[CONTRIBUTION FROM THE DEPARTMENT OF MEDICAL RESEARCH, DETROIT COLLEGE OF MEDICINE AND SURGERY]

Technical Refinements for the Micro Colorimetric Method of Iodine in Blood

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Several investigators are studying the determination of small amounts of iodine by use of the blue color formed with iodine and starch. Leitch and Henderson[1] and Aitken[2] oxidize the iodide to iodate with bromine, liberate the iodine by addition of an excess of potassium iodide and titrate with thiosulfate using the blue color produced by starch as the indicator. Aitken modified the method in that he devised a titration vessel in the form of a short tapering tube in which solutions can be brought with ease to a volume of 0.2 cc. Titration is made with $N/1000$ thiosulfate. In a later report[3] he recommends the use of a micro buret in which the standard solution floats on a thread of mercury, the position of the latter being adjusted by means of a fine screw projecting into a mercury reserve. Reith[4] and the author[5] have studied the same reaction, omitting the titration, comparing the color produced against known standards. Reith prepares several standards and matches these with the unknown, while in our method one standard is prepared and the unknown compared in a micro colorimeter. The stability of the color and its proportionality to the amount of iodine present have been found by the author[6] to be sufficiently accurate for the estimation of small amounts of iodine ranging from 0.0005 to 0.005 mg.

During the course of our study certain difficulties have arisen. At times we have encountered a complete failure, either slight or no color developing or an intense black color resulting. These two difficulties have been overcome by the use of special purified reagents. The purpose of the paper is to give in detail the factors which we have found to cause failures and the necessary procedures to overcome these difficulties.

Experimental

The first difficulty encountered with the method was a partial or complete failure of color development. Reith (personal communication) wrote that iodine is lost if added to alcohol and the alcohol removed by ignition instead of evaporation. To 10 cc. of 94% alcohol he added potassium iodide sufficient in amount to bring the iodine content to 10γ. The alcohol was burned off and the iodine recovered was 0.7γ. Three

(1) Leitch and Henderson, *Biochem. J.*, **20**, 1003 (1926).
(2) Aitken, *ibid.*, **24**, 1456 (1930).
(3) Aitken, *ibid.*, **25**, 446 (1931).
(4) Reith, *Biochem. Z.*, **216**, 249 (1929).
(5) Turner, *J. Biol. Chem.*, **88**, 497 (1930).
(6) Turner, THIS JOURNAL, **52**, 2768 (1930).

other experiments like the above were carried out with the exception that he added 2, 5 and 20 mg. of potassium carbonate. The results found were, respectively, 7.0, 7.4 and 10.1γ iodine. Therefore, he assumes that the alkalinity must be very marked before no loss of iodine occurs from ignition.

In the method described by one of us[5] it was stated that the absolute alcohol must be freed from traces of iodine by redistillation over potassium hydroxide. This practice worked successfully with some grades of alcohol but at times when new supplies of alcohol were used the development of color failed to appear.

The following experiments were undertaken to determine whether the failure encountered was due to ignition of the alcohol.

To two centrifuge tubes containing 50 cc. of water each, 0.001 and 0.002 mg. of iodine in the form of potassium iodide were added. Potassium sulfate and barium chloride were added as described in our method.[6] This was centrifuged and extracted and the solution evaporated to dryness in an oven. The dry residue was extracted with the non-redistilled alcohol and the alcohol burned in a platinum crucible as described in the publication.

The above was duplicated in the same manner but instead of igniting the alcohol in the crucible, they were placed in an oven at 100° and allowed to evaporate. The residue in the platinum crucibles was then extracted with water as in the method given and a color developed. In both cases our results showed only a slight color not comparable with a 0.001-mg. standard.

Since both tests gave negative results it was thought that possibly some interfering substance such as aldehydes in the alcohol might have an inhibiting effect on color production. Alcohol was treated by the Winkler method[7] and by the method of Castille and Henri.[8] A modified form of the latter method has been adopted.

Procedure for Purification of Alcohol.—One-half gram of iodine crystals is added to 500 cc. of absolute alcohol. The mixture is allowed to stand for twenty-four hours. Distil off the alcohol, discarding the first 25 cc. of distillate, and retain 50 cc. of the residue. Shake the distillate with 100 g. of granulated zinc until the yellow color disappears. Distil off the alcohol again as above. To this distillate add as before 100 g. of granulated zinc and shake continuously for fifteen minutes. Distil as before. The alcoholic distillate thus obtained is free from iodine and interfering substances.

TABLE I

THE EFFECT OF PURIFICATION OF ALCOHOL ON COLOR PRODUCTION

Tube no.	Alcohol used for extraction	Results
1	Not purified	Negative
2	Purified by Winkler's method	Slight color but less than standard
3	Purified by modified method of Castille and Henri	Color equivalent to twice the standard or 0.002 mg. of iodine

If aldehydes are responsible for the interfering substance, they are not removed completely by the Winkler method. At present no claim is made

(7) Winkler, *Ber.*, **28**, 612 (1905).

(8) Castille and Henri, *Bull. soc. chim. biol.*, **6**, 299 (1924).

as to the identity of the interfering substance. Results have been satisfactory if the alcohol is treated by the iodine–zinc method as described. The experiment conducted to determine the possibility of loss of iodine by ignition of alcohol as described (on page 4) was repeated with alcohol purified by the iodine–zinc method. Complete recovery was obtained in both cases. Therefore, the failure of color production is not due to loss of iodine by ignition of the alcohol but to impurities which may be present in the alcohol and which are removed by the method presented.

The second difficulty in which the final reaction turned black due to oxidation of excess potassium iodide involves two factors. First, different samples of potassium carbonate may vary as to the amount of iodine present. The following method has been adopted for purification of all potassium carbonate used in the determination of small amounts of iodine.

Method for Purification of Potassium Carbonate.—To 100 g. of potassium carbonate in a liter beaker add 500 cc. of 95% alcohol. Stir with an electric stirrer for ten hours, decant the alcohol and dry the residue by pressure between filter paper. Dissolve the dried residue in 200 cc. of boiling water. Filter and evaporate the filtrate to one-third its original volume. Place in an ice-box to crystallize. When equilibrium is maintained filter and place the residue in an oven at 110° to dry. When dry powder and dissolve the contents in 100 cc. of hot water. Cool and pour the solution into a 500-cc. separatory funnel with an equal volume of absolute alcohol and shake continuously for thirty minutes. Transfer the aqueous layer to a 250-cc. beaker and evaporate over a flame to one-third its volume. Place in an ice-box until crystallization is complete, filter, and dry in an oven at 110°. Under these conditions no iodine could be detected in the purified potassium carbonate. 4 *N* potassium hydroxide may be used satisfactorily in place of the 4 *N* potassium carbonate solution. If chemically pure potassium hydroxide is used no purification is necessary.

Second, the iron content of the concentrated sulfuric acid used in preparation of the 2 *N* sulfuric acid solution must be taken into account. It is known that oxidized iron will set free iodine from potassium iodide. An investigation was made regarding the iron content of sulfuric acid and its effect on the starch–iodide reaction as used for the estimation of iodine in blood. One of the experiments is given as an example of the mode of investigation.

The following three samples of sulfuric acid diluted to the strength as used in the method (2 *N* H_2SO_4) were taken for examination.

Sample No. 1. Baker and Adamson containing 0.0002% iron

Sample No. 2. Baker and Adamson containing 0.00008% iron

Sample No. 3. Baker and Adamson reagent three months old. Iron content unknown. Tested negative when first prepared by the following method.

Method for Testing Purity of Sulfuric Acid.—Three drops of purified potassium iodide solution (1%) was added to each of three test-tubes (125 × 15 mm.) graduated to 1 cc. Distilled water (iodine-free) was added to the 1-cc. mark and 5 drops of the starch solution introduced. Two drops of sulfuric acid solution (No. 1 above) was added to tube No. 1. Likewise the same quantity of sulfuric acid solution (No. 2 above) was added to tube No. 2 and the sulfuric acid solution (No. 3 above) to tube No. 3. Another set of three tubes was prepared in a like manner with the exception that the 1% potassium iodide solution added had not been prepared with potassium iodide purified as described in our first publication.[5] These were numbered 1A, 2A and 3A, respectively.

The potassium iodide used in preparing the above solutions was Baker and Adamson (Lot 3A, Code Ba 4220). The results are shown in Table II.

TABLE II

THE EFFECT OF IMPURE REAGENTS ON COLOR PRODUCTION

Reagent no.	Color development: Time in minutes 15	30	After addition of excess sulfuric acid reagent thirty minutes after development of the color Excess, 10 drops
1	+	++	+++
1A	++	+++	++++
2	−	sl +	sl +
2A	+	+	+
3	+	+	++
3A	++	++	+++

+ Equivalent to blue color produced with 0.001 mg. of iodine standard prepared as directed in the method. ++ Equivalent to a 0.002 mg. standard. +++ Dense, not matched against standards. ++++ Black, not matched against standards. sl + slight, not sufficient color to match against 0.001 mg. standard diluted to 2 cc.

The results show that if the sulfuric acid solution is fresh and sufficiently iron-free, no color is developed within fifteen minutes after adding the acid to the purified potassium iodide solution containing starch. The slight color produced at the end of thirty minutes with acid No. 2 (low in iron content) is believed to be due to the effect of the atmosphere on the oxidation of potassium iodide solution. This slow oxidation was recognized as stated in our publication[6] but if standard and unknown are prepared at the same time, the change is proportional for at least an hour and the colorimetric reading remains the same. The above experiment shows the effect of foreign oxidizable substance, presumably oxidized iron, on potassium iodide in acid solution. Potassium iodide is in excess when the final reaction for production of the color is carried out, therefore, the purity of the reagents used in this step must be watched. If foreign oxidizable substances are eliminated, iodine is not liberated from potassium iodide in acid solution within thirty minutes.

Conclusions

This investigation includes necessary procedures to avoid certain factors which cause failures in the author's method[5] for the estimation of iodine in blood.

To obtain satisfactory results it is essential to treat the absolute alcohol by the modified iodine–zinc method of Castille and Henri for removal of aldehydes.

Potassium carbonate must be freed from iodine by the method described or chemically pure potassium hydroxide used in its place to produce the desired alkalinity.

The sulfuric acid used for preparing the 2 *N* solution should not contain

over 0.00008% iron, and at all times this reagent should be tested with the 1% potassium iodide solution for the presence of oxidizable foreign substance, assumed to be oxidized iron.

Under these conditions the above mentioned difficulties do not arise and the method of analysis can be carried through with the accuracy mentioned in the former publication.[5]

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Carbohydrate-Fatty Acid Linkings in Corn Alpha Amylose[1]

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The fatty acids associated with corn starch occur entirely in the α-amylose[2] or insoluble portion of the starch, while the soluble β-amylose is pure carbohydrate. Thus the presence of the fatty acids affords one means of differentiating between the two corn amyloses. In the interests of obtaining more data on chemical make-up of each of the corn amyloses, an examination of the linkage between the fatty acid groups and the carbohydrates in corn α-amylose is desirable. This investigation is concerned with that problem.

It has been shown that the fatty acid compounds in corn α-amylose are derived from palmitic, oleic and linolic acids,[3] which are chemically combined with carbohydrate for they are not extracted by solvents, but are liberated only after relatively long aqueous acid hydrolysis of the amylose.

In spite of the fact that these acids are in chemical combination with the carbohydrate, no corn α-amylose has been prepared which contains all that is in the original starch. Some is always lost in the process of separating the α-amylose from the β-amylose. This applies also to the derivatives of starch. Acetylated and methylated products of corn starch containing fatty acids have been prepared,[4] but in neither case do the products contain more than half of the original acids. This suggests that the fatty acids may not all be linked to the carbohydrate molecule in the same manner.

As starch is a polyhydroxylated compound, the fatty acids can be combined to the carbohydrate through an oxygen linking at any one or more of the otherwise free hydroxyl groups of the glucose residues which form the amylose molecule.

(1) An abstract of a dissertation presented by Ruth T. Sherman to the Faculty of Pure Science of Columbia University in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

(2) (a) Taylor and Nelson, THIS JOURNAL, **42**, 1726 (1920); (b) Taylor and Iddles, *Ind. Eng. Chem.*, **18**, 713 (1926).

(3) Lehrman, "The Fatty Acids in Corn Starch and Synthesis of Corn Beta Amylose Palmitate," Columbia Dissertation, 1925.

(4) Werntz, "Studies of the Corn Amyloses," Columbia Dissertation, 1926.