A New Method for the Quantitative Determination of Monosaccharides, Amino Sugars and *N*-Acetylneuraminic Acid and of 6-Deoxyhexose (Fucose) in the Presence of other Sugars

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(Received 7 June 1968)

1. Monosaccharides, amino sugars and *N*-acetylneuraminic acid were determined by using an original colorimetric assay procedure, based on the detection of formaldehyde released after periodate oxidation. A range of these compounds was investigated by this method and they were all found to obey Beer's law within the concentration range 0–0.6 μ mole/ml. 2. A simple method for the determination of 6-deoxyhexose concentration in the presence of other monosaccharides is also described. 3. The optimum pH for the release of formaldehyde from sugars by periodate oxidation was 7.0–7.5. 4. The methods described have considerable advantages over existing assay systems and their particular value in automatic colorimetry, where the use of concentrated acids is undesirable, is discussed.

Existing methods of determining the so-called total carbohydrate in a solution utilize very concentrated acids and vigorous reaction conditions. The orcinol reaction (Brückner, 1955), which is at present commonly used, does not detect glucosamine or sialic acid.

A method for the colorimetric assay of formaldehyde was described by Nash (1953) based on the condensation of the aldehyde with acetylacetone in the presence of ammonium salts. The 3,5-diacetyl-1,4-dihydrolutidine so produced was shown to be a yellow substance, absorbing at 412 $m\mu$. This reaction is used as a means of measuring periodate reduction in the structural investigation of glycoproteins (Neuberger & Marshall, 1966). Nash (1953) also noted that, under the same conditions, acetaldehyde gives rise to 3,5-diacetyl-1,4-dihydrocollidine, with an absorption maximum at 388 $m\mu$.

The method of Nash (1953) was later applied by Schwarz (1958) to the chromatographic location of serine residues after periodate oxidation. Subsequently, Weiss & Smith (1967) modified the reaction and extended it to the identification of reducing and non-reducing sugars, amino sugars and sialic acid, on paper and thin-layer chromatograms.

We have now adapted the method of Weiss & Smith (1967) for the quantitative assay of small quantities of monosaccharides and sialic acid, and have extended it to enable the determination of fucose in the presence of other monosaccharides to be carried out.

EXPERIMENTAL

Materials. D-Galactose and D-mannose were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks. D-Fructose, L(–)-fucose, D(+)-glucosamine hydrochloride and D(+)-galactosamine hydrochloride were obtained from British Drug Houses Ltd., Poole, Dorset. *N*-Acetylneuraminic acid was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Acetylacetone (British Drug Houses Ltd.) was redistilled and stored in a dark bottle at 4°; under these conditions it was stable for 1 month. Periodic acid (50%, w/v; British Drug Houses Ltd.) was diluted appropriately with water immediately before use. All other chemicals were of A.R. grade.

Reagents. Reagent A consisted of 15 g. of ammonium acetate and 3.0 ml. of acetic acid made up to 100 ml. with water. Reagent B consisted of a 5% (v/v) solution of redistilled acetylacetone in reagent A. This reagent was unstable and was therefore made up immediately before use.

Procedure. A 0.9 ml. sample of aq. 20% (v/v) pyridine was pipetted into 1.0 ml. of a solution in water of sample or standard containing 5–100 μ g. of sugar, amino sugar or *N*-acetylneuraminic acid. After mixing, 0.1 ml. of 1% (w/v) HIO_4 (0.042 *M*) was added, and the solution was mixed by gentle rotation and left for 10 min. at 20° in the dark. At this stage 1.0 ml. of reagent B was pipetted into the mixture and this was again gently rotated and left for a further 2 hr. at 20° in the dark, after which E_{412} was measured in a Unicam SP.600 spectrophotometer.

When fucose was to be determined in the presence of other sugars, extinctions were measured after 4 hr. at both 412 $m\mu$ and 390 $m\mu$, and values of the quotient E_{412}/E_{390} were calculated. Hüfner's method (Heilmeyer, 1943) of calculating the partition of two pigments mixed in solution

was used to determine the relative proportion of fucose and other monosaccharide present. Experimental results were substituted in the following equation:

Quotient for a mixture of fucose and other sugar =

$$\frac{E_{412}}{E_{390}} = \frac{\frac{x}{A} + \frac{(100-x)}{B}}{\frac{x}{a} + \frac{(100-x)}{b}}$$

where A and a are the absorption ratios of pure fucose at 412 m μ and 390 m μ respectively (absorption ratio is extinction divided by concentration in g./ml.), B and b are the corresponding values for the other sugar and x is the percentage of fucose in the mixture of fucose and the other sugar. If the extinctions are measured on pure sugars of equal concentration, $1/E$ can be substituted for absorption ratio in the above equation, providing an easier computation.

Vierordt's procedure (Heilmeyer, 1943) was used to determine the concentration, as opposed to the proportion, of fucose in a mixture with another monosaccharide:

Concn. of fucose in mixture (g./ml.) =

$$y = \frac{Aa(E_{412} \cdot B - E_{390} \cdot b)}{Ba - Ab}$$

This equation can also be simplified by measuring the extinctions of the pure sugars on solutions of the same concentration.

A comparison of theoretical and experimental results was carried out for mixtures of fucose and glucose (Table 1).

RESULTS

3,5-Diacetyl-1,4-dihydrolutidine is a bright-yellow compound, having a maximum extinction between 410 m μ and 415 m μ , and in the concentration range used in the present work it was shown to obey Beer's law (Fig. 1). 3,5-Diacetyl-1,4-dihydrocollidine is a pale-yellow compound, having a maximum extinction at 390 m μ . It was also found to obey Beer's law.

Overall sensitivity of the reaction. The sensitivity of the method for each individual sugar or sugar compound tested is related to the amount of formaldehyde or acetaldehyde released under the conditions used for periodate oxidation. This is shown clearly in Fig. 2, where the amount of colour produced by glucose, glucosamine and fructose was plotted against time of periodate oxidation. In each case formaldehyde production appeared to be complete within 10 min.

The sensitivity of the reaction was increased by 35% when the concentration of the periodate in the solution added was decreased from 5% to 1% (w/v). The molar extinction coefficients, relative to glucose, were 4650 and 6300 respectively.

The overall sensitivity of the reaction was increased when the concentration of pyridine in the solution added was increased from 0 to 20% (v/v)

(Fig. 3). This was due mainly to increase in periodate oxidation.

Effect of pyridine on periodate oxidation. The effect of pyridine on the efficacy of the oxidation is shown in Fig. 3. The aberrant point with 5% (v/v) pyridine is reproducible. It is due to the fact that very little change in pH occurs between 2% and

Fig. 1. Plots of E_{412} against concentration for: ▲, fructose; ●, glucose, mannose, galactose, *N*-acetylneuraminic acid; ○, glucosamine, galactosamine.

Fig. 2. E_{412} values after oxidation of glucose (●, 100 μ g.), glucosamine (○, 100 μ g.) and fructose (Δ, 50 μ g.) by the addition of a 5% (w/v) HIO_4 solution for various times.

Fig. 3. Effect of pyridine on the sensitivity of the reaction. (a) Solutions (0.9 ml.) containing different concentrations of pyridine were added to 1 ml. samples containing 50 μ g. of glucose, before addition of periodate (●). (b) Solutions (0.9 ml.) containing different concentrations of pyridine were added to the reaction mixtures immediately after the addition of reagent B (○). In (a) and (b), all tubes were read against water blanks with corresponding pyridine concentrations. (c) This curve gives the numerical difference between curves (a) and (b), which represents the effect of pyridine on periodate oxidation only (▲).

Fig. 4. Effect of pH on periodate oxidation. (a) The data of Fig. 3(c) are given, replotted against the pH produced during oxidation by different concentrations of pyridine (○). (b) Glucose standards (50 μ g./ml.) were prepared in water and phosphate-citrate buffers, pH in the range 5.0–7.5 (0.2 M- Na_2HPO_4 adjusted with 0.1 M-citric acid). A portion (0.9 ml.) of 20% (v/v) pyridine was added to each test after reagent B, producing pH 6.2–6.3 in every case during colour development (Δ). (c) The same glucose standards as in (b) were used. Water (0.9 ml.) was added to each test after reagent B, instead of pyridine, producing pH 5.5–5.6 in every case during colour development (●).

5% (v/v) pyridine concentrations in the added solution. This was confirmed when the results for this experiment were replotted as a function of the pH produced by varying the concentrations of pyridine during periodate oxidation (curve a, Fig. 4). The close similarity of the curve to curves (b) and (c), in which the pH differences during oxidation were created by use of appropriate phosphate-citrate buffers, demonstrates the importance of the buffering effect of pyridine; pH 7.5 seems ideal for formaldehyde production.

Effect of pyridine on the condensation of acetylacetone with formaldehyde. As much as a 50% increase in the production of 3,5-diacetyl-1,4-dihydrolutidine from formaldehyde and acetylacetone was observed when pyridine was present (Figs. 3 and 4). However, the pH during the condensation step was 6.2–6.3 for curve (b) where pyridine was added and 5.5 for curve (c) where no pyridine was added. Fig. 5 shows the effect of the final pH on the condensation reaction.

Effect of varying the concentration of acetylacetone. The concentration range 1–8% (v/v) acetylacetone in reagent B was studied. At 5% concentration in reagent B, maximum colour developed.

Effect of time on colour development. At 2 hr. the condensation of acetylacetone with formaldehyde reached completion. The colour was stable during a further 2 hr. period. Longer time studies for the stability of the colour were not undertaken.

The condensation of acetylacetone with acetaldehyde took place more slowly, and was not complete after 4 hr.

Fig. 5. Effect of pH on the condensation reaction. Glucose standards (50 μ g./ml. of 1.6 M-ammonium acetate, pH 7.35) were oxidized by the addition of a 1% (w/v) HIO_4 solution. Differences in pH during condensation were achieved by using different concentrations of acetic acid in reagent B. ●, 0.9 ml. of 20% (v/v) pyridine added after oxidation step. ○, 0.9 ml. of water added after oxidation step. In every case tests were read against water blanks treated with the corresponding reagent B.

Table 1. Comparison between the calculated and the absolute quantities of fucose in a binary mixture of fucose and glucose

Experimentally determined extinctions of mixture			Actual quantity of fucose in mixture		Calc. quantity of fucose in mixture	
E_{412}	E_{390}	E_{412}/E_{390}	(%)	($\mu\text{g./ml.}$)	(%)	($\mu\text{g./ml.}$)
1.16	0.920	1.26	0	0	0	0
0.950	0.810	1.17	20	20	22.0	20.7
0.865	0.790	1.09	40	40	39.0	39.0
0.767	0.745	1.03	50	50	50.6	50.2
0.712	0.720	0.99	60	60	57.5	57.6
0.530	0.630	0.84	80	80	80.6	79.5
0.404	0.590	0.69	100	100	99.7	99.5

Comparative sensitivity of the method for different sugars. Glucose, galactose, mannose and *N*-acetylneuraminic acid gave identical plots of extinction against concentration (Fig. 1). Fructose was the most sensitive sugar tested. The amino sugars were slightly less sensitive than glucose.

Determination of fucose in the presence of other monosaccharides. A comparison of the actual proportions and concentrations of fucose, in binary mixtures with glucose, with those calculated from the experimental values of E_{412} and E_{390} for the same mixtures is shown in Table 1.

Although completion of the reaction is desirable for the determination of fucose alone in solution, time was not found to be a critical factor in determining fucose in the presence of glucose, provided that the extinctions of pure glucose and pure fucose at 412 $m\mu$ and 390 $m\mu$ required for the calculation were measured at the same time as the quotient for the mixtures.

DISCUSSION

Although determination of formaldehyde by various means has often been applied in periodate-reduction studies (Reeves, 1941; O'Dea & Gibbons, 1953), it is surprising that it has not previously been used as a simple method for sugar determination.

Determinations of formaldehyde by precipitation with dimedone and by the chromotropic acid method necessitate prior reduction of excess of periodate in the oxidation mixture. According to Nash (1953), periodate destroys dihydrolutidine, and we indeed observed that increasing the concentration of the periodate solution added from 0.042 *M* to 0.209 *M* depressed the colour production by 25%. However, reproducible high extinctions were obtained with 0.042 *M*-periodic acid, and the molar extinction coefficient relative to glucose and formaldehyde was only 21% lower than that recorded by Nash (1953) in the absence of periodate. We believe that the advantage of the simplification

of the assay technique by omitting the removal of the excess of periodate far outweighs the small loss of sensitivity.

The sensitivity of the method described in the present paper reflects both the number of formaldehyde residues that are produced by individual sugars after periodate oxidation and, with the amino sugars, the steric hindrance to the oxidation presented by the amino group.

Fructose is expected to yield 2 moles of formaldehyde and the aldohexoses and hexosamines 1 mole of formaldehyde/mole of sugar, and the theoretical yield from fucose is 1 mole of acetaldehyde/mole. Experimentally, however, the yield of formaldehyde from fructose was only 72% of the theoretical relative to glucose, mannose and galactose (Fig. 1). A possible explanation may be a choice of two reactions at one point in the stepwise oxidation procedure, only one of which yields the second formaldehyde molecule (Bobbitt, 1956). Reeves (1941) noted a similar, though slightly smaller, discrepancy between the theoretical and experimental formaldehyde release from fructose.

That the effect of pyridine on periodate oxidation was mainly due to pH is not surprising; pH exerts a remarkable influence on the course of periodate oxidation. The pH value most commonly used for periodate-reduction studies of carbohydrates is pH 3–5 (Bobbitt, 1956; Dyer, 1956), alkaline media often resulting in overoxidation (Cantley, Hough & Pittet, 1959). However, pH 7.5 has often been more successful than lower pH values for formaldehyde production (Reeves, 1941; Jeanloz, 1944; Bell & Greville, 1950). Hough & Perry (1956) showed that for maltose the time required for maximum liberation of formaldehyde was greatly shortened by increasing the pH of the oxidizing medium. This is due to the fact that in acidic solution the final stages of periodate oxidation can be slow for sugars such as glucose, which yield *O*-formyl esters as products of incomplete oxidation. Hydrolysis of these esters proceeds much more rapidly under

slightly alkaline conditions than it does in mildly acidic media. It is logical therefore that the optimum concentration of pyridine for the reaction was that which created a pH of about 7 for periodate oxidation. At this pH value, oxidation of the simple compounds tested was complete in 10 min.

The pH-dependence of the reaction of acetylacetone with formaldehyde (Fig. 5) suggests that the effect of pyridine on this part of the reaction is also a pH effect. It may be possible to utilize buffer systems instead of pyridine for maintaining the pH during periodate oxidation, provided that care is taken to use the concentration of acetic acid in reagent B that produces the optimum pH for the condensation step.

The final concentration of acetylacetone (approx. 0.16M) that gave maximum colour development in the shortest time was higher than that employed by Nash (1953), who obtained optimum colour after 16 hr. with 1mM-acetylacetone, and after 4 hr. with 0.1mM-acetylacetone.

The method described here is highly sensitive for small quantities of sugars (less than 0.5 μ mole) and variations between individual sugars, with the exception of fructose, are less than in the more usual orcinol method. It has the additional advantage that it is also applicable to amino sugars and sialic acids.

Determinations of 6-deoxyhexoses in the presence of other sugars based on the liberation of acetaldehyde after periodate oxidation have been described previously (Nicolet & Shinn, 1941; Folkes, 1953). In these methods an extraction procedure for acetaldehyde was involved, after which a separate analysis of formaldehyde in the residual oxidation mixture could be performed. In the present work extinctions were measured simultaneously at two wavelengths and the mathematical calculations of Hufner and of Vierordt (Heilmeyer, 1943) applied to determine the proportion and the actual concentration respectively of fucose in mixtures with glucose. Percentages and concentrations calculated by these methods were in good agreement with theoretical values. The technique is applicable to mixtures of

fucose or other 6-deoxyhexose with any sugar that gives formaldehyde on periodate oxidation, and the method is essentially simpler than those previously described.

Application of the method described to the quantitative determination of sugar residues in glycoproteins would depend on hydrolysis conditions being such that free serine and threonine were not liberated, as these would interfere in the reaction.

At present there is a considerable trend towards automated analysis. The orcinol method for neutral sugars does not lend itself easily to automation, both because of the heating conditions required and because the final acidic mixture is too viscous for easy manipulation through automatic colorimeters. The method described in the present paper can easily be adapted to mechanization.

We thank Professor J. H. Kellgren for his interest in and support of this project.

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