

found that the no pretreatment sections at 60-minute water wash are as clear as the 30-minute treated sections; however, the original problem of inconsistent staining is not corrected by the longer water wash. Excessively long water washes before silver nitrate treatment or after treatment but before exposure to light result in diminution or complete absence of the line of healing. The ether-acetone is the most important pretreatment. Further steps only tend to improve the contrast between calcified and uncalcified areas.

How soon after staining must the sections be scored? In Figure 4 are pictures of a series of bones, treated with ether-acetone solution followed by a water wash of 7, 15, or 30 minutes, taken within 2 hours of staining and the same bones photographed again approximately 30 hours after staining. These bones were exposed to ordinary room lighting for at least half of the 30 hours. It is obvious that rapid discoloration of the bone surfaces is not a problem. Again there is a definite improvement with increased water-wash time until at 30 minutes there is little, if any, change during the 28-hour time lapse shown.

It has been observed that if the prepared slide is adequately washed, then allowed to dry, the line appears unchanged upon rehydration, even after several months.

Other investigators have followed procedures in which the bone sections are placed in either 10% formalin or 95% ethyl alcohol for 3 hours to several days (2). These variations were studied, but were found to provide staining not as clear as the extraction procedure described here.

Figure 1 shows a comparison of results of the old technique and of the revised procedure just described. The two sets of bones shown here were taken from the same animals, those above from the right leg, and those below from the left leg. The greater clarity and more precise stain greatly reduce the required judgment and result in a more reproducible score bringing the variation in the method closer to that of the rat itself.

#### LITERATURE CITED

- (1) Dyer, F. J., *Quart. J. Pharm. Pharmacol.*, **4**, 503 (1931).
- (2) Scott, H. T., Baird, F. D., Bunker, J. W. M., Krauss, W. E., Nelson, E. M., and Russel, W. C., *Am. J. Pub. Health*, **29**, Suppl. 54 (1939).
- (3) Shipley, P. G., and Park, E. A., *J. Biol. Chem.*, **51**, 41 (1922).
- (4) U. S. Pharmacopeia, XIV Revision, p. 793.

RECEIVED for review June 6, 1952. Accepted August 5, 1952. Presented before the Division of Biological Chemistry at the 121st Meeting of the AMERICAN CHEMICAL SOCIETY, Buffalo, N. Y.

## A Simple Volumetric Assay for Sodium Borohydride

D. A. LYTTLE, E. H. JENSEN, AND W. A. STRUCK

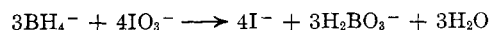
Research Laboratories, The Upjohn Co., Kalamazoo, Mich.

THE increasing importance of sodium borohydride as a reducing agent has made a rapid and convenient assay procedure desirable. A volumetric method has been developed in which sodium borohydride is oxidized with potassium iodate which must be present in excess. Unreduced potassium iodate is determined by the usual iodometric procedures. The method is reproducible, faster than methods based upon measurement of the volume of hydrogen evolved on acidification of sodium borohydride, and can be carried out with simple volumetric equipment.

One published method (1) for the determination of sodium borohydride involves a measurement of the volume of hydrogen evolved upon acidification of an aqueous solution. This method appears to be accurate, but has several disadvantages, inasmuch as it is somewhat slow, and cannot be used conveniently for kinetic studies or similar applications where the amount of sodium borohydride present at any instant in a solution of changing concentration must be determined. The gasometric determination also requires more specialized equipment than may be available to many workers.

A second method (2) has been described which consists of the titration of sodium borohydride with a solution of iodine in buffered solution at pH 7.0. No details are given in the publication, but the method seems to be limited to pH 7.0, a pH at which sodium borohydride is less stable than in alkaline media. Furthermore, the method suffers from the disadvantage of utilizing a relatively unstable reagent.

The present method is based upon the stoichiometry shown in the following equation:



and involves the addition of a sample of sodium borohydride, either solid or in alkaline solution, to an excess of standard potassium iodate. If solid sodium borohydride is used, alkali must be added to the iodate. Potassium iodide is then added, followed by acid, and the liberated iodine is titrated with standard thiosulfate.

While the equation accurately expresses the over-all stoichi-

ometry of the reaction, considerable difficulty was experienced in early experiments, inasmuch as results tended to be low and erratic. A systematic study was therefore made of the following variables:

1. Concentration and total amount of iodate required
2. Effect of time in the different phases of the procedure
3. Influence of pH in the sodium borohydride-iodate reaction
4. Stability of sodium borohydride in alkaline solution

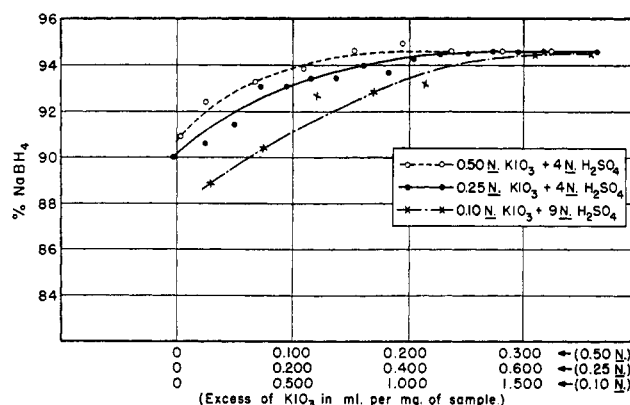


Figure 1. Titration of Commercial Sodium Borohydride

All experiments were carried out with commercial sodium borohydride of about 95% purity. Comparison of the present method with the "hydrogen evolution" method was made using aliquots of the same alkaline solution of sodium borohydride. Agreement between the two methods was very good. Values obtained by the iodate method were slightly higher than those obtained by the "hydrogen evolution" method.

As a result of the study, the following conclusions were reached:

1. A large excess of iodate is required for a quantitative determination. The absolute excess necessary is smaller when higher concentrations of iodate are used (Figure 1).

2. The borohydride reduction of iodate appears to be an instantaneous reaction.

3. The presence of alkali in amounts greater than that necessary to neutralize all boric acid formed has no effect on the reaction between iodate and borohydride.

4. Commercial sodium borohydride deteriorates rapidly in aqueous solution, but is fairly stable in basic solutions—the higher the pH the greater the stability (Figure 2).

#### REAGENTS

Sodium hydroxide, reagent grade, 0.5 *N*  
Sulfuric acid, reagent grade, 4 *N*  
Potassium iodate, standard, 0.25 *N*  
Sodium thiosulfate, standard, 0.10 *N*  
Potassium iodide, reagent grade  
Amylose starch indicator

#### PROCEDURE

A 20- to 25-mg. sample of sodium borohydride is dissolved in 25 ml. of 0.5 *N* sodium hydroxide. Standard potassium iodate (35.00 ml., 0.25 *N*) is added immediately, and the flask is swirled vigorously for 30 seconds. Two grams of potassium iodide are added, followed by 20 ml. of 4 *N* sulfuric acid. The flask is allowed to stand in darkness for 2 or 3 minutes before the liberated iodine is titrated with 0.10 *N* sodium thiosulfate (starch indicator). Sodium borohydride is hygroscopic, so suitable precautions must be taken in handling it. A dry box may be used, or a larger sample may be weighed quickly and dissolved in a proportionately larger amount of 0.5 *N* sodium hydroxide, from which an aliquot containing approximately 20 to 25 mg. of sodium borohydride is taken. The weight of sodium borohydride can be calculated by means of the following formula:

$$\text{Mg. of NaBH}_4 = (\text{ml. of KIO}_3 \times \text{normality} - \text{ml. of Na}_2\text{S}_2\text{O}_3 \times \text{normality}) \times 4.731$$

The equivalent weight of sodium borohydride is equal to one

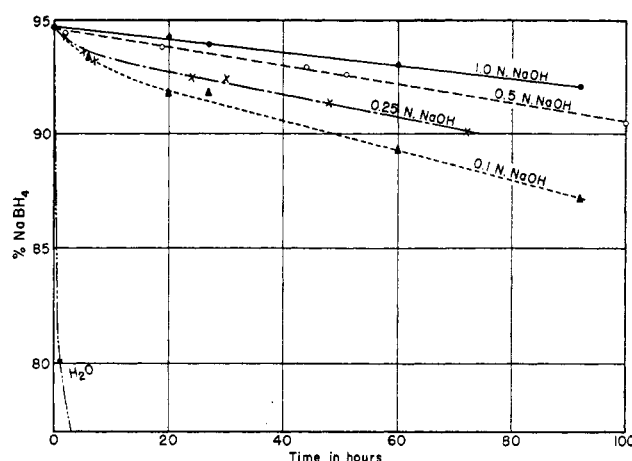


Figure 2. Deterioration of Commercial Sodium Borohydride in Different Solutions

eighth of the molecular weight ( $\text{BH}_4^- + 3\text{H}_2\text{O} \rightarrow \text{H}_2\text{BO}_3^- + 8\text{H}^+ + 8e$ ).

#### ACKNOWLEDGMENT

The authors are grateful to Howard S. Clark, Illinois Geological Survey, Urbana, Ill., for his interest and valuable suggestions.

#### LITERATURE CITED

- (1) Chaikin, S. W., and Brown, W. G., *J. Am. Chem. Soc.*, **71**, 122 (1949).
- (2) Mathews, M. B., *J. Biol. Chem.*, **176**, 229 (1948).

RECEIVED for review July 7, 1952. Accepted August 4, 1952.

## Use of Anthrone Reaction for Determination of Carbohydrates in the Presence of Serum Protein

M. R. SHETLAR

Research Laboratory, Veterans Administration Hospital, and The Oklahoma Medical Research Foundation, Oklahoma City, Okla.

WHEN compared to the tryptophan method of Shetlar, Foster, and Everett (5), the anthrone method for the determination of the polysaccharides in serum recently proposed by Graff, Greenspan, Lehman, and Holeczek (1) has an advantage of greater specificity for carbohydrates. However, the absorption curves resulting from the reaction of serum proteins with anthrone show an absorption maximum at 530  $m\mu$  as well as the maximum at 620 to 625  $m\mu$  which is characteristic of the hexose-anthrone reaction. Morris (3) noted that a red color was obtained with certain proteins, and Seifter, Dayton, Novic, and Muntwyler (4) reported that tryptophan reacted with anthrone to produce a solution with a maximum absorption at 515  $m\mu$ . None of these investigators apparently realized that some reaction between tryptophan and carbohydrate would also undoubtedly occur as reported in the work of Shetlar, Foster, and Everett (5). The maximum absorption for this reaction is at 500  $m\mu$ .

The following work was undertaken in order to clarify the question as to what the interfering reaction or reactions might be and to determine whether this reaction might interfere with the determination of polysaccharides in serum and in tissue.

#### REAGENTS

Anthrone reagent, 0.15% anthrone in 95% sulfuric acid. As recommended by Viles and Silverman (6), this reagent was

aged at least 4 hours and was discarded after it became more than 9 days old. The solution was kept in the refrigerator when not in use.

Sulfuric acid, 95% by volume. Nine hundred and fifty milliliters of concentrated sulfuric acid (Du Pont, c.p. reagent grade, specific gravity, 1.84 at 15° C.), were added to 50 ml. of distilled water.

#### EQUIPMENT

All measurements of optical density were made in a Model 14 Coleman spectrophotometer.

#### EXPERIMENTAL

**Method of Heating after Adding Anthrone Solution.** Graff *et al.* (1) employed a method by which the anthrone was added to the carbohydrate solution at room temperature. The heat evolved due to hydration of the sulfuric acid is sufficient to cause the reaction. However, Seifter *et al.* (4) reported that considerable discrepancies occurred among identical samples of glucose by this procedure, and devised a method consisting of adding the anthrone to cooled sugar solutions, followed by a heating period in a boiling water bath. The question remained as to which of these procedures would favor the development of the reaction characteristic of carbohydrate-anthrone and retard the development of the reaction characterized by the absorption at 530  $m\mu$ . Consequently, human serum was diluted with twice the volume