DETERMINATION OF PROTEIN-BOUND IODINE*

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The growing interest in chemical evaluation of thyroid function by use of the plasma level of protein-bound iodine (PI) is attested by the increasing numbers of reports in which this criterion is employed, both chemically and experimentally. However, the specific techniques available for such a determination do not yet seem sufficiently reliable. The purpose of this communication is to describe a procedure evolved in this laboratory which is consistent and sensitive in our hands.

The complete determination of plasma PI involves four distinct steps: (1) precipitation and washing of the plasma proteins; (2) digestion of the protein, leaving the PI in inorganic form; (3) distillation of the inorganic iodide; (4) actual determination of the inorganic iodide. The shortened technique of Salter (4), in which a dry ashing with NaOH and Na₂CO₃ was used to eliminate the need for distillation, has not proved satisfactory in our experience, and no other short cuts have been proposed.

In 1940, Chaney (1) described an all-glass still for use with a chromic-sulfuric acid digestion mixture; iodide catalysis of the reduction of ceric ions by arsenious acid was employed for the ultimate colorimetric determination of iodine. Although other workers have adopted the Chaney still (7), the highly sensitive ceric-arsenious catalysis method was generally avoided until Taurog and Chaikoff reported an entire procedure for plasma iodine (8). The iodide catalysis reaction had been extensively studied in 1937 by Sandell and Kolthoff (5), who demonstrated a marked enhancement of the iodide effect by the presence of a relatively high concentration of chloride. We have found that the use of chloride in optimum concentration improves the sensitivity 30 to 80 per cent with no sacrifice in reproducibility. None of the recently reported techniques has taken advantage of this appreciable increase in sensitivity.

EXPERIMENTAL

Reagents—

Distilled water. Although in the past it has been widely reported necessary to take the extraordinary precautions of freshly distilling the water from alkali, we have found it quite satisfactory merely to redistil once

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distilled water in an all-glass still. Since this is a low iodine region, the
deficiency of iodine in the water may facilitate this. Furthermore, water
so prepared and stored in a stoppered Pyrex flask for as long as 2 months
at summer temperatures did not show any change from its extremely low
iodine content or any effect on the recovery of iodide quantitatively added
to it. All other reagents are made up in the double distilled water.

Somogyi precipitating reagents (6). The acid zinc sulfate solution
contains 12.5 gm. of ZnSO₄·7H₂O and 125 ml. of 0.25 N H₂SO₄ per liter.
NaOH is made up to 30 gm. per liter, and the two solutions are balanced
until 50 ml. of the acid zinc sulfate require between 6.7 and 6.8 ml. of the
alkali to show a permanent pink to phenolphthalein. A blank must be
run on these reagents, carried through the washing procedures to be de-
scribed.

Sulfuric acid solutions. 70 per cent by weight. 780 ml. of concentrated
sulfuric acid, special, As- and N-free, are slowly added, with cooling, to
600 ml. of water in a 2 liter Pyrex flask. Thorough mixing should then
be carried out.

1.0 N. 28 ml. of concentrated acid are carefully added to 900 ml. of water,
and the solution cooled and made to 1 liter.

Chromic oxide. 600 gm. are dissolved in water, and made to 1 liter.
This material has often been found to be contaminated; we have tested
several batches of technical grade CrO₃ and found some to be very low in
iodine. These particular samples are far less expensive than the high
purity material, which is equally apt to be contaminated.

Sodium sulfate. A 10 per cent solution is used, which is made up fresh
for each series of distillations by dissolving 2 gm. in 20 ml. of water.

Phosphorous acid, 50 per cent. 250 gm. are dissolved in about 200 ml. of
water and made to 500 ml. If necessary the solution can be freed of
iodine by boiling for ½ hour (with frequent addition of water); when cool, it
is made to 500 ml. We have used the reagent from Fisher without boiling.

Arsenious acid. 3.71 gm. of As₂O₃ are dissolved in 50 ml. of N NaOH
with stirring. 200 ml. of water are added and the solution neutralized
with H₂SO₄ (requiring about 2.5 ml. of the 70 per cent solution). Then
54 ml. of the 70 per cent H₂SO₄ are added, and the solution made to 500 ml.
3.125 gm. of iodide-free NaCl are dissolved in the 500 ml. of reagent to
avoid the need of another solution.

Ceric sulfate. 12 gm. of ceric ammonium sulfate (G. Frederick Smith)
are stirred into 500 ml. of 3.5 N H₂SO₄. This will be turbid at first, clearing
up within ½ hour upon occasional stirring.

Iodide standards. Pure NaI is carefully dried in a desiccator; 118.1 mg.
are then dissolved and diluted to 1 liter. This stock solution contains
100 γ of I per ml., and must be appropriately diluted to yield the 0.005
to 0.100 γ of I desired as standards.
Special Apparatus—The Riggs modification of the Chaney still was employed as described by Talbot et al. (7) p. 481) except that a 250 ml. flask was substituted for the 500 ml. flask. All grease was cleaned from both stop-cocks, and water was used as the only lubricant. Care must be taken to prevent freezing of the stop-cocks due to drying.

Procedure

This section falls logically into the four categories mentioned earlier.

1. Protein Precipitation and Washing—2 ml. of oxalated or heparinized plasma are precipitated by Somogyi's zine sulfate reagent in a 50 ml. round bottom centrifuge tube. After a 10 minute period of centrifuging, the supernatant is poured off, and the precipitate washed free of inorganic iodide by four successive washings each with 25 ml. of iodine-free distilled water. After the last washing and centrifuging, the protein is dissolved in 5 ml. of 70 per cent H₂SO₄ and transferred to the digestion flask. Four further 5 ml. portions of acid and one final 5 ml. portion of water are employed to insure complete transfer.

2. Digestion—3 ml. of 60 per cent CrO₃ are added, plus a few glass beads, carborundum particles, or other antibumping agent. The digestion is carried out over a small flame until sulfuric acid fumes appear. The flask is allowed to cool, 15 ml. of water are added, and the digestion repeated.

3. Distillation—25 ml. of water and a few fresh beads are added and the chromic acid crystals dissolved by rotation just before distilling in order to make use of the heat generated by dilution. The flask is attached to the still and a micro burner flame placed beneath. Enough water is added through the upper opening of the trap to fill the region of attachment of the stop-cock, and then 0.5 ml. of a 10 per cent Na₂SO₃ solution is allowed to drain down the walls of the bulge above the trap. Most of this will collect in the low portion of the trap, and soon after the distillation of water vapor has begun, condensation results in the trap being completely closed with fluid.

The water-cooled condenser is connected and 5 ml. of 50 per cent H₃PO₄ are placed in the dropping funnel after it has been inserted into the free opening in the flask. All ground glass joints are lubricated with water before being assembled. After boiling has continued until water vapor has entered the condenser and has started to drip into the return tube, the H₃PO₄ is slowly blown into the flask by gentle pressure. The distillation is continued for 10 minutes after reduction is completed. To terminate the distillation, the flame is turned off and the trap is immediately drained into a 22 X 175 mm. test-tube calibrated at 25 ml. The condenser is raised clear and the walls of the trap rinsed down with five successive 2.5 ml. quantities of water, each washing being added to the distillate in the test-tube.
1 ml. of \( n \) H\(_2\)SO\(_4\) is added to each combined distillate and washings. The resulting sulfurous acid is decomposed and SO\(_2\) blown off by aeration at about 2.5 liters per minute while the tube is in boiling water. The tube is then cooled, the volume made to the 25 ml. mark, and the solution thoroughly mixed.

4. Colorimetric Determination of Iodide—5 ml. aliquots are pipetted from the 25 ml. total volume into Klett-Summerson colorimeter tubes. 0.4 ml. of arsenious acid is added, and the tubes are placed in a water bath accurately regulated to 37°. Two tubes containing 5.0 ml. of water and 0.4 ml. of arsenious acid should be routinely used for blank determinations in each series of twenty. Ceric ammonium sulfate is next added, but this must be done on a definite time schedule, since only one measurement is to be made of a rate of reaction. Incubation for the ceric sulfate-arsenious acid reaction is carried out for 15 minutes, and \( \frac{3}{4} \) of a minute is allowed for each tube to be read in the colorimeter. At zero time, 0.5 ml. of the ceric ammonium sulfate solution is added to the first tube, the contents are quickly mixed, and the tube replaced in the water bath. A 45 second interval is allowed, and then the procedure is repeated for the rest of the tubes. Thus, a maximum of twenty individual tubes or ten duplicate determinations can be handled in one series. 15 minutes after addition of the ceric solution to the first tube, it is removed from the bath, the outside quickly wiped clean and dry, and a reading obtained in the photoelectric colorimeter with No. 42 blue filter. The same 45 second interval should be ample for making each reading.

Because of the necessity for rigid adherence to a time schedule, performance must be checked at the start by including standards in each series of determinations. Once the routine has been thoroughly established, it is adequate to run only one set of standards, ranging from 0.01 to 0.10 \( \gamma \), as part of one series each time several sets of determinations are being performed.

The standard curve can be considered straight over only a restricted portion, and iodine values are best judged from an actual plot of reference values (Fig. 1). Blank values should be established for each new batch of reagents, and should be repeated occasionally as a check on contamination. After the blank has been deducted, calculations from these values should include the factor of 5 to cover the aliquot of 5 ml. out of the 25 ml. total volume and a factor of 50 to express the plasma PI in terms of micrograms per 100 ml. of plasma.

**Comments and Precautions**

1. **Protein Precipitation and Washing**—Somogyi's zinc precipitation procedure as described has been found by far the most convenient. Heat
coagulation in a weakly acid medium is satisfactory, but requires constant attention. Trichloroacetic acid has been found unsatisfactory, at least partly because of the large amount of additional organic matter requiring digestion.

The precipitation technique has been applied successfully to tissues other than plasma by using the Potter homogenizer (3). 500 mg. of liver, kidney, heart, or skeletal muscle are homogenized in 8 ml. of the acid zinc sulfate reagent. This is poured into a 50 ml. centrifuge tube, followed by four rinsings of the grinder, each with 4 ml. of the reagent. 0.75 N NaOH is carefully stirred in, to a permanent pink with phenol red (about 3 ml. are required). When a normal thyroid gland is to be analyzed, it is homogenized in water or dilute H₂SO₄, the total volume of suspension plus rinsings being 100 ml. 1 ml. of the thoroughly mixed suspension is taken as an aliquot and is added to 2 ml. of dog plasma stock in a 50 ml. centrifuge tube. The Somogyi zinc precipitation is carried out as usual, followed by the four washings. In this case, the PI value of the dog plasma must be determined and deducted, together with the reagent blank value.

The four washings described have been found adequate to eliminate 99.92 per cent of 1000 γ of inorganic iodide added per 100 ml. of plasma and thus should offer ample routine protection. With the same procedure 5 γ per cent of added thyroxine iodine are retained completely by the precipitate, and 5 γ per cent of added diiodotyrosine iodine are retained to 75 per cent of completion. This curious situation has previously been noted by Man and coworkers (2).

2. Digestion—The amount of chromic acid has been increased over that recommended by Taurog and Chaikoff in order to insure an adequate excess for lipemic plasmas as well as for proteins of other tissues. Potassium or sodium dichromate can be used as well as chromic acid, but they are so much less soluble that the amount to be added would need to be used in solid form. Care should be taken that the second digestion is not prolonged beyond the stage of definite appearance of fumes, since excessive heating often results in considerable loss of the iodine present.

3. Distillation—In this laboratory the digestion of organic material has usually been carried out in ordinary 250 or 300 ml. Pyrex Florence flasks so that many determinations could be carried out without the expense entailed in an equal number of the special flasks used on the distillation apparatus. The principal inconvenience resulting from this economy is the insertion of an extra transfer from digestion flask to distillation flask by means of the 25 ml. of water, used in 5 ml. portions. 10 per cent sodium sulfite is used as the absorbing solution in the trap as a simple substitute for the Na₂CO₃-NaHSO₃ combination utilized by Talbot et al. Results in this laboratory with NaOH alone, as proposed by
Taurog and Chaikoff, have shown uniformly unsatisfactory recoveries, of the order of 0 to 10 per cent. The need for a reducing solution suggests that the volatile iodide may actually be iodine, instead of HI.

The duration of the distillation period is a compromise between the desire for a 100 per cent recovery and for as short a reaction time as possible. In one series of determinations, the recoveries of 0.10 γ of inorganic iodide added to digests of dog plasma were found to be as follows for various times: 5 minutes, 56 per cent; 7½ minutes, 78 per cent; 10 minutes, 85 per cent; 12.5 minutes, 89 per cent; 15 minutes, 92 per cent. At that rate, about 25 to 30 minutes would be required for a 98 to 100 per cent complete distillation. Since other series with 0.05 to 0.25 γ of I have shown 87 to 95 per cent recoveries in 10 minutes, this time has been selected. A correction factor covering the 5 to 15 per cent loss may be introduced if desired.

Although the sodium sulfite has been found essential, as noted earlier, it must be decomposed to SO₂ by acid and this blown off in order not to cause a complete and non-specific decolorization of the ceric solution later. Compressed air has been used, since the substitution of N₂ did not alter the results. A simple and effective set-up consists of a four or six outlet manifold, with finely drawn out glass tubing which reaches nearly to the bottom of each tube. The tubes specified are long enough so that vigorous bubbling can be accommodated. A screw clamp control should be provided for each nozzle to prevent excessive air currents. This aeration step also results in some volatilization of water; so that the level in each tube should be less than 25 ml. even after adequate rinsing of the fine glass tubes. Thus, ample allowance is made for the addition of water to a consistent final volume.

4. Colorimetric Determination of Iodide—This part of the determination is the one most apt to present difficulties, owing to the need for rigorous control of nearly every phase. However, with the requisite amount of care, highly dependable results can be obtained, as is shown by Fig. 1.

Time must be controlled to a far greater extent than is usual with colorimetric procedures, inasmuch as differing rates of a reaction eventually proceeding to completion are involved. Those who have previously used the ceric sulfate-arsenious acid reaction have taken two, and often more, readings in order to establish definitely the rate of decolorization. However, in the interests of simplification, it has seemed desirable to acknowledge the arbitrariness of all colorimetric procedures and to make only a single routine reading at 15 minutes. This should be supplemented by a 30 minute reading whenever the first reading shows a decolorization amounting to less than 40 units on the Klett-Summerson colorimeter (yielded by 0.005 γ of I) if one desires the most accurate results possible in the lower range of iodide values.
Since accurate temperature control is also necessary during the incubation, it has been found convenient to employ a test-tube rack of appropriate size suspended from hooks hung on the opposite sides of a constant temperature water bath maintained at 37°. Although this was done to avoid the nuisance of continually resetting the thermoregulator, any temperature in the region of 30–40° is satisfactory, provided it is regulated within ±0.1°.

The ceric ammonium sulfate solution is measured with the greatest possible accuracy, since it contributes the initial amount of color. The 0.5 ml. required is delivered from a fine tipped, small orifice pipette, between two marks. There is little need for accurate standardization of the solution from an oxidation-reduction standpoint, because each new stock

<table>
<thead>
<tr>
<th>Iodine</th>
<th>NaCl, mg.</th>
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<tbody>
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<td></td>
<td>0.0</td>
<td>1.0</td>
<td>2.0</td>
<td>3.0</td>
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<td></td>
<td>Colorimeter reading</td>
<td>Colorimeter reading</td>
<td>Increase*</td>
<td>Colorimeter reading</td>
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<tr>
<td>γ</td>
<td>per cent</td>
<td>per cent</td>
<td>per cent</td>
<td>per cent</td>
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<td>490</td>
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<td>486</td>
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<td>0.010</td>
<td>445</td>
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<td>23</td>
<td>184</td>
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<tr>
<td>0.100</td>
<td>86</td>
<td>50</td>
<td>27</td>
<td>43</td>
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* This was calculated on the basis of the iodine values of the colorimeter readings without NaCl.

will require that a blank be established and that the iodine effect be standardized.

The 45 second interval between tubes has proved the most valuable, 30 seconds being too short an allowance for all colorimeter readings to be taken and 60 seconds unnecessarily long. One control blank in duplicate plus nine determinations in duplicate on distillates (twenty tubes in all) can thus be handled successfully in each series. The photoelectric colorimeter to be used should be adequately tested for adjustment immediately before one starts the addition of ceric sulfate.

Table I shows the considerable enhancement of the iodide catalysis caused by the inclusion of 1 to 3 mg. of sodium chloride per tube. The maximum effect appears to be exerted on the smaller amounts of iodide, being as great as 82 per cent with 0.010 γ of iodine. Quite satisfactory, reproducible results can thus be obtained with 0.005 γ of I. That contamination of the salt with iodide is not giving a spurious "catalysis" is evident
from inspection of the data in Table I: the blank values on the reagents show only a slight extra decolorization with increased amounts of chloride; the results with 2.0 and with 3.0 mg. of added NaCl are essentially the same.

**Results**

Although the standardization of the iodide-catalyzed arsenious acid decolorization of the yellow ceric solution requires the extraordinary precautions already discussed, remarkably consistent results are obtained when these precautions are scrupulously observed. Fig. 1 shows the mean and the maximum deviation from the mean of points on a calibration curve; ten duplicate determinations were performed on each point over a period of 3 months. From the 30 minute curve in Fig. 1 it can be seen that reliable results have even been obtained with 0.005 γ of iodide under these circumstances.

In order to test the application of this sensitive analytical procedure to biological material, aliquots of dialyzed, pooled dog plasma were analyzed as such and after the addition of amounts of iodide, thyroxine, or diiodotyrosine ranging from 0.05 to 0.50 γ (as I). Table II shows that recoveries were 87 to 95 per cent complete when the addition was made before dis-
tillation was carried out, whereas they were 98 to 101 per cent when iodide was added to the distillate. These findings strongly indicate some loss of iodide during distillation, probably due to the arbitrary termination of

<table>
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<th>TABLE II</th>
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<tr>
<td>Recovery of Iodine from Compounds Added to Dog Plasma</td>
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<td>Substance added</td>
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<td>NaI, before digestion</td>
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<td>Thyroxine, before digestion</td>
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<td>Diiodotyrosine, before digestion</td>
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<td>NaI, after digestion</td>
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<tr>
<td>KIO₃,</td>
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<td>NaI, distillation</td>
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* All figures are the averages of at least four duplicate determinations.

<table>
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<th>TABLE III</th>
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<td>Protein-Bound Iodine in Tissues of Various Species</td>
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<td>Species</td>
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<td></td>
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<tr>
<td>Rat, normal</td>
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<td>&quot; on thiouracil</td>
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<tr>
<td>&quot; thyroxine</td>
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<td>Dog, normal</td>
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<td>Human, normal</td>
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* Standard deviation.
† Micrograms of PI in the entire thyroid gland (10 to 20 mg.).

this step at 10 minutes. As previously mentioned, it was not thought worth while to continue the distillation to the point of a more complete recovery. In this laboratory, a 10 per cent correction is routinely made.

Another fact which should be pointed out is the poor recovery of inorganic iodide in the absence of organic material to be digested. Re-
coveries under such circumstances range from 70 to 85 per cent. Taurog and Chaikoff also noted this, and reported the use of iodine-free wheat as an organic carrier. In this laboratory we have found dialyzed dog serum much more convenient; it can be stored in the frozen condition for long periods of time or it can be lyophilized and redissolved when needed. The iodine values are low and remain stable over long periods of storage in the frozen or dried state.

Studies on the various solutions obtained at different stages of the procedure have revealed unexpected keeping qualities, provided bacterial contamination does not occur. Plasmas and final distillates have been kept in the refrigerator without preservative for as long as 3 months without a detectable loss of iodide. Simple water solutions of NaI containing as little as 0.01 γ of I per ml. have remained stable for 10 months.

Table III contains the results of estimations of protein-bound iodine in blood plasma of three species, as well as in various tissues of the rat. In all instances, inorganic iodide was washed out. When thyroxine or diiodotyrosine was added in amounts equivalent to 1.0 to 5.0 γ per 100 ml. of plasma before the washing, recoveries of approximately 100 and 75 per cent (corrected) respectively were obtained. These findings indicate adsorption of thyroxine and, to a somewhat lesser extent, diiodotyrosine on the zinc proteinate precipitate. The binding must be strong to withstand four washings.

SUMMARY

A procedure has been reported for the determination of protein-bound iodine in various tissues, including blood plasma. The principal steps are precipitation, washing and then oxidation of the protein, distillation of the iodine, and colorimetric determination of the iodine by means of its catalytic effect on the reduction of ceric ions by arsenious acid.

The present method, combining and modifying previous methods, permits a satisfactory analysis to be performed on 2 ml. of plasma, one-hundredth of a normal rat thyroid gland, or 500 mg. of rat liver, kidney, heart, or skeletal muscle.

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