

THE COLORIMETRIC DETERMINATION OF GLUCOSE

BY

E. J. KING AND R. J. GARNER

British Postgraduate Medical School, London

(RECEIVED FOR PUBLICATION, APRIL 25, 1947)

Several new descriptions of colorimetric methods for the determination of blood sugar have been described in recent years (for example, Polis and Sortwell, 1946; Somogyi, 1945; Nelson, 1944; Haslewood and Strookman, 1939). These procedures are all based on the use of alkaline copper reagents and on the production of molybdenum blue by the action of cuprous copper on complex molybdc acid solutions. With a view to finding the method or combination of methods which gives the most reliable results, particularly in the very low range of blood sugar, the following combinations of reagents were compared: (a) Harding's Schaffer-Hartmann copper reagent with the Folin-Wu phosphomolybdc acid; (b) Harding's copper reagent with the Nelson arsenomolybdc acid; (c) Somogyi's copper reagent with the Folin-Wu phosphomolybdc acid; and (d) Somogyi's copper reagent with the Nelson arsenomolybdc acid.

Much difficulty has occurred during the war years and subsequently in this country with colorimetric methods for blood sugar. Biochemists have repeatedly complained of the rapid fading of the blue colours. This fault has been more manifest because of the recent introduction of photo-electric instruments in which the rapid falling-off of the intensity of the blue colour has been noted by numerous workers. With Duboscq colorimeters the fading would not have been so readily noticed, since the rate of fading in standard and test is very similar. It has frequently been impossible during the last several years to obtain chemicals of Analytic Reagent quality, and this abnormal behaviour has been traced to the use of inferior chemical reagents. The ordinary or technical grade of sodium carbonate, for instance, contains considerable sodium bicarbonate as well as the sodium carbonate. The amount of this inferior reagent specified for the preparation of an alkaline copper solution contains insufficient sodium carbonate to produce the desired properties in the final solution, and an abnormal behaviour consequently results.

Experimental

REAGENTS

Harding's copper reagent.—The modified Harding-Schaffer-Hartmann reagent is described by Haslewood and Strookman (1939). This reagent is identical with that described by Harding and Downs (1933), except for the omission of the potassium iodide. Its preparation is also described by King (1947).

Somogyi's copper reagent.—Prepared according to Somogyi (1945).

Folin and Wu's phosphomolybdc acid.—Prepared according to Folin and Wu (1920).

Nelson's arsenomolybdc acid.—Prepared according to Nelson (1944), King (1947).

Glucose standards.—A stock solution is made by dissolving 0.1 g. anhydrous glucose in saturated benzoic acid solution and making up to 100 ml.; 2, 3, and 5 ml. of the stock solution are diluted to 100 ml. with saturated benzoic acid to give working standards of 0.02, 0.03, and 0.05 mg. per ml. respectively (equivalent to 80, 120, and 200 mg. glucose per 100 ml. of blood, according to the following method).

Isotonic sodium sulphate-copper sulphate solution.—3 g. Na_2SO_4 , 10 H_2O , and 0.6 g. CuSO_4 , 5 H_2O are dissolved in water and made up to 100 ml.

Sodium tungstate.—10 g. per 100 ml.

PROCEDURE

Preparation of blood filtrate.—0.05 ml. of blood is added to 1.85 ml. of the isotonic sodium sulphate-copper sulphate solution in a conical centrifuge tube, and 0.1 ml. of sodium tungstate is added. The mixture is shaken, and the precipitated proteins and copper tungstate are spun down in the centrifuge, or filtered through a small paper.

Estimation of glucose.—1 ml. of the above filtrate and 1 ml. each of the glucose standard solutions are measured into 3/4-in. diameter test tubes; 1 ml. of copper reagent is added and mixed. The test tubes are stoppered with cotton-wool and heated in a boiling water bath for exactly 10 minutes. They are cooled in running water, and 3 ml. of Folin-Wu phosphomolybdc acid, or 1 ml. of Nelson's arsenomolybdc acid, with water to 5 ml. (for Duboscq; 10 ml. for photo-electric) are added. The colours are allowed to develop for 10 minutes and are then compared.

Results

REAGENTS PREPARED FROM "ORDINARY GRADE" CHEMICALS

Comparison of colorimetric methods.—Several dilutions (in addition to the above) were made of the stock standard glucose solution. One ml. samples were treated according to the above procedure and the colours measured in a photo-electric absorptiometer with the Ilford spectrum red filter. The zero adjustment was made with water in the cuvette. The results (Fig. 1) showed that the com-

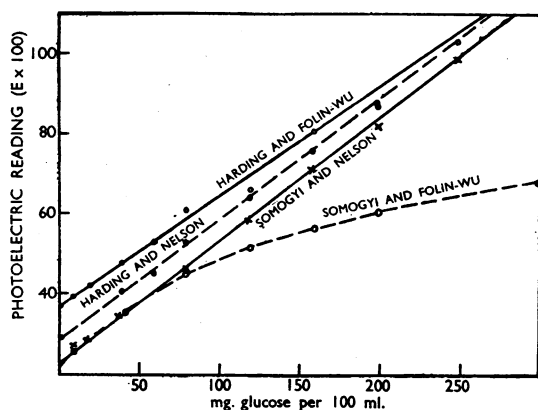


FIG. 1.—Photo-electric readings ($E \times 100$) of glucose solutions estimated by different methods. Chemicals of ordinary quality (not analytical reagent grade) were used in preparing the reagents. Note the high "blank" colours.

binations of Harding with Folin-Wu, Harding with Nelson, and Somogyi with Nelson, furnish procedures capable of giving good conformability with Beer's Law; but that the Harding-Folin-Wu mixture produced a considerably larger blank than the others. The Somogyi-Folin-Wu combination yielded much less satisfactory figures whose proportionality fell off rapidly with increasing concentrations of glucose.

Stability of colours produced.—Standard solutions containing amounts of glucose equivalent to 60 and 120 mg. glucose per 100 ml. and a water blank were prepared with the Harding copper and Folin-Wu phosphomolybdic acid. Rapid fading was found to occur in the final blue solutions with loss of proportionality (Fig. 2). It will be seen that a considerable amount of reduction occurred in the blank. This was, in fact, visible to the naked eye, there being a greenish precipitate of cuprous oxide when the tube was removed from the water bath.

The reagents used in the above experiments had been prepared when no Analytical Reagent chemicals were available. It had previously been pointed out from this laboratory (King, Haslewood, and Grant, 1936) that impure sodium carbonate cannot be used for the titrimetric method for glucose, and it is apparent now that it is equally dangerous to use it for the colorimetric procedures. It was observed that when the Folin-Wu phosphomolybdic acid was prepared from wartime molybdic acid, a considerable amount of ammonia was evolved when the sodium hydroxide was added in the preparation of the reagent.

REAGENTS PREPARED FROM ANALYTICAL REAGENT CHEMICALS

Fresh copper reagents were now prepared in which Analytical Reagent chemicals were used throughout. The sodium carbonate was Kahlbaum pro. anal. (Griffin and Tatlock); the other chemicals were from British Drug Houses.

Fresh phosphomolybdic acid was prepared using an equivalent amount of sodium molybdate (instead of molybdic acid and sodium hydroxide). 44.3 g. $\text{Na}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$ and 5 g. $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ were dissolved in 250 ml. distilled water containing 2.8 g. NaOH, and the solution boiled for 30 minutes. Water was added to approximately 350 ml., followed by 125 ml. H_3PO_4 (89 per cent w/v; sp. gr. 1.75) and the volume made up to 500 ml.

Blank colour.—The use of these reagents resulted in the elimination of the high blank

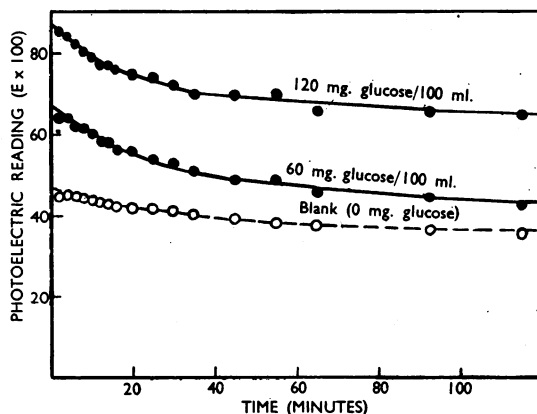


FIG. 2.—Photo-electric readings ($E \times 100$) of glucose solutions estimated by the modified Harding copper reagent and the Folin and Wu phosphomolybdic acid reagent. Ordinary quality chemicals—not Analytical Reagent grade. Note the high "blank" readings and the rapid fading of the colours.

readings with the Harding with Folin-Wu combination (Haslewood and Strookman method). Blanks prepared from fresh copper reagents heated with water (instead of glucose solution) gave a deflection of approximately a division on the absorptiometer when the zero adjustment was made with distilled water.

Stability of colour.—Water blanks and standard solutions containing an amount of glucose equivalent to 80 mg. per 100 ml. were prepared, using fresh copper reagents, and the colours developed with either phosphomolybdic or arsenomolybdic acid. The colours obtained were compared in a Hilger Spekker absorptiometer, using an Ilford spectrum red 608 filter. The zero adjustment was made with the blank solutions. Results are shown in Table I. Except in the case of the Somogyi with Folin-Wu combination, the colours remained substantially stable over a three-hour period.

TABLE I
STABILITY OF COLOUR IN AN 80 MG. GLUCOSE
STANDARD DURING THREE HOURS
(Figures represent photo-electric readings, $E \times 100$)

Time (mins.)	Harding and Folin-Wu	Harding and Nelson	Somogyi and Folin-Wu	Somogyi and Nelson
2	22.5	24	23	24.5
10	22.5	25	22.5	25.2
20	22.5	25	21.5	26
30	22.5	25	20.5	26
60	22.2	25	19.5	26
180	22	25	17.5	25

The development of colour in a similar series, in which the colour development was followed for eighteen hours and the blank colours were read with a water adjustment of the instrument zero, is illustrated in Table II. In every case, and particularly in the blanks, the colour becomes more intense with the lapse of time; but the difference between the standard and the blank readings becomes less except in the case of the Harding with Nelson combination, where it becomes greater. Thus, although there is an actual intensification of colour in every case, there is, except with the Harding-Nelson, a "relative fading" (that is, a lessening of the difference between standard and blank readings), which is most marked in the Somogyi with Nelson combination.

Proportionality.—The relation between colour development and concentration of glucose with

the fresh copper reagents is shown in Fig. 3. Beer's Law is closely adhered to, even in the low range of sugar values, particularly with the Harding-Nelson combination. The Harding-Folin-Wu and the Somogyi-Nelson combinations also give satisfactory results, but the Harding-Nelson combination is preferred because of the greater stability of its blank colours.

Discussion

The above results emphasize the necessity of preparing sugar reagents from the best chemicals obtainable. Only Analytical Reagent chemicals should be employed. The use of reagents prepared from impure chemicals can only lead to the production of unreliable results.

The purity of sodium carbonate seems to be the most important factor in the production of satisfactory copper reagents. If Analytical Reagent sodium carbonate cannot be obtained, the ordinary sodium carbonate should be heated in a crucible to red heat and maintained at that temperature for about thirty minutes. The cooled product is powdered in a mortar and stored in a bottle with a tightly fitting stopper. This treatment ensures that any contaminating sodium bicarbonate is decomposed into sodium carbonate. The procedure,

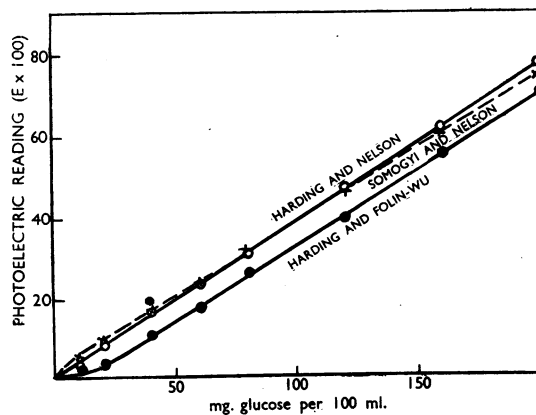


FIG. 3.—Calibration curves for glucose methods with reagents prepared from Analytical Reagent grade chemicals. Photo-electric readings ($E \times 100$) with the Hilger Spekker absorptiometer, 1 cm. cells and Ilford spectrum red filters.

however, is only recommended when Analytical Reagent sodium carbonate is not available.

Some samples of Nelson arsenomolybdic acid, prepared strictly according to Nelson's instruc-

TABLE II
STABILITY OF COLOUR OF A 200 MG. GLUCOSE STANDARD DURING EIGHTEEN HOURS
(Figures represent photo-electric readings, E × 100)

Time	Harding and Folin-Wu			Harding and Nelson			Somogyi and Folin-Wu			Somogyi and Nelson		
	200 mg. std.	Blank	Diff.	200 mg. std.	Blank	Diff.	200 mg. std.	Blank	Diff.	200 mg. std.	Blank	Diff.
(min.) 5	63	3.5	59.5	77.5	14	63.5	76	18.5	58.5	80	19.5	60.5
(hr.) 1	64.5	5	59.5	78.5	14.5	64	77.5	22.5	55	83	23	60
18	72	21.5	50.5	127	18	109	78	40	38	107	85	22

tions, give a persistent green colour with either the Harding or Somogyi copper reagents. Other batches give a green colour which with Harding copper reagent develops into a blue colour within 5 minutes, while with the Somogyi reagent the colour retains a definite greenish tint for about one hour. It has also been found that the readings of blanks prepared from the Harding or Somogyi reagents increase slightly with the age of the reagent. Neither of these points affects the accuracy of the results when the readings of the colours are made with a red light filter, and when, with a photo-electric instrument, the zero setting is made with a blank solution.

Summary

1. The Harding copper reagent (without the iodate) proposed by Haslewood and Strookman gave better colour development both with Folin and Wu phosphomolybdic acid and with Nelson arsenomolybdic acid than did the corresponding Somogyi reagent.

2. The colour development obtained with Harding reagent is of the same order as that obtained with Somogyi reagent.

3. The Harding-Folin-Wu and Harding-Nelson combinations are found to give the more stable colours, the Somogyi-Nelson and the Somogyi-Folin-Wu combinations being subject to varying degrees of fading.

4. With low sugar concentrations the Harding-Nelson combination is found to give more reliable results than the Harding-Folin-Wu.

5. Only chemicals of Analytical Reagent (A.R.) quality should be used for the preparation of blood sugar reagents.

REFERENCES

Folin, O., and Wu, H. (1920). *J. biol. Chem.*, **41**, 367.
 Harding, V. J., and Downs, C. E. (1933). *J. biol. Chem.*, **101**, 487.
 Haslewood, G. A. D., and Strookman, T. A. (1939). *Biochem. J.*, **33**, 920.
 King, E. J. (1947). *Micro-Analysis in Medical Biochemistry*. London : Churchill.
 King, E. J., Haslewood, G. A. D., and Grant, G. A. (1936). *Nature*, **137**, 872.
 Nelson, N. (1944). *J. biol. Chem.*, **153**, 375.
 Polis, B. D., and Sortwell, M. (1946). *Arch. Biochem.*, **11**, 229.
 Somogyi, M. (1945). *J. biol. Chem.*, **160**, 61.