

PROTEIN-CARBOHYDRATE INTERACTION

VIII. A TURBIDIMETRIC METHOD FOR THE ANALYSIS OF D-MANNOSE

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The quantitative analysis of a specific sugar in the presence of other carbohydrates has always been an involved and time-consuming process. Only with the advent of the glucose oxidase-peroxidase colorimetric method¹⁻³ has it been possible to analyze routinely for D-glucose in the presence of other sugars. Similarly, D-galactose can be estimated by using galactose oxidase⁴. Both of these oxidases show a high degree of specificity for their respective substrates and have proved to be extremely efficient analytical tools. Enzymic procedures are also available for several other carbohydrates (*cf.* ref. 5).

The determination of D-mannose, especially in a mixture of sugars, remains a tedious process. It usually involves paper-chromatographic or -electrophoretic separation of the components of the mixture, followed by elution of developed chromophores obtained by a spray reagent, or alternatively, by elution of the appropriate sugar followed by colorimetric or reductometric analysis (*cf.* ref. 6). Gas-liquid chromatography has also found wide application in the analysis of sugar mixtures, although this usually requires further analysis of the various anomeric forms of individual sugars (*cf.* ref. 7).

Morgan and Watkins⁸ have shown that certain mono- and oligo-saccharides specifically inhibit the interaction of the phytohemagglutinins from various plant-seeds with red blood-cells of types A and B. Similarly, it has been shown in this laboratory⁹ that D-mannose and D-glucose, and some of their derivatives, inhibit the interaction of concanavalin A (a phytohemagglutinin from jack bean) with certain branched α -D-glucans and α -D-mannans.

This communication reports, for the analysis of D-mannose, a procedure that depends upon estimation of the degree to which the turbidity that is formed on interaction of concanavalin A with glycogen is inhibited by D-mannose. The application of this procedure to the determination of D-mannose in a variety of polysaccharides is described.

MATERIALS AND METHODS

Concanavalin A was extracted from jack-bean meal, and purified by the method of Agrawal and Goldstein¹⁰. It was stored as a stock solution (2.8 mg/ml) in

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saturated sodium chloride. The protein was stable for long periods of time when stored at 4°. A diluted solution that contained 60 $\mu\text{g}/\text{ml}$ of protein in 0.1N sodium phosphate buffer, pH 7.0, and that was approximately 0.1N with respect to sodium chloride, was used for the analysis.

Carbohydrates. — Oyster glycogen was purchased from Distillation Products Industries (Rochester, New York). Chromatographically “pure” D-mannose and D-glucose were purchased from Mann Research Laboratories, Inc. (New York, New York). The D-mannose, which contained impurities migrating as oligosaccharides on paper chromatograms, was further purified by repeated recrystallization from acetic acid followed by cellulose-powder, column chromatography [elution solvent: 10:4:3 (v/v) ethyl acetate–pyridine–water]. The remaining sugars were obtained commercially, and were used without further purification.

The following polysaccharides were gifts from the sources noted: yeast mannan from *Saccharomyces cerevisiae* (Prof. S. Peat); galactomannan from *Leucaena glauca* (Dr. A. M. Unrau); glucomannan from Aspen wood and galactoglucomannan from Eastern Hemlock wood (Prof. T. E. Timell); galactoglucomannan from wood of *Picea abies* Karst (Prof. B. Lindberg); and guar gum (Meer Corporation, New York).

Reagents. — Phenol (analytical reagent) and Amberlite IR-45 anion-exchange resin were purchased from Mallinkrodt Chemical Works (St. Louis, Missouri). The phenol was redistilled, and prepared a an aqueous solution (80% w/w). Concentrated sulfuric acid (reagent grade) was a product of E. I. DuPont de Nemours and Co. (Wilmington, Delaware); Glucostat and Galactostat were products of the Worthington Biochemical Corp. (Freehold, New York).

PROCEDURE

Turbidimetric analysis. — To a Bausch and Lomb (Rochester, New York) Spectronic 20 tube (0.5 inch diameter) were added 0.95 ml of an aqueous solution of D-mannose (320–740 $\mu\text{g}/\text{ml}$) and 0.05 ml of glycogen solution (8.60 mg/ml). The same amount of glycogen, diluted to 1.0 ml with water and containing no inhibitor, was used as a control (hereafter called the uninhibited reference). The reaction was initiated by the rapid addition of 2.0 ml of concanavalin A solution (60 $\mu\text{g}/\text{ml}$). The solution was stirred immediately with a polyethylene rod, and incubated at 25°. After exactly 20 min, the resulting turbidity was measured at 420 nm in a Bausch and Lomb Spectronic 20 spectrophotometer, and recorded as optical density units (O.D.). Blank readings were obtained by clarifying the reaction mixture by the addition of crystalline methyl α -D-mannopyranoside (ca. 0.5 mg), an excellent inhibitor of the turbidimetric reaction⁹. The optical density of the clear solutions was determined after 5 min, and this value was subtracted from the optical density of the solutions prior to the addition of methyl α -D-mannopyranoside, to give a value referred to as corrected O.D.

Each analysis of a single, unknown solution was performed at three different dilutions, each in duplicate. A similar protocol was followed for standard solutions of D-mannose and D-glucose. The percent inhibition was calculated as follows:

Percent inhibition =

$$\frac{\text{Corrected O.D. (uninhibited reference)} - \text{Corrected O.D. (inhibited)}}{\text{Corrected O.D. (uninhibited reference)}} \times 100.$$

Hydrolysis of polysaccharides. — Hydrolysis of polysaccharides (5–8 mg/ml) was conducted in sealed tubes in 1.5*N* sulfuric acid for 5–6 h in a boiling water-bath. The hydrolyzates were neutralized with Amberlite IR-45 (OH⁻) resin (*ca.* 1 g/ml of hydrolyzate), the resin was washed thoroughly, and the solutions were evaporated to dryness *in vacuo* in a rotary evaporator. The residue was dissolved in sufficient water to produce a concentration of 4–6 mg of total sugar/ml, based upon the weight of the starting material.

During the course of these studies, it was noted that flocculation occurred during the 20-min incubation period of the analysis, when barium carbonate was used for neutralizing the sulfuric acid in the hydrolyzates. Therefore, the hydrolyzates were deionized with Amberlite IR-45 (OH⁻) resin prior to analysis for carbohydrates. It was also noted that sodium chloride in concentrations up to 2*N* had no effect on the estimation of D-mannose. Additional information concerning the effect of various factors on the concanavalin A–glycogen interaction will be reported elsewhere¹¹.

Quantitative paper-chromatography. — Whatman No. 1 chromatographic filter paper was used for the quantitative, chromatographic separation of sugar mixtures, and was irrigated with either 10:4:3 (v/v) ethyl acetate–pyridine–water or butanone–water azeotrope. The D-mannose was eluted with water, and analyzed by the phenol–sulfuric acid method¹². D-Glucose was analyzed by the Glucostat procedure, performed according to the micro method of Worthington Biochemical Corp. (manufacturer's directions) and D-galactose was analyzed by the Galactostat method (same manufacturer's directions).

RESULTS

Standard curves of the log of the weight (in mg) of D-mannose and D-glucose *versus* percent inhibition are shown in Fig. 1. Standard curves were prepared within the

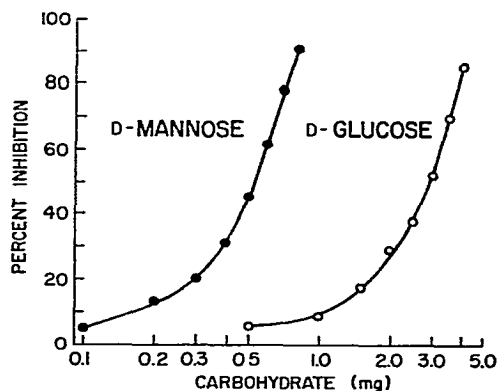


Fig. 1. Inhibition of concanavalin A–glycogen interaction by D-mannose and D-glucose. Concanavalin A, 120 μ g; glycogen, 430 μ g; in a total volume of 3.0 ml.

range of 10–90% inhibition by using eight different concentrations of D-mannose and D-glucose. It may be seen that, as an inhibitor, D-mannose is 5.4 times as effective as D-glucose. At least three concentrations of D-mannose (chosen to produce 25–75% inhibition), each in duplicate, were used as standards and were included with each analysis of unknowns.

When D-mannose was the sole sugar present, inhibition analysis, followed by reference to the standard curve for D-mannose, gave directly the quantity of this sugar present. The presence of noninhibitory sugars did not affect the determination of D-mannose by this method (*cf.* Table I).

TABLE I

TURBIDIMETRIC DETERMINATION OF D-MANNOSE IN THE PRESENCE OF D-GLUCOSE OR D-GALACTOSE

<i>Synthetic mixtures, mg</i>			<i>Total D-mannose-inhibiting equivalents^a, mg</i>	<i>D-Glucose, in D-mannose-inhibiting equivalents, mg</i>	<i>D-Mannose found, mg</i>	<i>Recovery, %</i>
D-Mannose	D-Glucose	D-Galactose				
0.200	0.500	—	0.290	0.093	0.197	98.5
0.300	0.500	—	0.401	0.093	0.308	102
0.400	0.500	—	0.489	0.093	0.396	99
0.300	—	1.95	0.312	—	0.312	104
0.400	—	1.65	0.408	—	0.408	102
0.500	—	1.35	0.500	—	0.500	100

^aConverted from the percent inhibition, directly from the standard graph (Fig. 1).

It was observed that the inhibiting power of each sugar in a mixture of D-mannose and D-glucose was unaffected by the presence of the second sugar in the mixture. Thus, the inhibiting power of a mixture of the two sugars was additive. Since, as an inhibitor, D-mannose was 5.4 times as effective as D-glucose (as observed from the standard curves at the 50%-inhibition level), it was possible to calculate the theoretical inhibition of a mixture of these two sugars. Thus, a mixture of 0.30 mg of D-mannose and 1.62 mg of D-glucose would yield an inhibiting power equivalent to 0.60 mg of D-mannose alone: [0.30 mg of D-mannose + (1.62 mg of D-glucose)/5.4 = 0.60 mg of D-mannose].

Synthetic mixtures of D-glucose and D-mannose were readily analyzed in the following manner. A Glucostat determination gave the quantity of D-glucose present. (D-Mannose did not interfere in this determination.) The amount of D-glucose present was then converted into "D-mannose-inhibiting equivalents" (M.I.E.), expressed as mg of D-mannose, by employing the following relationship:

M.I.E. (mg of D-mannose) =

$$\text{mg of D-glucose} \times \frac{(\text{mg of D-mannose required for 50\% inhibition})}{(\text{mg of D-glucose required for 50\% inhibition})}$$

Values for the 50%-inhibition levels were taken from the respective standard curves.

An inhibition determination was then performed on the D-glucose–D-mannose mixture by employing the standard curve for D-mannose. The amount of D-mannose present in the mixture is calculated by subtracting the contribution of D-glucose, in terms of M.I.E., from the total amount of D-mannose.

Table I shows the results obtained for the analysis of D-mannose in synthetic mixtures of D-mannose and D-glucose. It is evident that, after correcting for the known amount of D-glucose, the values obtained for D-mannose are in excellent agreement with the amounts present. Table I also demonstrates that D-galactose, a common constituent of many heteromannans, has no effect on the estimation of D-mannose.

A comparison of the analysis of D-mannose in a variety of mannans, by the combination of quantitative, paper-chromatographic separation and the phenol–sulfuric acid method¹², and by the concanavalin A, turbidimetric assay, is shown in Table II. There is excellent agreement between these two empirical procedures.

TABLE II

COMPARISON OF THE TURBIDIMETRIC AND PHENOL–SULFURIC ACID METHODS FOR THE DETERMINATION OF D-MANNOSE IN VARIOUS MANNANS

Mannan	Constituents	Percent of D-mannose in hydrolyzates	
		Concanavalin A method	Phenol–H ₂ SO ₄ ¹² method
<i>Saccharomyces cerevisiae</i>	D-mannose	94.5	100.0 ^b
<i>Leucaena glauca</i>	D-mannose, D-galactose ^c	59.3	58.3
Guar gum	D-mannose, D-galactose ^c	57.4	59.9
Aspen wood	D-mannose, D-glucose ^d	62.0	64.8
Eastern Hemlock wood	D-mannose, D-galactose ^c , D-glucose ^d	68.7	69.5
<i>Picea abies</i> Karst ^e	D-mannose, D-galactose ^c , D-glucose ^d	70.6	71.4

^aPaper-chromatographic separation, followed by elution of the D-mannose and analysis by the phenol–H₂SO₄ method (see ref. 11). ^bNormalized to 100.0. ^cDetermined by the Galactostat method. ^dDetermined by the Glucostat method. ^eThis mannan was initially dissolved in 0.3N NaOH, and H₂SO₄ was added to yield a final acid concentration of 1.5N.

The effect, on the analysis of D-mannose, of some common carbohydrate constituents found in polysaccharides and glycoproteins is shown in Table III. Only slight interference occurs at high concentrations of D-arabinose and 2-amino-2-deoxy-D-glucose.

DISCUSSION

The concanavalin A turbidimetric procedure for the analysis of D-mannose is a simple, rapid, and precise method that makes use of the Landsteiner inhibition technique in which a species of low molecular weight (hapten) competes with an antigen for the binding sites on a specific antibody. The resemblance of concanavalin A–polysaccharide interaction to the antibody–antigen system has been discussed previously^{9,13–15}. Here, the extent to which a specific inhibitor of the system

(D-mannose) inhibits the turbidimetric reaction between concanavalin A and glycogen is exploited for the quantitative determination of D-mannose.

TABLE III

EFFECT OF SOME ALDOSES ON THE TURBIDIMETRIC DETERMINATION OF D-MANNOSE

Sugar, mg	D-Mannose, mg		Recovery %
	Present	Found	
D-Arabinose (1.00)	0.500	0.522	104
D-Arabinose (1.50)	0.500	0.536	107
D-Xylose (2.00)	0.500	0.511	102
D-Xylose (3.00)	0.500	0.513	103
L-Fucose (5.00)	0.500	0.505	101
2-Amino-2-deoxy-D-glucose (1.00)	0.500	0.522	104
2-Amino-2-deoxy-D-glucose (1.50)	0.500	0.536	107
2-Amino-2-deoxy-D-galactose (3.00)	0.500	0.510	102
L-Rhamnose (5.00)	0.500	0.505	101

By this procedure it is possible to estimate D-mannose in the range of 300–750 μg . Modification of the assay by proportionally decreasing the components and the volume of the system could result in its use as a microanalytical tool for the determination of D-mannose.

A typical analysis required approximately one h. The length of time used for the incubation period may be decreased, but this causes a decrease in the resultant turbidity. Concurrent studies on the turbidimetric technique for studying polysaccharide–concanavalin A interaction has revealed the time-dependency of the reaction. Thus, with a longer incubation period, a parallel decrease in the percentage inhibition was observed¹¹; this necessitated the use of larger aliquots of sample to produce a given percentage inhibition.

Because the amount of sugar needed to cause a given degree of inhibition is a function of the ratio of concanavalin A to glycogen, it was necessary to establish the conditions for maximum sensitivity to inhibition by D-mannose. This was accomplished by studying the initial rate of development of turbidity when various amounts of glycogen were allowed to react with a given amount of concanavalin A (120 μg). The ratio that yielded the highest initial rate of formation of turbidity (430 μg of glycogen: 120 μg of concanavalin A, commonly called the ratio of optimum proportions) was chosen for this study (*cf.* ref. 11).

It has been suggested^{9,13,14} that the 2-deoxy-D-arabino-hexopyranose or D-arabinofuranose moiety is the minimal structural requirement for the binding of a monosaccharide to concanavalin A. Of the sugars in Table III, only 2-amino-2-deoxy-D-glucose and D-arabinofuranose possess these configurational features. Indeed, of the sugars tested, only 2-amino-2-deoxy-D-glucose and D-arabinose caused somewhat higher results than expected, when in concentrations equivalent to approximately three times that of D-mannose. Compensation for the presence of large proportions of such interfering substances may be made in a manner similar to that

used for the D-glucose correction. Thus, if the amount of an interfering substance is known, the D-mannose-inhibition equivalent, namely,

$$\text{mg of interfering substance} \times \frac{\text{mg of D-mannose needed for 50\% inhibition}}{\text{mg of substance needed for 50\% inhibition}},$$

of this substance may be subtracted from the total amount of sugar obtained directly from the standard graph. The consistent agreement that is obtained for the analysis of D-mannose by the concanavalin A method (with the above correction) and the method employing paper-chromatographic separation and phenol-sulfuric acid assay, in heteromannans containing D-glucose, is illustrated in Table II.

To the best of the authors' knowledge, this is the first report of the use of plant lectins for the specific, quantitative analysis of a sugar. It suggests the possibility that other carbohydrates may be analyzed in a similar manner by the use of other lectins, or of possible antihapten antibodies specific for a particular sugar or other haptens.

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SUMMARY

A unique, spectrophotometric method for the quantitative estimation of D-mannose in the presence of other sugars is described. The procedure depends on the degree to which the turbidity formed on interaction of concanavalin A with glycogen is specifically inhibited by D-mannose. The analysis, whose useful range is 300 to 750 $\mu\text{g/ml}$, gives essentially the same results as other methods commonly used. A correction for the presence of D-glucose, which also inhibits glycogen-concanavalin A interaction, is readily achieved by use of the Glucostat reagent.

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