A METHOD FOR THE DETERMINATION OF MONOSACCHARIDES IN THE PRESENCE OF DISACCHARIDES AND ITS APPLICATION TO BLOOD ANALYSIS*

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While attempting to determine the presence of lactase in mammary tissue, it became evident that no satisfactory method for the determination of the hydrolytic products of lactose in the presence of the substrate was available. We, therefore, sought to modify the Barfoed test in order to make it practical for quantitative, as well as qualitative, procedures.

Since the publication of Barfoed's test many other attempts have been made to improve it. Barfoed's reagent consists of copper acetate and acetic acid. The modifications vary as to the proportions of these two constituents used and as to the time of boiling (1). Too high acidity and prolonged boiling both result in disaccharides giving positive tests (2, 3). The difficulties which arose were due chiefly to the volatilization of the acetic acid. Sieben (4), in order to avoid the loss of the acetic acid, used sealed containers and heated the fluid at 40°. Legrand (5), employing Barfoed's reagent in volumetric work, collected the Cu_2O which formed on oxidation of the sugars, on filter paper, treated it with $Fe_2(SO_4)_3$, and then titrated with $KMnO_4$. This and the interesting study of Hinkle and Sherman (6) gave promise of the usefulness of this reagent for the colorimetric determination of monosaccharides in the presence of disaccharides.

The principle of the new method is the same as of those devised by Folin and Wu and by Benedict, for the determination of reducing sugars, the only difference being that the solution containing the

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sugars is heated with a non-volatile acid copper solution instead of an alkaline one. The cuprous oxide which forms on reduction is treated as in the methods mentioned, with an acid molybdate solution and the blue color obtained compared with a standard.

We found that the ordinary Barfoed's reagent (copper acetate and acetic acid) when boiled with glucose solutions gave amounts of cuprous oxide not proportional to the quantity of sugar used. This was found to be due to the volatilization of the acetic acid and the consequent formation of basic salts. We replaced the acetic acid with lactic acid, and found that the cuprous oxide formed was proportional to the amount of monosaccharide used. The reagent as modified in this manner did not give a positive test with fairly large amounts of disaccharides even after much longer boiling than was usual in earlier modifications.

This new colorimetric method is also useful for the study of saccharases and glucosides. Of a large number of glucosides, which we tested, nearly all gave reductions with alkaline copper solutions but none with the lactic acid reagent. The usefulness of this method would seem to be very wide. Phlorhizin was long considered to be an α-glucoside and only recently (7) was it found to be hydrolyzed by emulsin. This was due to the very slow velocity at which its splitting takes place. Phlorhizin seems to be one of the most slowly hydrolyzed β-glucosides. By our method the effect of emulsin on phlorhizin may be detected within a few hours. Polarimetric methods, which have been used to determine glucoside hydrolysis in most investigations, present certain difficulties. The method about to be described is simple, rapid, and requires very little material. It may be used qualitatively as well as quantitatively.

Solutions

New Acid Copper Monose Reagent—Dissolve 24 gm. of copper acetate (Merck, normal, C.P.) in 450 cc. of boiling water. If a precipitate forms do not filter. Add immediately 25 cc. of 8.5 per cent lactic acid (Mallinckrodt, United States Pharmacopœia X, 85 per cent) to the hot solution. Shake; nearly all the precipitate will dissolve. Cool, dilute to 500 cc., and after sedimentation filter off the impurities.

Standard Monose Solutions—Two standard monose solutions

are needed, one containing 0.15 mg. of monose per cc. and one containing 0.3 mg. of monose per cc. Toluene serves as a good antiseptic and does not interfere with the test.

Color Reagent—This reagent is the same as described recently by Benedict (8). It was found to be extremely useful for this method. For convenience we give the preparation of the reagent. Place 150 gm. of pure molybdic acid (we used Eimer and Amend's "free of ammonia") in an Erlenmeyer flask and add 75 gm. of pure anhydrous sodium carbonate. Add water in small portions, with shaking (about 500 cc.). Heat to boiling or until all of the molybdic acid has been dissolved. Filter off the insoluble matter. At this point we noticed only a small trace of insoluble substances. We therefore omitted the washing of the precipitate with hot water as suggested by Benedict. Add 300 cc. of 85 per cent phosphoric acid to the filtrate, cool, and dilute to 1 liter.

When fresh, and even after 4 months, the new copper monose reagent gives no color after 8 minutes boiling with 2 mg. of lactose and the addition of the color reagent and a very slight color with 3 mg. It gives a very good color with 0.1 mg. of glucose, the intensity of which is the same whether lactose is present or not (see Table I). The smallest amount of the various monoses which can be accurately determined is 0.1 mg. With pure bioses the minimum amount which gives reduction should be determined whenever the monose reagent is freshly prepared.

Tubes—Since there is no reoxidation in this method, any tubes marked at 25 cc. may be used. Benedict's sugar tubes serve the purpose.

Determination—Place in a tube 2 cc. of the *neutral* monose-biose solution (or the solution to be tested)[1] having a *total* reducing value[2] of not more than the equivalent of 2.5 mg. of glucose and not less than the equivalent of 0.1 mg. of glucose, in the case of glucose-lactose mixtures; or not more than the equivalent of 1 mg. of glucose in glucose-maltose mixtures. With sucrose, even 5 mg. of this sugar do not interfere. Transfer to two separate

[1] The solution should contain a minimum of inorganic salts; *e.g.*, more than 3 mg. of NaCl per 2 cc. of the solution to be tested interfere (see also Welker (9)). The pH should be as close to 7.0 as possible.

[2] The total reducing value may be determined by any method, such as the Folin-Wu or Benedict (8) method.

tubes 2 cc. of each of the two standards. Add 2 cc. of the monose reagent to each of the three tubes. Heat in boiling water for 8 minutes (time is an important factor). Cool for 2 minutes. Add 2 cc. of the color reagent. Mix the contents and after 2 minutes further standing add water to the 25 cc. mark in the case of the standards and dilute the unknown to a suitable mark. Mix thoroughly and compare with the standard colorimetrically.

Calculation— $\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 0.3$ or 0.6 (*i.e.*, mg. monose in standard) $\times \frac{\text{volume of unknown}}{\text{volume of standard}}$ equals mg. monose in unknown.

TABLE I

Mixtures of Glucose and Lactose†*

The total volume of all samples is 2 cc.

Experiment No.	Glucose	Lactose	Glucose found by new method	Experiment No.	Glucose	Lactose	Glucose found by new method
	mg.	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*
1	0.10	0.90	0.10	8	0.80	0.20	0.81
2	0.20	0.80	0.20	9	0.90	0.10	0.92
3	0.30	0.70	0.30	10	1.00	0.00	1.02
4	0.40	0.60	0.39	11	0.00	2.00	0.00
5	0.50	0.50	0.51	12	0.20	2.50	0.21
6	0.60	0.40	0.60	13	0.00	3.00	Very slight
7	0.70	0.30	0.72				trace

* Merck, C.P.; other pure preparations gave similar results.

† Mallinckrodt, C.P.; other pure preparations gave similar results.

In Table I is shown the result of glucose determination in glucose-lactose mixtures. It will be seen that 2.5 mg. of lactose are the maximum amount which may still be present in the mixture without causing interference. In Table II, Experiment 6 shows that 2 mg. of maltose interfere and in Table III it is shown that as much as 50 times as much sucrose as fructose allows the exact determination of the monose present. In all three sets of experiments the determination of a monose in the presence of a biose was accomplished with reasonable accuracy, great ease, and rapidity.

Application to Determination of Monose in Blood

Since this method yields excellent results when employed for the determination of monoses in aqueous solutions, it seemed desirable to attempt to apply it to blood sugar determination.

TABLE II

*Mixtures of Glucose and Maltose**

The total volume of all samples is 2 cc.

Experiment No.	Glucose	Maltose	Glucose found by new method
	mg.	*mg.*	*mg.*
1	0.10	0.90	0.11
2	0.20	0.80	0.20
3	0.40	0.60	0.41
4	0.80	0.20	0.80
5	1.00	0.00	0.98
6	0.00	2.00	Slight trace

* Kahlbaum; other pure preparations gave similar results.

TABLE III

Mixtures of Fructose and Sucrose†*

The total volume was 2 cc. in all samples.

Experiment No.	Fructose	Sucrose	Fructose found by new method
	mg.	*mg.*	*mg.*
1	0.10	5.00	0.10
2	0.20	5.00	0.21
3	0.40	5.00	0.39
4	0.80	5.00	0.82
5	1.00	5.00	1.03
6	0.00	1.00	0.00
7	0.00	5.00	0.00

* Kahlbaum; other pure preparations gave similar results.
† Commercial.

For this purpose it is only necessary to have a neutral filtrate with a minimum of electrolytes. Such a filtrate is obtained by the iron precipitation method of Steiner, Urban, and West (10). This yields automatically a filtrate having a neutral pH, with no

increase in electrolytes, and is claimed to yield only true sugar values. By using this precipitation procedure and our monose method, results were obtained which appeared to be very satisfactory.

Qualitative Test for Monosaccharides

Method A—Determine the total reducing value of the solution of reducing sugars to be tested by the volumetric sugar method of Benedict (11), or by any other quantitative method. Dilute so as to contain 0.1 per cent of total reducing sugars (mono- and disaccharides). If the sugar mixture is in dry form make a 0.1 per cent solution. Transfer to a test-tube 1 cc. of the 0.1 per cent solution. Place in a separate tube 1 cc. of distilled water, to serve as a control. Add 1 cc. of the new acid copper reagent to each of the two tubes. Heat in boiling water for 3 minutes. Cool for 2 minutes. Add 1 cc. of the color reagent to each. Mix. A blue color will be obtained if a monose is present; but the solution will have the same color as the control if only bioses are present.

Chlorides interfere with the test (9), but we found up to 5 mg. of NaCl in 1 cc. of the 0.1 per cent sugar solution to be without effect.

Under these conditions exact results may be obtained easily by this new method. The smallest amount of glucose (or other monosaccharide) which can be detected in the presence of a disaccharide is 0.1 mg. in 1 cc. of the solution. There may be 10 times this amount of maltose, or 25 times this amount of lactose present in the monose-biose mixture, without causing interference.

Method B—A more rapid but less exact procedure may be performed in the following manner. Transfer to a test-tube 1 cc. of the solution to be tested. Add 1 cc. of the new acid copper reagent. Heat in boiling water for 3 minutes. A red precipitate (Cu_2O) will indicate the presence of 0.1 per cent or more of monose if not more than 4 per cent of lactose, 2 per cent of maltose, or 10 per cent of sucrose is present.

SUMMARY

1. A new colorimetric method for the determination of monoses in the presence of reducing bioses has been described. It is rapid,

accurate, and simple. The method is also useful in the study of saccharases and glucosidases respectively.

2. The method has been applied to the determination of sugar in suitable blood filtrates.

3. An exact qualitative method for the differentiation of monosaccharides and disaccharides has also been given.

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