

# A PHOTOMETRIC ADAPTATION OF THE SOMOGYI METHOD FOR THE DETERMINATION OF GLUCOSE

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The reliability of the various Somogyi-Shaffer-Hartmann (1, 2) copper reagents for glucose determination in biological material has been established. Adaptation of these reagents to colorimetric use may be accomplished by omission of the iodide and iodate in their preparation, since these interfere with the molybdate color reagents. This omission produces no especial change in the character of the reagents. KI, however, inhibits the autoreduction of the copper and in its absence an unstable reagent results. Nevertheless, if the copper is added to the rest of the reagent on the day of its use, this difficulty is avoided.

When the Somogyi micro reagent (2) is used in this way with almost any of the various phosphomolybdate reagents, very satisfactory proportionality is found between color density and glucose taken over a wide range of values. However, all of the phosphomolybdate reagents tried left much to be desired in reproducibility from time to time and lacked the desired stability of color.

We therefore tried various color reagents, which led to the development of a new arsenomolybdate reagent. When this reagent was used with Somogyi's micro reagent, it gave satisfactory stability and reproducibility of color. By this means it has been possible to utilize the copper reagents in a photometric procedure for practically all the uses to which the titrimetric procedures are adapted. These include tissue sugar, glycogen, urine reduction equivalent, maltose, glucuronic acid, etc. However, diastase determinations have not been successful because of the effect of the undigested starch on the clarity of the final colored solution.

The reactions involved in the molybdenum blue reaction are uncertain and beyond the scope of this report. Woods and Mellon (3) discuss and give references to the various interpretations of the reaction.

*Reagents*—Analytical reagent grade or the equivalent.

1. Copper Reagent A. Dissolve 25 gm. of  $\text{Na}_2\text{CO}_3$  (anhydrous), 25 gm. of Rochelle salt, 20 gm. of  $\text{NaHCO}_3$ , and 200 gm. of  $\text{Na}_2\text{SO}_4$  (anhydrous) in about 800 ml. of water and dilute to 1 liter. Filter if necessary.<sup>1</sup> This

<sup>1</sup> The effective linearity of results in the very low range of reduction has been improved over that secured in the ordinary Somogyi micro blood sugar procedure. This

solution should be stored where the temperature will not fall below 20°. A sediment may form after a few days. This may be filtered off without detriment to the reagent.

Copper Reagent B. 15 per cent  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  containing one or two drops of concentrated sulfuric acid per 100 ml.

2. Arsenomolybdate color reagent. Dissolve 25 gm. of ammonium molybdate in 450 ml. of distilled water, add 21 ml. of concentrated  $\text{H}_2\text{SO}_4$ , mix, add 3 gm. of  $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$  dissolved in 25 ml. of  $\text{H}_2\text{O}$ , mix, and place in an incubator at 37° for 24 to 48 hours. Fig. 1 shows the progress of formation of the chromogenic compound during incubation at 37°. If a reagent is needed quickly, an alternative procedure is to heat to 55° for about 25 minutes. However, stirring must be adequate to prevent local overheating; otherwise decomposition of the chromogen may occur. This is accompanied by the precipitation of a bright yellow compound. The first procedure has been uniformly successful and is the recommended one; the second is inconvenient, and with certain preparations of sodium arsenate yields reagents which, though useful, are inferior in potential color development to those prepared by the first procedure. This reagent should be stored in a glass-stoppered brown bottle.<sup>2</sup>

3. 5 per cent  $\text{ZnSO}_4 \cdot 6\text{H}_2\text{O}$ .

4. Approximately 0.3 N  $\text{Ba}(\text{OH})_2$ . The zinc and barium solutions should be adjusted so that 5 ml. of zinc require between 4.7 and 4.8 ml. of barium to produce a definite pink to phenolphthalein. The zinc should be diluted to 20 or 25 ml. with  $\text{H}_2\text{O}$  and the  $\text{Ba}(\text{OH})_2$  added dropwise with constant mixing during the titration. Store in a bottle protected by soda lime from the carbon dioxide of the air. It is convenient to have an arrangement for direct delivery of the  $\text{Ba}(\text{OH})_2$  into a 5 ml. burette graduated to 0.01 or 0.02 ml.

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is done by using 1 volume of 1:20 blood filtrate per volume of Reagent A instead of 1 volume of 1:40. In order to extend the useful range of the reagent to compensate partially for this change, 0.6 per cent copper sulfate is used instead of 0.4 per cent. This procedure has an additional advantage in that a smaller amount of reagent will suffice, and, consequently, the blank (which arises chiefly from the reagents other than copper sulfate) will be reduced correspondingly.

<sup>2</sup> Reagents prepared by either of the procedures described probably will have considerably more of the active chromogen per ml. than will be needed for 0.3 mg. of glucose (our arbitrary upper limit of estimation). To economize on the reagent, one may determine in a titration against the maximum amount of glucose which it is desired to estimate how much reagent is required to give maximum color. On the basis of this, the reagent may be diluted with 1.5 N  $\text{H}_2\text{SO}_4$  to allow about a 20 per cent excess concentration of the chromogenic compound over the maximum needed.

*Procedure*

The blood filtrates are prepared as follows:<sup>3</sup> Add 1 volume of blood to 15 volumes of water, mix, add 2 volumes of  $\text{Ba}(\text{OH})_2$ , mix, and after the mixture has turned brown add 2 volumes of  $\text{ZnSO}_4$  and mix. After a few minutes the mixture may be filtered; somewhat more filtrate may be secured by a preliminary centrifugation. For finger-tip blood, one may wash 0.1 ml. of blood from a pipette calibrated "to contain" into 1.5 ml. of water contained in a small vial or test-tube. After 0.2 ml. each of  $\text{Ba}(\text{OH})_2$  and  $\text{ZnSO}_4$  is added as described above, the mixture is centrifuged. The filtrate is then drawn into a 1 ml. pipette tipped with washed cotton.

1 ml. of filtrate is pipetted into a narrow test-tube graduated at 25 ml. The ordinary Folin-Wu blood sugar tubes are convenient but not essential.<sup>4</sup> 1 ml. of a mixture (prepared the day of use) of 25 parts of Reagent A to 1 part of Reagent B is added. Since this latter volume is not critical, a burette or measuring pipette may be used. 1 ml. portions of appropriate standards and 1 ml. of distilled water, to serve as a blank, are set up in the same way. The solutions are mixed and heated for 20 minutes in a boiling water bath. At the end of 20 minutes the tubes are cooled in a pan of cold water. 1 ml. of the arsenomolybdate reagent is then added to each; a measuring pipette is convenient and adequate for this measurement. The color develops very rapidly and will be completed by the time thorough mixing and evolution of  $\text{CO}_2$  are completed. The mixture is then diluted to the mark, mixed, and read in a photoelectric colorimeter at 500 or 520  $\mu$ . The photometer is adjusted so as to read 100 per cent transmission through the blank. The color is very stable and may therefore be read at convenience. The stability of the color is absolute and not relative; the density of the blanks as well as of the more deeply colored solutions remains unchanged with time.

It has been our practice to run duplicate determinations on all blood

<sup>3</sup> The deproteinization procedure given is one suggested to us by Dr. Somogyi several years ago. It has been well known (Benedict (4), Somogyi (2)) that the  $\text{ZnSO}_4$ - $\text{NaOH}$  deproteinization procedure leaves a small amount of Zn remaining in the filtrate and that this trace apparently accelerates the reoxidation of cuprous oxide. The extent of this is small and relatively unimportant in a macro blood sugar estimation, but decidedly significant in a microdetermination. We had encountered this difficulty and devised a procedure similar to but more cumbersome than the one Somogyi was using to overcome the same difficulty. We have found his method a very useful technique for various purposes, since it has the advantage of yielding a filtrate practically free of the deproteinizing reagents.

<sup>4</sup> The high  $\text{Na}_2\text{SO}_4$  concentration of the reagent gives adequate protection against reoxidation for most purposes; so that neither the constricted Folin-Wu tube nor covered tubes are essential. However, if high accuracy with quantities of glucose below 5  $\gamma$  is needed, these precautions should be taken.

sugars and to run each urine determination at two levels of dilution. In our records for a 6 months period, covering over 2000 determinations, a deviation of 3 per cent between duplicates is rare. In most cases the spread was less than 1.5 per cent of the amount determined. We routinely carry through one pair of standards and blanks with each set and once each day three pairs of standards are included; for this we use 0.05, 0.15, and 0.3 mg. of glucose per ml., corresponding to 1 ml. of a 1:20 dilution of blood containing 100, 300, and 600 mg. per 100 ml. respectively. The variation

TABLE I

*Optical Density (Log  $I_0/I$ ) at 500  $M\mu$  at Various Intervals after Color Development*

Glucose taken	Time			
	0	25 min.	100 min.	18 hrs.
mg.				
0.050	0.169	0.169	0.169	
0.300	0.979	0.979	0.987	
0.025	0.089			0.087
0.050	0.175			0.173
0.150	0.520			0.522

TABLE II

*Relationship between Density (Log  $I_0/I$ ) and Glucose Taken (Average of Duplicate Determinations)*

Glucose taken, g	Optical density, $d$	$\frac{g}{d}$
mg.		
0.005	0.0175	0.286
0.025	0.0865	0.289
0.050	0.1760	0.284
0.150	0.5140	0.292
0.300	1.015	0.295

in the density values for these standards from one batch of reagents to another is not essentially greater than that encountered from day to day with the same reagents. The maximum spread of the density values has been about 6 per cent. Usually the density coefficient of the standards can be predicted with a 1 or 2 per cent precision.

Representative data indicating the stability of the color developed are given in Table I. The optical density was occasionally checked as late as 3 days after development, when only small changes from the initial values were found.

Table II shows a typical calibration indicating the essential proportionality between optical density and glucose taken.

The color densities were read in a photoelectric spectrometer (based on a Gaertner model 227 monochrometer) in Evelyn colorimeter tubes at a wavelength of  $500\text{ m}\mu$ , with slit widths of  $0.1\text{ mm}$ . However, other laboratories have used Filter 520 with the Evelyn colorimeter successfully for this purpose. At these wave-lengths the light absorption is far from the maximum, which lies at  $660\text{ m}\mu$  (Fig. 1). The wave-length  $500\text{ m}\mu$  was chosen because it represented a satisfactory compromise between the sensitivity desired

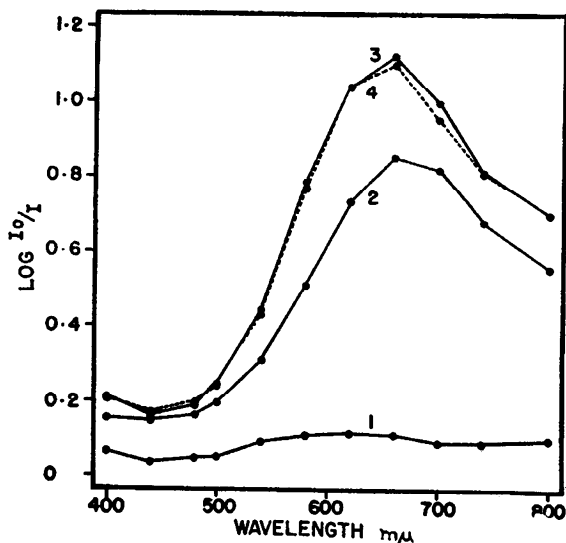


FIG. 1. Development of the arsenomolybdate chromogenic compound at  $37^\circ$ . 1 ml. of a 1:10 dilution in  $0.5\text{ N H}_2\text{SO}_4$  of the reagent described in the text was added to excess cuprous oxide. The monochromator slits were set at  $0.05\text{ mm}$ . for wave-lengths of  $600\text{ m}\mu$  and above, and at  $0.10\text{ mm}$ . for wave-lengths below  $600\text{ m}\mu$ . Curve 1, 0 time; Curve 2, 12 hours; Curve 3, 24 hours; Curve 4, 48 hours.

and the advantages gained by reducing to a minimum the effect of variation in such factors as blank due to reagents, reoxidation of cuprous oxide, etc. The sensitivity can be increased over 4 times merely by reading the light transmission at  $660\text{ m}\mu$ .

Use of the color reagent for measurement of the reduced copper formed in the Somogyi reagent is regarded merely as an alternative to iodometric titration. Within the limits of error of the two procedures, equivalent results have been secured in all applications but measurement of diastatic

activity. The characteristics of the reagents are available in the original articles (1, 2).

Thirty-nine blood specimens run in parallel by the above procedure and by the Somogyi titrimetric method showed an average of 155.7 mg. per cent for the titration against 155.9 mg. per cent for the photometric technique. The standard error of the difference of the means was 0.007. The blood sugar ranged from 45 to 585 mg. per 100 ml. and included specimens from normal and diabetic owls, dogs, monkeys, and humans. The same blood filtrates were used for both determinations.

Although we have not used the procedure with a Duboscq colorimeter, there appears to be no reason why it should not offer some advantages over the usual methods. A yellow filter or light source such as that used by Folin (5) would probably facilitate the comparison of standard with unknown.

#### SUMMARY

A photometric method has been described for the estimation of glucose (or reduction equivalent) with copper reagents and an arsenomolybdate reagent. The optical density of the color developed is proportional to the glucose taken and is stable over long periods of time.

#### BIBLIOGRAPHY

1. Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, **100**, 695 (1933).
2. Somogyi, M., *J. Biol. Chem.*, **117**, 771 (1937).
3. Woods, J. T., and Mellon, M. G., *Ind. and Eng. Chem., Anal. Ed.*, **13**, 760 (1941).
4. Benedict, S. R., *J. Biol. Chem.*, **92**, 141 (1931).
5. Folin, O., and Malmros, H., *J. Biol. Chem.*, **83**, 121 (1929).