THE DETERMINATION OF IODINE IN PROTEIN COMBINATIONS.

(SECOND PAPER.)

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During the past three years the study of the iodine content of protein substances, particularly those of the thyroid gland and various thyroid extracts, has been pursued quite steadily in this laboratory with several objects in view. Besides an attempt to determine the relation, if any, of the quantity of iodine to normal and various pathological conditions of the gland, the studies have led to the establishment of certain standards, based on the iodine content, for the grading of thyroid preparations used medicinally. Fundamental to the accomplishment of the objects is an accurate method for the determination of iodine in protein combinations.

During this investigation over three hundred analyses have been made not including duplicates. About one hundred of these were of thyroid glands from as many different human beings. Over eighty were of animal thyroids from dog, beef, pig, and sheep. About sixty were of thyroid extracts such as "thyroglobulin," "thyronucleoproteid," "thyroidine," and medicinal preparations of the gland. A dozen analyses were made of tissues other than thyroid, and about sixty were of mixtures of iodide or iodate with fibrin, heart tissue, pancreas tissue, casein, or other non-iodine containing protein.

The author was led to question the Baumann process by failure to obtain any iodine in nine thyroid glands from patients whose cases were reported as exophthalmic goiter, although iodine was found in glands from twenty similar cases, and also by failure in some instances to obtain concordant results by duplicate analyses of samples from the same gland.

In my previous paper[1] it was shown that more or less of the iodine might be converted to iodate during the fusion process, and consequently not recovered by shaking out with carbon tetrachloride, if the proportion of iodate to iodide be greater than a certain amount, which is perhaps one molecule of iodate to five molecules of iodide. It was also shown that iodate left by the application of Baumann's process could be recovered by reduction.

The present paper furnishes much additional experimental evidence of the necessity for using the reduction process. The objection[2] urged against this process is considered; and analytical data are exhibited from a considerable variety of material.

The essential points of the Baumann process are given in detail in my first paper. In outline they are: fuse the protein with sodium hydroxide and saltpeter to a homogeneous melt, extract the fused mass with water,

[1] THIS JOURNAL, 31, 710 (June, 1909).

[2] Seidell, *Ibid.*, 32, 1326 (Dec., 1909).

filter, acidify with sulphuric acid, and shake out with chloroform. The chloroform solution of the free iodine is matched in color by a chloroform extract of a mixture consisting of sodium sulphate, sulphuric acid, sodium nitrite, and a known quantity of iodine as potassium iodide.

The most important mod fications of the Baumann process suggested in my first paper are: Use of carbon tetrachloride in place of chloroform. Use of 10 cc. Nessler tubes of *white* glass in which 10 cc. of liquid makes a layer 10 cm. deep. Use of a fusion extract of a non-iodine containing protein instead of plain sodium sulphate for the preparation of standards. After the completion of the analysis by Baumann's method, the acid aqueous liquid, from which free iodine had been removed, was made alkaline, reduced with Devarda's alloy, filtered, acidified with sulphuric acid, sodium nitrite added, any free iodine shaken out with carbon tetrachloride and read against standards made from the reduction products of Devarda's alloy and a known quantity of iodine.

Several investigators have reported the finding of thyroid glands free from iodine, and theories have been constructed with reference to the function of "iodine-free thyroid." In the analysis of over one hundred and eighty thyroid glands in this laboratory I have yet to find one free from iodine. If, however, I had used the Baumann process *without* the reduction feature, I could have reported more than a dozen glands as iodine-free. Table I exhibits some of these results.

In Tables I, II, III, IV, and VI the figures have been calculated from the readings to milligrams of iodine per gram of fresh gland or fresh proteid. In Table V, the figures mean total quantity of iodine in milligrams in the sample used for analysis. The term "trace" wherever used in this paper means less than 0.01 mg. of iodine in 10 cc. of carbon tetrachloride. The untrained eye can readily appreciate 0.01 mg. of iodine in 10 cc. of carbon tetrachloride when looking through a column of the liquid 10 cm. deep at a white ground. Where percentages are given, traces are discarded in the calculations.

TABLE I.

No.	Before reduction.	After reduction.	No.	Before reduction.	After reduction.
1	trace	0.032	7	0.0	0.035
2	0.0	0.011	8	0.0	0.0025
3	0.0	0.013	9	0.0	0.01
4	trace	0.022	10	trace	0.034
5	trace	0.03	11	0.0	0.043
6	0.0	0.01	12	trace	0.026

Nos. 1 to 9 represent pathological human thyroids analyzed in 1907, using zinc dust as a reducing agent. It is possible that if Devarda's alloy had been used larger quantities of iodine would have been recovered. Some of these glands weighed from 150 to 200 grams so that although

the weight of iodine per gram of fresh gland was small, the total quantity was in some cases equal to that frequently found in normal glands. Nos. 10 to 12 represent sheep thyroids very poor in iodine.

Seidell suggests that the iodine which I found by reduction was that left in the aqueous liquid by imperfect extraction, although in the thirty analyses reported in my previous paper I used two portions of carbon tetrachloride of 10 cc. each, while he claims complete extraction with three portions of two or three cc. each. A few careful analyses in which more iodine was found after reduction than before would be sufficient to show the error of the foregoing suggestion. In twenty-five analyses I find more than 50 per cent. of the iodine by reduction.

Again if the iodine obtained by reduction be due to imperfect removal of the free iodine by the *first* extraction with carbon tetrachloride, then the greater the quantity found before reduction, the larger the quantity left by imperfect extraction to be recovered by reduction. Table II exhibits the analytical results upon glands rich in iodine in which none or but traces were found by reduction.

TABLE II.

No.	Before reduction.	After reduction.	No.	Before reduction.	After reduction.
1	0.47	trace	7	0.36	0.0
2	0.30	0.0	8	1.67	trace
3	0.34	0.0	9	0.97	trace
4	0.70	trace	10	0.30	0.0
5	0.65	0.0	11	0.71	trace
6	1.33	trace	12	1.04	0.0

In these cases where the largest absolute quantity of iodine should be found by reduction, if Seidell's assumption be well founded, I find none or but traces.

In my previous paper it was stated that the most accurate readings were obtained when 10 cc. of carbon tetrachloride contained from 0.02 to 0.15 mg. of iodine. (It is my practice to select such an aliquot part of the fusion extract as will bring the readings within these limits mentioned.) But four of the readings of the thirty analyses of thyroids reported in that paper were above 0.15. Two were 0.16, one 0.20, and one 0.21. The average reading for the thirty was 0.11 mg. In each of these thirty analyses the acid aqueous liquid, which generally measured about 20 cc., was extracted with a second 10 cc.[1] of carbon tetrachloride to remove possible traces of iodine left by the first extraction before applying to reduction process. This detail was overlooked by Seidell in his reference to the reduction process.

In order to determine the quantity of iodine left in 20 cc. of the acid aqueous liquid after *one* extraction with 10 cc. of carbon tetrachloride

[1] THIS JOURNAL, 31, 712, line 7 and line 39.

the following experiment was performed: Ten cc. of an iodine-free fusion extract were placed in each of six separators marked respectively *A*, *B*, *C*, *D*, *E*, and *F*. To each separator were added 0.2 mg. of iodine as potassium iodide, 1 cc. of a 1 per cent. solution of sodium nitrite, 10 cc. of carbon tetrachloride, and about 7 cc. of 10 per cent. sulphuric acid which was enough to cause a strong acid reaction. The separators were then vigorously shaken with care to prevent loss by the escape of carbon dioxide. After three shakings at intervals of ten to fifteen minutes the carbon tetrachloride was drawn off from each separator as completely as possible. Ten cc. of fresh carbon tetrachloride were then added to the contents of separator *A*, thoroughly shaken, filtered into a 10 cc. Nessler tube, compared with standards and found to contain much less than 0.01 mg. of iodine. This portion of carbon tetrachloride, which had made the *second* extraction of the acid aqueous liquid in *A*, was then added to the contents of separator *B*, shaken out and added to *C* and so on until the acid aqueous liquid in each of the six separators had been subjected to its *second* extraction by the same 10 cc. of carbon tetrachloride. From the last separator (*F*) the carbon tetrachloride was filtered into a Nessler tube, compared with standards, and found to contain 0.03 mg. of iodine, from which I conclude that approximately 0.005 mg. of iodine is left in 20 cc. of acid aqueous liquid, originally containing 0.2 mg. of iodine, after iodine is removed by the *first* ten cc. of carbon tetrachloride. This result was obtained before the publication of my previous paper. It has since been confirmed by two repetitions with concordant results.

The acid aqueous liquid in the six separators was then subjected to a *third* extraction with 10 cc. of carbon tetrachloride, performed in the same manner as the second extraction already described, and found to contain *no iodine*.

If 20 cc. of acid aqueous liquid contain *less* than 0.2 mm. of free iodine, less will be left after the first extraction thus making a second extraction quite superfluous. Experiment confirms this statement. Several samples of acid aqueous liquid containing from 0.1 to 0.15 mg. of iodine, when extracted with a second 10 cc. of carbon tetrachloride, gave a liquid that could not be distinguished from 10 cc. of the pure reagent by several observers each accustomed to colorimetric reading.

The results of the application of this process to other glands, in which the presence of iodine might be suspected, are shown in Table III.

TABLE III.

No.	Before reduction.	After reduction.	No.	Before reduction.	After reduction.
1	0.0	0.0	4	0.0	0.0
2	0.0	0.0	5	0.0	0.0
3	0.0	0.0	6	0.0	trace

No. 1 was a thymus gland from a child one year old, 2 and 3, thymus

glands from adults, 4 and 5, beef parathyroids, and 6 a human hypophysis. The record exhibited in Table III at least serves as a check on the purity of the reagents used in this investigation.

The analyses shown in Tables IV and V were made by Miss Van Alstyne of this laboratory and to whom I wish to express my thanks. The determinations of iodine recorded in Table IV were incidental to physiological studies on the storing of iodine in the thyroid gland by dogs; and those of Table V to the standardizing of thyroid preparations used medicinally.

TABLE IV.

No.	Before reduction.	After reduction.	No.	Before reduction.	After reduction.
1	3.59	0.0	6	trace	0.50
2	0.87	0.0	7	0.05	0.03
3	0.46	0.0	8	0.06	0.19
4	9.32	0.0	9	0.52	0.17
5	4.37	0.0	10	0.25	0.16

Twenty-four other determinations of iodine were made in the course of this investigation, fourteen of which gave other 5 per cent. of the iodine by reduction, while four gave all of the iodine *before* reduction. The extremely large quantities of iodine in Nos. 1, 4, and 5 were produced by previous iodine feeding.

TABLE V.

No.	Before reduction.	After reduction.	No.	Before reduction.	After reduction.
1	0.58	0.30	4	2.10	0.36
2	trace	0.18	5	0.38	0.025
3	trace	0.02			

Table VI shows the results of the analysis of various fractions of human thyroproteins by Dr. Beebe whom I again wish to thank for many favors.

TABLE VI.

No.	Before reduction.	After reduction.	No.	Before reduction.	After reduction.
1	0.68	0.19	4	0.30	0.43
2	0.80	0.20	5	0.27	0.18
3	0.22	0.39			

The figures exhibited in Tables IV, V, and VI clearly demonstrate that when 10 cc. of carbon tetrachloride are used with about 20 cc. of the acid aqueous liquid, imperfect extraction of iodine as suggested by Seidell in no way accounts for the results.

A partial summary of the author's work on the determination of iodine in the thyroid presents the following points: 14 out of 40 glands containing *more* than 0.25 mg. of iodine per gram of fresh gland gave above 5 per cent. of their iodine by reduction. Average of the 14 was 14.2 per cent. 38 out of 59 glands which contained *less* than 0.25 mg. of iodine per gram of fresh gland gave above 10 per cent. of their iodine by reduction. Average

of the 38 was 55.2 per cent. Excluding from the 38, 13 glands which gave all of their iodine by reduction and the average percentage of iodine obtained by reduction from the remaining 25 glands was 31.9 per cent. While this summary emphasizes the necessity of applying the reduction process to glands poor in iodine, particular analyses show that glands rich in iodine may sometimes yield a considerable quantity of their iodine by reduction.

The experimental results of this paper thoroughly confirm the statement of my previous paper, namely: that more or less iodate is usually present and that in an accurate determination of iodine in protein combinations it is never safe to omit the reduction feature. These results are not explained by the statement "at extreme dilution the constancy of distribution coefficients disappears," which Seidell invokes.

In attempting to follow Seidell's interpretation of Baumann's process, I find it impossible to be certain of several details. Seidell refers to Baumann's earlier process,[1] instead of his later improved process.[2] A very important and essential part of the process as described in both of Baumann's papers is made optional; thus, Seidell directs, "If much charred organic matter is present a little sodium nitrate may be added," etc., from which one would infer that the addition of a nitrate during the fusion is not required, and in the absence of "much charred organic matter" unnecessary. I find a loss of about one-half the total iodine to follow the omission of nitrate during the fusion process in two analyses of sheep thyroid rich in iodine.

Baumann, Oswald, Anten, Marine and Wells, working with similar quantities of protein material to those used by Seidell, employed 10 cc. of solvent (chloroform or carbon disulphide) to extract the iodine from the acid aqueous liquid. If this liquid be extracted with only two or three cc. of solvent instead of 10 cc., it would be expected that a considerable portion of iodine would remain to be extracted by further applications of fresh solvent. Although it is well known that two portions, say 5 cc., of solvent successively applied will dissolve slightly more solute than one portion of 10 cc., it is not clear wherein three successive applications of 2 or 3 cc. of solvent confers accuracy upon this process, especially in view of the fact that one application of 10 cc. of solvent removes 97.5 per cent. of the iodine from the maximum concentrations used by the present writer.

The statements by Seidell that "the reduction process as suggested by Riggs leads to greater errors in the determination of iodine than are inherent in the Baumann process" and "any iodine not removed before

[1] *Z. physiol. Chem.*, 21, 489.
[2] *Ibid.*, 22, 1.

the reduction process leads to a positive error" are totally unsupported by experimental evidence.

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AN APPARATUS FOR ABSOLUTE ALCOHOL.

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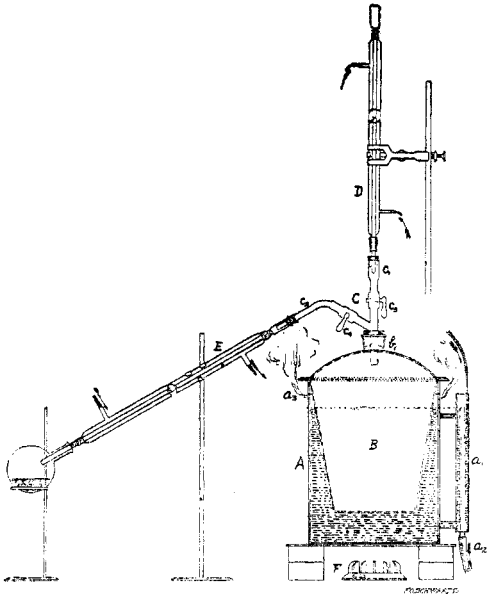
Freshly prepared absolute alcohol is more reliable than the article supplied by dealers and much cheaper. One never feels quite sure without making a test that he is getting an alcohol which is really absolute. For preparing small quantities of this solvent there is no need of suggesting any modification of the usual procedure of boiling ordinary alcohol over burnt lime in a glass flask under a return-condenser and distilling.



But large classes of students often require considerable absolute alcohol, the preparation of which has to be left to persons more or less inexperienced. Under these conditions there is frequent breakage of glass together with loss of alcohol, and another form of apparatus is desirable. For some time the apparatus shown in the drawing has given satisfactory results in this laboratory. It is constructed of copper and was made according to specification by the firm of Eimer and Amend, New York. A tinsmith can make a less expensive apparatus of heavy tin which will answer every purpose.

The outer flanged copper vessel A has an inside diameter of 28 cm. and is 30 cm. high. It is tinned on the inside and the bottom is reinforced on the outside with iron for protection against heat. This vessel serves as a water-bath in which water is kept at constant level by means of the side tubulus a_1 provided with the overflow pipe a_2. The water which passes through condenser D runs into a_1. Several openings a_3 at the top allow steam to escape. This bath rests upon bricks and heat is applied from the Fletcher burner F.

Within A and resting quite free upon its flange is a second flanged