urines. In this test, preservation of the urines for a longer period than sixteen days resulted in a decrease of the uric acid.

The authors wish to acknowledge the valuable assistance of Messrs. F. G. Allison and A. F. D. Wussow in connection with the analytical work of this investigation.

URBANA, ILL.

THE DETERMINATION OF IODINE IN PROTEIN COMBINATIONS.

BY LOUIS W. RIGGS.

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The recent progress of thyroid therapy, and the apparent relation between the quantity of iodine in thyroid preparations and their physiological action, have emphasized the importance of greater accuracy in the quantitative determination of iodine in protein combinations.

The method used by nearly all investigators for the determination of the amount of iodine in protein combination is that of Baumann,[1] certain features of which were previously suggested by Rabourdin,[2] and is briefly as follows:

The iodine-containing protein is dried, powdered, and mixed in a large silver crucible with from two to three times its weight of solid sodium hydroxide and sufficient water to make a paste. The contents of the crucible are then cautiously heated to complete carbonization when sodium nitrate, in amount approximately equal to one-half the weight of the sodium hydroxide used, is added to completely oxidize the carbon. The fused mass is extracted with water, filtered, acidified with sulphuric acid, and shaken out with chloroform. The chloroform solution has a purple color. The quantity of iodine is estimated colorimetrically by adding to solutions of sodium sulphate known quantities of potassium iodide, a few drops of a dilute solution of sodium nitrite, sulphuric acid to acid reaction, shaking out with an equal volume of chloroform, and comparing colors in equal sized cylinders.

Oswald,[3] upon Baumann's advice, employed a nickel instead of a silver crucible for the fusion, while Anten[4] used carbon disulphide in place of chloroform on account of the rapid fading of the color of dilute chloroform solutions of iodine.

The author's determinations of iodine in about sixty human thyroid glands, according to the foregoing process, indicated the desirability of further improvements of Baumann's method.

Unless otherwise stated, in all determinations to which reference is

[1] *Z. physiol. Chem.*, **22**, 1.

[2] *Ann. Chem.*, **76**, 375.

[3] *Z. physiol. Chem.*, **23**, 265.

[4] *Arch. Exp. Path. Pharm.*, **48**, 331.

made in the following investigation, the protein, if dry, was fused with from two to two and one-half times its weight of solid sodium hydroxide in a nickel crucible. About one-fifth of the sodium hydroxide was added after the carbonization, and immediately before adding sodium nitrate. The same weight of sodium hydroxide was used with four or five grams of fresh tissue as with one gram of dried tissue or dried protein. The fusion was conducted at a barely perceptible red heat and the powdered sodium nitrate added gradually. The fused mass was cooled, extracted with water and filtered, the crucible and filter repeatedly washed with hot water, the washings added to the extract, the latter made up to 100 cc., and thoroughly mixed. An aliquot part of this extract, usually ten cc., was placed in a small separator with ten cc. of carbon tetrachloride[1] and cautiously acidified with 25 per cent. sulphuric acid. After several thorough shakings with all possible care to avoid loss due to the escape of carbon dioxide, the carbon tetrachloride extract was filtered through a small filter previously moistened with carbon tetrachloride into a 10 cc. Nessler tube of carefully selected white glass, giving a column of liquid 10 cm. in length.

At first standards were made according to Baumann's method, but it was found that the quality of the color of the carbon tetrachloride solution from the fused protein was often slightly different from that of the standards. In order that the standards might be prepared under conditions more nearly parallel to those of the extracts under examination, about 100 grams of beef heart tissue were fused with sodium hydroxide and sodium nitrate, the fused mass extracted with water, filtered and diluted. Standards were prepared by placing five or ten cc. of this extract in a separator with 10 cc. of carbon tetrachloride and a known quantity of potassium iodide, sulphuric acid was then added to acid reaction, the carbon tetrachloride solution of the free iodine shaken out and filtered into a 10 cc. Nessler tube, which must be precisely the same in shape and color of glass as the tube containing the carbon tetrachloride solution of iodine from the protein under examination.

Is a Measurable Portion of Iodine Oxidized to Iodate during the Fusion Process?—This possibility was recognized by Baumann, but he stated that loss from such action would be avoided by removing the flame when carbonization was completed and immediately adding finely powdered sodium nitrate to the contents of the hot crucible. In attempting to follow this part of Baumann's procedure it was found necessary to heat the crucible to bright redness in order to obtain complete combustion of the carbon. Such a degree of heat, and the violent deflagration which occurs on the addition of the nitrate, although tending to reduce iodate

[1] At the suggestion of Dr. Beebe, carbon tetrachloride was used instead of chloroform or carbon disulphide.

to iodide, would cause a loss of the latter by volatilization. This procedure also requires a larger quantity of nitrate than is necessary if the latter be added gradually to the contents of the crucible maintained at a dull red heat.

In order to determine if any iodine as iodate remained in the acid aqueous liquid after shaking out with carbon tetrachloride, the liquid was again shaken with another 10 cc. of carbon tetrachloride to remove any traces of free iodine. This second portion of carbon tetrachloride was drawn off, the acid aqueous liquid placed in a small Kjeldahl flask with about 0.5 gram of Devarda's alloy[1] and made strongly alkaline with sodium hydroxide. A brisk action usually began at once, but in all cases heat was applied for about 30 seconds to insure a vigorous and complete reduction, and the flask with its contents allowed to stand over night. (Experiments upon solutions of sodium iodate of known concentration showed reduction to be complete in about three hours.) The liquid in the flask was then filtered into a separator. To the filtrate were added 10 cc. carbon tetrachloride, one cc. of a one per cent. solution of sodium nitrite, and 25 per cent. sulphuric acid to *strong* acid reaction. The acid should be added until the precipitate which first formed is completely dissolved. After thorough shaking, the carbon tetrachloride was filtered into a 10 cc. Nessler tube and read against standards made from a liquid containing the reduction products of Devarda's alloy, one cc. of a one per cent. solution of sodium nitrite and known quantities of potassium iodide.

For comparison tubes Baumann used cylinders 20 cm. in length and of 100 cc. capacity. Ten cc. of chloroform would make a layer of liquid in such a cylinder about two cm. deep. Accordingly he stated that he obtained the most satisfactory readings when 10 cc. of chloroform contained from 0.2 to 1.5 mg. of iodine. With the smaller tubes used by the writer, the best readings were obtained when 10 cc. of carbon tetrachloride contained from 0.02 to 0.15 mg. of iodine. Quantities of iodine within these limits, in 10 cc. of carbon tetrachloride, were much more accurately read by the small Nessler tubes than by a Duboscq colorimeter.

The purpose of the analyses, of which the results are given in the following table, was to determine if iodine as iodate was left in the acid aqueous liquid after shaking out with carbon tetrachloride. Before applying the reduction process the acid aqueous liquid was shaken out with a second 10 cc. of carbon tetrachloride to remove any trace of free iodine left by the first extraction. This second extraction would be omitted in analyses if the object be to determine the total iodine, as any traces

[1] *Z. anal. Chem.*, **38**, 55. (Al, 59; Cu, 39; Zn, 2 per cent.)

of free iodine left by the first extraction would be recovered along with that obtained by reduction.

One entire lobe of the gland was used for each analysis. They varied in weight as follows: Beef thyroid lobes from 3.5 grams to 33.0 grams. Pig thyroid lobes from 2.5 grams to 13.5 grams. Sheep thyroid lobes from 2.1 grams to 11.8 grams. The glands were selected at random from lots of 10 to 25 pounds received from abattoirs. Column (I) gives the number of the analysis; column (II) the iodine found in milligrams per gram of fresh gland; and column (III) the percentage of total iodine found by the reduction process.

		Beef thyroid.			
I.	II.	III.	I.	II.	III.
1	0.03	27.0	8	0.41	7.2
2	0.19	20.0	9	1.02	2.0
3	0.04	30.7	10	0.61	6.6
4	0.10	20.0	11	0.70	2.3
5	0.17	13.5	12	0.45	5.6
6	0.17	9.1	13	1.47	2.6
7	0.26	5.4	14	1.07	6.3
		Pig thyroid.			
1	1.04	0.0	5	1.54	3.0
2	0.65	0.0	6	0.31	4.0
3	1.34	trace	7	0.65	2.4
4	0.12	trace	8	0.08	25.0
		Sheep thyroid.			
1	0.03	77.0	4	0.034	58.0
2	0.013	72.0	5	0.062	57.0
3	0.034	55.0	6	0.41	10.2

While our analytical results generally show that the less the quantity of iodine per gram of fresh gland the greater the proportion oxidized to iodate, exceptions sometimes occur. Thus a sample of tissue residue from human thyroid glands, which had been ground with sand and extracted several times with 0.8 per cent. sodium chloride solution, and therefore was very poor in iodine, was roughly divided into two nearly equal portions, A and B, and the iodine determined.

	Iodine before reduction, mg.	Iodine after reduction, mg.	Total, mg.
A	1.4	0.55	1.95
B	2.0	0.05	2.05

Although the total quantities of iodine in A and B agreed as closely as could be expected, it being impossible to divide the tissue equally; 28 per cent. of the iodine in A was obtained by reduction, while only 2.4 per cent. in B.

Iodides reduce iodates in the presence of sulphuric acid according to the reaction

$$5NaI + NaIO_3 + 3H_2SO_4 \longrightarrow 3I_2 + 3Na_2SO_4 + 3H_2O.$$

Disregarding at this point the action of nitrous acid, which is always present in the acidified fusion extract, it is evident that if the proportion of iodate be greater than one molecule of iodate to five molecules of iodide, some of the iodine will remain as iodate after shaking out with carbon tetrachloride.

In the fusion process sodium nitrate is added in a quantity sufficient to produce a homogeneous melt free from particles of carbon. Under these conditions the extent of oxidation is entirely unmanageable.

From the results of these and many other analyses we conclude that more or less iodate is usually present, and that in an accurate determination of the iodine in protein combination it is never safe to omit the reduction feature of the method outlined in the foregoing pages. Inasmuch as the reduction process is applied to the acidified fusion extract *after* the determination of iodine has been made by the usual method, it in no way interferes with this method. Any iodine recovered by the reduction process would otherwise be thrown away.

Accuracy of the Reduction Process.—The reliability of the reduction process was tested as follows: An alkaline fusion extract was prepared by fusing a protein free from iodine with sodium hydroxide and sodium nitrate. Eight solutions containing this fusion extract mixed with known quantities of iodide and iodate were made up to 100 cc. and numbered by a member of the laboratory staff. These solutions were examined for iodine precisely as in the case of aqueous fusion extracts from iodine containing proteins, with the following results:

	Given.			Found.		
No.	Iodine as iodide, mg.	Iodine as iodate, mg.	Total, mg.	Before reduction, mg.	After reduction, mg.	Total, mg.
1.....	0.35	0.3	0.65	0.41	0.22	0.63
2.....	0.25	0.4	0.65	0.3	0.33	0.63
3.....	0.4	0.5	0.9	0.55	0.3	0.85
4.....	0.5	0.8	1.3	0.75	0.55	1.3
5.....	0.15	0.4	0.55	0.35	0.2	0.55
6.....	0.9	0.2	1.1	0.85	0.2	1.05
7.....	0.45	0.55	1.0	0.65	0.3	0.95
8.....	1.0	0.25	1.25	0.95	0.28	1.23

That the reduction feature of this method is quite accurate is shown by a comparison of the figures in the fourth and seventh columns of the foregoing table.

Attempt to Reduce Iodate with Nitrous Acid.—Twenty-five grams of beef heart tissue were fused with 17 grams of sodium hydroxide and sufficient sodium nitrate to oxidize the carbon. The fused mass was extracted

with water, the extract filtered and made up to 250 cc. Ten cc. of this fusion extract gave by the permanganate method 0.15 gram of sodium nitrite. Ten cc. of the fusion extract with 10 cc. of carbon tetrachloride and 0.2 mg. of iodine as sodium iodate were placed in each of four separators; the contents of each separator strongly acidified with 25 per cent. sulphuric acid and shaken at frequent intervals for one and one-half hours. On comparison with standards it was found that the largest reduction of any of the four samples was equivalent to 0.05 mg. of iodine, or only one-fourth of the iodine present.

Ten cubic centimeters of a fusion extract—prepared as in the last experiment but containing 0.21 gram of sodium nitrite in 10 cc.—were placed with 10 cc. of carbon tetrachloride in each of eight separators. Iodine as sodium iodate was added to the contents of the separators as follows: Nos. 1 and 2 received 0.05 mg. 3 and 4 received 0.1 mg., 5 and 6 received 0.15 mg., and 7 and 8 received 0.2 mg. The contents of each separator were made strongly acid with 25 per cent. sulphuric acid, and shaken vigorously at frequent intervals. Ten minutes after the first shaking Nos. 1–4 gave no color; Nos. 5–8 gave a very slight color. One hour later, with frequent shaking, Nos. 1–4 showed a slight color; Nos. 5 and 6 gave a color equivalent to 0.04 mg. of iodine; Nos. 7 and 8 gave 0.05 mg. of iodine. All smelled of nitrous fumes. After three hours there was no apparent change in color. The next day all colors had faded as compared with standards made from solutions of potassium iodide, sodium sulphate, and 0.01 gram of sodium nitrite in 10 cc., and which had been allowed to stand the same length of time. Nos. 2, 4, 6, and 8 were then made alkaline with sodium hydroxide, reduced with Devarda's alloy, and the balance of the iodine recovered without difficulty.

Ten cubic centimeters of a fusion extract containing 0.15 gram of sodium nitrite were mixed in a separator with 10 cc. of carbon tetrachloride, 0.2 mg. of iodine as sodium iodate, and 25 per cent. sulphuric acid to strong acid reaction. Immediately after shaking, the carbon tetrachloride showed but a trace of color equivalent to not more than 0.01 mg. A one per cent. solution of sodium nitrite was then added to the contents of the separator in portions of 1 cc. each, with vigorous shaking between each addition. Three cc. of sodium nitrite developed a color equivalent to about 0.05 mg. of iodine, or one-fourth of the quantity present. The color of the tetrachloride was brownish and impossible to read accurately. Two cc. more of the nitrite solution and one cc. of 25 per cent. sulphuric acid caused dense brown fumes above the liquid in the separator.

Nitrous acid fails to reduce iodates quantitatively to iodine so that the latter can be measured colorimetrically.

Ten cubic centimeters of a fusion extract containing 0.15 gram of sodium nitrite were mixed in a separator with 10 cc. carbon tetrachloride, 0.1 mg. of iodine as potassium iodide, and 25 per cent. sulphuric acid to acid reaction. The color immediately after shaking was equivalent to 0.1 mg. of iodine. To the contents of the separator 1 cc. of a 20 per cent. solution of sodium nitrite was added and mixed thoroughly, whereupon the color of the tetrachloride changed to salmon. Another cubic centimeter of the sodium nitrite caused a further loss of color, and 4 cc. of 20 per cent. sodium nitrite completely discharged the purple color of the carbon tetrachloride. A repetition of this experiment with double the quantity of iodine gave parallel results.

Not only does nitrous acid fail to reduce iodates so that the iodine can be read colorimetrically, but sufficient excess of the acid will decolorize a solution of iodine in carbon tetrachloride. It was also found that nitrous acid would modify or discharge the color of a carbon disulphide solution of iodine..

Analyses of Mixed Iodide and Proteid.—Baumann tested his process by mixing known quantities of potassium iodide with fibrin and subjecting the mixture to analysis by the method employed for the determination of iodine in thyroid glands. About 90 per cent. of the iodine was recovered by analysis.

In order to test the details of the process as employed by the author, analyses were made of thirty mixtures of potassium iodide with fibrin. The concentration varied from 0.45 to 4.0 mg. of iodine as potassium iodide to one gram of dried fibrin. In the majority of these analyses, ninety per cent. or more of the iodine was recovered *before* reduction, and traces or none by the reduction process. In general, the greater the concentration of the iodine the less obtained by reduction. Mixtures containing two or more mg. of iodine as potassium iodide with one gram of fibrin yielded 95 per cent. of their iodine without reduction. Two out of the thirty mixtures gave exceptional results, namely, 20 and 30 per cent. of their iodine by reduction.

The analyses of ten mixtures of fresh beef pancreas tissue with varying quantities of iodine as potassium iodide, gave results closely agreeing with those obtained from the analysis of mixtures of fibrin and potassium iodide.

It is evident that iodine as potassium iodide *mixed* with a proteid is not oxidized by fusion with sodium hydroxide and sodium nitrate to the same extent as iodine in *combination* with a proteid, such as the iodine-containing constituent of the thyroid gland.

The fact that iodine-containing proteids, especially those containing small quantities of iodine, do not yield all of their iodine by Baumann's

process, throws doubt upon the accuracy of results heretofore announced with reference to the iodine content of protein substances.

The modifications of Baumann's process suggested in this paper still leave much to be desired. It is therefore my intention to continue the study of the problem, especially the fusion and reduction features.

Summary.

Ten-cubic-centimeter Nessler tubes of clear *white* glass, and giving a column of liquid 10 cm. in length, yield more delicate readings with dilute solutions of iodine in carbon tetrachloride than larger-sized tubes or a Duboscq colorimeter.

A portion of the iodine is oxidized to iodate during the fusion and may be lost unless subsequently reduced. Devarda's alloy was used as the reducing agent. The reduction is particularly necessary in the analysis of proteins containing but a small proportion of iodine.

Excess of nitrous acid fails to reduce iodates so that the iodine can be estimated colorimetrically in carbon tetrachloride solution, and a sufficient excess of nitrous acid will modify or discharge the color of a carbon tetrachloride solution of iodine. Too great a quantity of sodium nitrate must not be added during the fusion, or an excess of nitrous acid will be formed upon acidifying.

Mixtures of protein substances and potassium iodide subjected to analysis by the foregoing process do not give results comparable with those obtained from the analysis of a protein substance containing *combined* iodine, such as thyroid gland tissue.

Extreme care must be used to make the conditions under which standards are prepared and read parallel to those to which the substance under examination is subjected. Reductions and colorimetric readings should be made in duplicate or triplicate, and repeated, if necessary, until concordant results are obtained.

The writer wishes to express his thanks to Dr. S. P. Beebe for much of the material upon which this investigation was made, and for his hearty encouragement throughout the work.

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THE DETERMINATION OF UREA IN URINES.

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Recent work on protein metabolism has established the fact that the ratio of urea nitrogen to total nitrogen in urine is a variable, depending on many physiological and pathological conditions and, furthermore,