$ 14
K	Spleen	0.04	KI, after pptn. and washing, before digestion	8	74 $\pm$ 19

* See Table 1 for the iodine content of the dog tissues used in these experiments.

† When 'digestion' alone is listed, a preliminary cold digestion was not used.

A preliminary 'cold' digestion was adopted after it was found that iodide added to certain tissues, especially skeletal muscle, could not be recovered reliably unless a preliminary 'cold' digestion was done. A comparison of the data for dog *K* skeletal muscle with those for dog *M* in Table 2 will make this clear. Although the recovery of the protein-bound iodine already present in the tissue may have been adequate (Table 1), even when the recovery of added iodide was poor, such an assumption was considered dangerous. We cannot explain why the recoveries of iodide added to certain other tissues were satisfactory in the absence of preliminary digestion (Table 2). It was decided that the safest course would be to subject all tissues to a preliminary 'cold' digestion. It seemed very unlikely that this would be detrimental with tissues which gave good recoveries without such digestion. This assumption was tested by comparing the recoveries from liver with and without 'cold' digestion. No significant difference was noted (Table 2).

Distillation. The size of the flasks has been increased to accommodate the increased volume of reagents. The small flasks used for serum analysis can be used for tissue analysis only if the H_2PO_4 is added very slowly, thus preventing the digest from boiling up into the trap.

End-point determination. The schedule used is arbitrary and may be modified as desired, provided each tube is treated in exactly the same way.

Since the reaction proceeds most rapidly directly after addition of the cerium, immediate thorough mixing is necessary. Shaking the tubes in the bath or stirring with an ordinary stirring rod gave poor results. Removing the tubes from the bath and shaking allowed satisfactory mixing but caused appreciable cooling. Satisfactory mixing without removal of the tubes was obtained with the stirrers described above. They are easy to make and to clean.

Originally, the end-point determination for both members of each pair of duplicates was performed at the same time. However, experiments in which a large number of analyses of the same serum or tissue were performed showed that the variation between results obtained on the same day was significantly less than the variation between results obtained on different days. The principal source of this day-to-day variation was the end-point determination, as the following experiment showed. On day 1, 0.10 $\mu g.$ of iodide was added to each of six blanks and the recoveries were determined. On day 2, a second set of six was similarly analysed. Colorimetric analysis of each distillate of the first set was also performed on day 2 together with the distillates of the second set. A significant difference ($P=0.01$) was found between the means of the results for the first set of distillates analysed on day 1 and day 2 in two different end-point determinations. No significant difference was found between the means of the results of the two different sets of analyses, digested and distilled on different days, when the same end-point determination was used for both sets. The same batch of reagents was used in both end-point determinations, and a standard curve was run for each determination. This experiment led us to adopt the system of splitting duplicates between two end-point determinations, with consequent decrease in error due to the differences between end-point determinations.

During any single end-point determination the temperature of the water bath has remained constant within $\pm 0.5^\circ$. This amount of variation does not affect the precision of the method appreciably, although we do not deny

the desirability of regulation to within $\pm 0.1^\circ$, as recommended by Barker (1948). The setting of our thermostat has varied spontaneously from day to day between extremes of 35 and 38° . However, this day-to-day variation is of little consequence since a separate standard curve is constructed for each end-point determination, and we have not been able to correlate the differences between the results obtained in different end-point determinations with the observed differences in temperature.

Throughout this paper the mean of each pair of analyses has been treated as a single result. In practice, whenever our results show a difference of more than $2.0 \mu g./100 g.$ between the members of a pair of analyses of serum or tissue, the analyses are repeated. In calculating the statistics listed in Tables 1 and 2, however, such data have not been discarded, and the statistics in certain instances therefore slightly overestimate the variation obtained in practice.

The reasons for changes in the arsenious acid solution have been explained in a previous paper (Carr, Graham, Ober & Riggs, 1950). Since then the concentration of NaCl has been further increased. With lower NaCl concentrations the sensitivity of an occasional end-point determination was very low. Increasing the NaCl concentration has eliminated this difficulty. It is conceivable that such poor results were due to contamination with traces of Ag, which is known to interfere with the iodide catalysis, presumably through formation of insoluble AgI (Sandell & Kolthoff, 1936). The solubility product of AgCl is almost 10^8 times that of AgI and it would therefore require a high Cl/I ratio to favour AgCl formation. It is even possible that the entire chloride effect is due to the presence of traces of Ag.

When the amount of iodine in the sample is high, it may be necessary to use less than 10/25 of the distillate. Although the standard solutions contain the same amount of trap solution as 10/25 of a distillate, they may still be used, since the differences in amount of trap solution have not been found important.

In another experiment equal samples of the same pooled distillate from tissue were placed in each of a series of tubes, amounts of iodide ranging from 0.00 to 0.08 $\mu g.$ were added, and an end-point determination performed. By comparison with the usual series of standard solutions, it was found that the distillate decreased the slope of the standard curve. Distillate from liver (9 ml.) gave results which were 19% too low and 8 ml. of distillate from muscle gave results which were 16% too low. The amount of trap solution was the same in all tubes. Failure to obtain 100% recovery of iodine added to tissue therefore does not necessarily mean loss during digestion or distillation.

RESULTS

The results of analyses of serum and tissues from normal and hyperthyroid dogs are shown in Tables 3 and 4. All dogs were fed a diet of chopped horse meat. HLP (heart-lung preparation) dogs were made hyperthyroid by the additional daily feeding of 0.8 g./kg. of U.S.P. thyroid for 5–8 weeks. Hyperthyroid HLP dogs showed weight loss or increased appetite or both. They also showed a marked tendency to develop hyperthermia during sodium pentobarbital anaesthesia, which was induced once a week for the performance of other

studies (Riggs, Stanbury & Carr, 1952). 'Bleeder' dogs were made hyperthyroid in the same manner, except that the thyroid powder was administered for 1-2 weeks. As a control, one dog was fed 1.6 mg. of inorganic iodide daily for 17 days. This amount was equivalent to the total iodine content of 0.8 g./kg. of the thyroid powder.

The feeding of powdered thyroid clearly increased the protein-bound iodine in the serum and tissues. The protein-bound iodine in the serum and tissues of the dog fed supplementary iodide also increased. To find whether the high concentrations in the latter dog actually represented protein-bound iodine or simply iodide which had not been removed by washing, the iodide in the supernatant fluid from the final wash of each sample of tissue was determined. In the supernatants from skeletal muscle, a mean of 0.052 μ g. of iodide was found. If one makes the conservative estimate that one-half this quantity remained in the precipitate, approximately 38 % of the supposed protein-bound iodine may actually have been iodide. For heart, the corresponding figure was 16 %. However, for all of the other tissues less than 10 % of the total iodine found in the precipitate could have represented residual iodide.

Analyses of brain have not been satisfactory, although our results suggest that the concentration of protein-bound iodine in dog brain is very low. Attempts were also made to determine the concentration of protein-bound iodine in the adrenal gland and ovary, but because of the small quantity of tissue available the results were not trustworthy and have been omitted.

In addition to comparing the concentrations of protein-bound iodine in different tissues, we studied the distribution of protein-bound iodine within the cell. These experiments were carried on in collaboration with Dr F. A. Lipmann and Dr R. K. Crane of the Massachusetts General Hospital, who have very kindly given us permission to include the results in this paper. In each of two separate experiments the livers of three normal adult rats were pooled, ground in a homogenizer in sucrose solution, and subjected to differential centrifugation according to the technique described by Hogeboom, Schneider & Pallade (1948) with minor modifications (Lipmann, 1951). Portions of the original suspension and of each fraction obtained by centrifugation were then analysed according to the procedure for tissue except that no grinding was necessary. The results are shown in Table 5. The concentrations of protein-bound iodine appeared to be about equal in the fractions of rat-liver cells. The total amount of protein-bound iodine, calculated from the sum of the three separate fractions, was 79 % of the amount actually found by analysis of the whole suspension in Exp. 1, and 90 % in Exp. 2. Assuming

Table 3. *Protein-bound iodine in serum and tissues of normal dogs*
(μ g./100 g. wet wt. tissue)

Dog	Serum	Heart	Skeletal muscle	Liver	Kidney	Spleen	Small intestine	Pancreas	Testis	Lung
<i>Q</i>	1.8	1.1	0.3	4.6	2.8	0.8	2.3	1.2	1.0	2.3
<i>R</i>	1.5	1.6	0.6	6.5	2.3	1.7	0.5	0.9	0.5	2.4
<i>U</i>	1.1	0.6	0.8	6.4	3.2	3.0	2.4	1.3	1.5	3.7
Mean	1.5	1.1	0.6	5.8	2.8	1.8	1.7	1.1	1.0	2.8
S.E.	± 0.20	± 0.29	± 0.15	± 0.62	± 0.48	± 0.64	± 0.62	± 0.12	± 0.29	± 0.45
<i>P</i> for serum v. tissue	—	—	—	—	—	—	> 0.1	> 0.1	> 0.1	> 0.05
Adult heart-lung preparation dogs (female)										
<i>B</i>	1.8	0.3	0.3	4.4	3.5	3.7	—	—	—	—
<i>G</i>	1.7	1.5	1.4	7.8	1.8	5.0	—	—	—	—
Mean, all adult dogs	1.6	1.0	0.7	5.9	2.5	2.8	—	—	—	—
S.E.	± 0.13	± 0.25	± 0.20	± 0.64	± 0.30	± 0.74	—	—	—	—
<i>P</i> for serum v. tissue	—	> 0.05	< 0.05	< 0.05	< 0.05	> 0.1	—	—	—	—
12-week puppies (female)										
<i>D</i>	2.6	2.6	1.6	6.9	—	—	—	—	—	—
<i>E</i>	3.0	2.5	2.3	3.3	—	—	—	—	—	—

Table 4. *Protein-bound iodine in serum and tissues of adult dogs fed thyroid or KI*

Drug	Weeks	Dog*	Serum	Heart†	(μg./100 g. wet wt. tissue)						
					Skeletal muscle	Liver	Kidney	Spleen	Small intestine	Pancreas	Testis
Thyroid	2	N	16.5	(48.5)	6.5	21.5	33.5	21.6	32.4†	9.2	9.9
	2	O	9.3	7.9	4.3	20.5	20.6	49.3	96.2	7.5	7.6
	1.5	P	10.6	13.3	3.6	10.8	12.2	8.0	17.5	4.3	3.4
	2.5	S	12.5	—	11.9	—	—	—	—	—	—
Mean, dogs N, O, P, S			12.2	10.6	6.6	17.6	22.1	26.3	48.7	7.0	7.0
Thyroid	5	11	7.8	8.4	(42.6)	—	—	—	—	—	—
	6	14	29.9	7.7	—	47.2	—	—	—	—	—
	6	15	10.5§	5.1	14.7	32.3	—	—	—	—	—
	6	16	15.0§	7.7	9.8	26.4	—	—	—	—	—
KI	5	19	17.9	4.3	7.3	20.5	15.1	—	—	—	—
	6	20	8.8	4.9	13.7	25.9§	9.7	6.1	—	—	—
	6	21	14.8§	12.8	3.1	24.9	14.4	17.2	—	—	—
	8	23	13.8	1.3	6.8	19.3	—	—	—	—	—
Mean all dogs fed thyroid	5	24	21.8	18.2	7.5	17.7	—	45.7	—	—	—
	6	25	9.9	5.8	3.0	11.6	—	—	—	—	—
	5	26	12.3	6.3	5.1	—	—	—	—	—	—
	2.5	T	14.1	8.0	7.5	23.2	17.6	24.6	48.7	7.0	24.9
Mean of normal dogs O, R, U			5.6	6.8	3.4	15.8	10.8	15.8	46.3	6.8	26.1
(Table 3)			1.5	1.1	0.6	5.8	2.8	1.8	1.7	1.1	1.0

* Dogs N, O, P and S were 'bleeder' dogs. All others were HLP dogs.

† Additional blood required in the heart-lung preparation was taken from hyperthyroid dogs for dogs 21-26, and from normal dogs for dogs 11-20. Only the heart was exposed to the added blood.

‡ Mean of the results of two pairs of samples, the means of the pairs agreeing within 2.0 μg. %.

§ The difference between duplicates was greater than 2.0 but less than 4.0 μg. %.

The two values in parentheses have been excluded in calculating the means.

Table 5. *Protein-bound iodine in rat-liver fractions*

(In each of two experiments the livers of three normal adult rats were pooled, dispersed in sucrose solution and differentially centrifuged.)

Fractions		Total protein (g.)	Protein-bound iodine ($\mu\text{g./g. of protein}$)	Total protein- bound iodine ($\mu\text{g.}$)
Nuclei	1	0.488	0.30	0.15
	2	0.426	0.26	0.11
Mitochondria	1	2.400	0.31	0.74
	2	2.192	0.38	0.83
Supernatant*	1	2.048	0.23	0.47
	2	1.982	0.35	0.69
Whole suspension (calculated)	1	4.936	—	1.36
	2	4.600	—	1.63
Whole suspension (found)	1	5.448	0.32	1.74
	2	5.320	0.34	1.81

* Chiefly cytoplasm and extracellular fluid.

that roughly 20% of the wet weight of liver is protein, the 0.32 and 0.34 $\mu\text{g.}$ of protein-bound iodine per g. protein found in the suspensions would correspond to protein-bound iodine concentrations of 6.4 and 6.8 $\mu\text{g./100 g.}$ in whole liver. These calculated concentrations are reasonable, since 4.8 and 6.9 $\mu\text{g./100 g.}$ of protein-bound iodine were actually found by analysis of whole, untreated livers from two rats in the same colony.

DISCUSSION

Protein-bound iodine was found in all tissues analysed, but not in equal concentrations. In normal dogs (Table 3) the concentration of protein-bound iodine in skeletal muscle was significantly lower, and in liver and kidney significantly higher than in serum. Indeed the concentration in liver was significantly greater than in any of the other tissues. Albert, Rall, Keating, Power & Williams (1949), Albert & Keating (1949) have reported accumulation of thyroxine in the human liver as judged by *in vivo* measurements of radioactivity, after the intravenous administration of radioactive DL-thyroxine. Although in our experiments the concentration of protein-bound iodine in heart tended to be lower, and in lung higher than in serum, the differences were not significant by Student's '*t*' test. Of all organs studied, the spleen showed the greatest variability. This was to be expected since erythrocytes lack protein-bound iodine (Riggs, Laviates & Man, 1942) and the quantity of erythrocytes may differ greatly from spleen to spleen.

In discussing the fate of radioactive DL-thyroxine in a cretin, Albert & Keating (1949) noted that on intravenous administration the initial volume of distribution of labelled thyroxine was approximately equal to the plasma volume. After 6 hr., however, a second volume of distribution was attained. 'Perhaps fortuitously this second volume

of distribution has approximately the dimensions of extracellular fluid, and is smaller than the volume of distribution of inorganic iodide' (Albert & Keating, 1949). Rough calculations based on the data for three dogs suggest that in these animals the volume of distribution of protein-bound iodine was substantially greater than the volume of extracellular fluid. Furthermore, the concentration of protein-bound iodine per gramme of wet tissue was greater in many tissues than in serum. Such high concentrations indicate that most of the protein-bound iodine in these tissues was extravascular and very probably that it was intracellular, since it is unlikely that a substance so extensively bound to protein would be more concentrated in interstitial fluid than in serum. Finally, the studies of distribution in rat liver (Table 5) strongly suggest that at least some protein-bound iodine is located within cells.

The feeding of U.S.P. thyroid in a dose of 0.8 g./kg. of body weight per day increased the concentrations of protein-bound iodine in serum and in most tissues roughly ninefold (Table 4). The average increase was somewhat less for liver, possibly because of its ability to excrete large quantities of exogenous thyroxine when given in unphysiological doses (Gross & Leblond, 1947). The average increase was somewhat greater for small intestine, very likely in part because of the oral route of administration and perhaps in part because of excretion of organic iodine in the bile. Unfortunately, the significance of the protein-bound iodine found in the serum and tissues of the hyperthyroid dogs is impossible to evaluate since very similar, although in general somewhat lower, concentrations were found in the tissues of the dog given only iodide in an amount equal to the total iodine content of the thyroid used. The only conclusion which may fairly be drawn is that in no tissue was there any striking differential accumulation of the exogenous hor-

mone. An increase in serum protein-bound iodine in humans due to the administration of iodide has been described by Danowski, Johnston & Greenman (1950). By extracting with butanol, Man *et al.* (1951) have been able to separate the protein-bound iodine fraction, which presumably represents hormone from the unknown protein-bound iodine compound and which appears during the administration of inorganic iodine. In future studies of the distribution of exogenous hormone in tissues it would probably be advisable to employ butanol extraction as an integral part of the method.

SUMMARY

1. A method for the determination of protein-bound iodine in extrathyroidal tissues is described. Data regarding the limits of error of the method are given.

2. In normal dogs the concentration of protein-bound iodine in the serum is higher than in skeletal

muscle and lower than in liver and kidney. No significant difference was found between the concentration of protein-bound iodine in the serum and that in the heart, lung, spleen, small intestine, pancreas and testis.

3. The feeding of powdered thyroid or iodide to normal dogs caused an increase in the concentration of protein-bound iodine of all tissues studied.

4. Protein-bound iodine is probably partly intracellular in the dog. In rat liver, protein-bound iodine occurs in approximately equal concentrations in the nuclear, mitochondrial, and supernatant (cytoplasm plus extracellular fluid) fractions.

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Studies in Detoxication

48. URINARY METABOLITES OF 4:6-DINITRO-*O*-CRESOL IN THE RABBIT

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4:6-Dinitro-*o*-cresol (DNOC) has been used as an insecticide and fungicide for many years, and more recently has become important in agriculture as a selective weed killer (Shepard, 1951). Its use for this purpose has been the cause of several deaths and its former use in the treatment of obesity has been discontinued because of its high toxicity (Bidstrup & Payne, 1951; Horner, 1942).

Little is known about the metabolism of DNOC; only a small amount is eliminated unchanged in the urine, although it is readily absorbed (Spencer, Rowe, Adams & Irish, 1948). In man, it seems to act as a cumulative poison and is eliminated slowly over a period of weeks after ingestion (Harvey, Bidstrup & Bonnell, 1951; King & Harvey, 1952). In laboratory animals, however, there appears to be no