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CONTINUOUS SPECTROPHOTOMETRIC ASSAY OF GLUCOSYLTRANSFERASE AND β-FRUCTOFURANOSIDASE ACTIVITY

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ABSTRACT

Sucrose 6-glucosyltransferase [(1→6)-α-D-glucan:D-fructose 2-glucosyltransferase, E.C. 2.4.1.5] converts sucrose into D-glucan and β-fructofuranosidase [β-D-fructofuranoside fructohydrolase, invertase, E.C. 3.2.1.26) hydrolyses sucrose, both releasing stoichiometric amounts of D-fructose. This study reports on a direct spectrophotometric method for measuring the kinetics of these enzymes. D-Fructose (in aqueous solution at 37°) has 2 absorption peaks at 188.5 and 278 nm (ε 133.6 and 1.12, respectively); D-glucose, which is also released by β-fructofuranosidase, has a λ_{max} at 186.0 nm (ε 123.0). The methods developed utilize the absorption peaks in the far u.v. range. Buffers exert a bathochromic shift and have a hypochromic effect. Glucosyltransferase was assayed in phosphate buffer (0.1mM, pH 6.8). From a plot of $\Delta(\varepsilon_{Dfructose} - \varepsilon_{sucrose})$ *vs.* λ, 195 nm was selected as the optimal wavelength since, at this wavelength, the contribution of sucrose was least ($\simeq 10\%$) and ΔA_{195}/min was linearly proportional to glucosyltransferase concentration. Determination of K_m by this method gave values comparable to those obtained by chemical assay of released reducing groups (2.05 *vs.* 2.00mM sucrose, respectively). β-Fructofuranosidase assayed at 207 nm in acetate buffer (0.5mM, pH 4.6) at 37° gave a K_m value of 12.3mM sucrose. This is in agreement with the results obtained by polarimetry and chemical assay of released reducing groups (14.6 and 12.3mM sucrose, respectively). The advantages of this method are simplicity, ability to measure initial reaction rates, and a continuous following of the course of the reaction.

INTRODUCTION

Sucrose 6-glucosyltransferases [(1→6)-α-D-glucan:D-fructose 2-glucosyltransferase, E.C. 2.4.1.5] are extracellular enzymes synthesized by a variety of microorganisms, including *Streptococcus mutans* and *Streptococcus sanguis*[1,2]. These glucosyltransferases require sucrose as a substrate which is utilized to form a D-glucan and release D-fructose. β-Fructofuranosidase [β-D-fructofuranoside fructohydrolase, E.C. 3.2.1.26], commonly known as invertase, releases D-fructose from substrates which possess an unsubstituted β-D-fructofuranosyl residue[3,4]. Previous measurements of the glucosyltransferase reaction have been colorimetric, based on determina-

tion of the reducing D-fructose released as a function of time[1,2]. Stoichiometric amounts of D-fructose released are measured with Somogyi's reagent[5], followed by the Nelson procedure[6]. The kinetics of the invertase reaction have been measured polarimetrically[7], by determination of reducing sugar formed, and more recently with D-glucose oxidase to estimate the D-glucose released[3]. The method reported here is based on the weak absorption transitions exhibited by the hexoses in the far u.v. range.

EXPERIMENTAL

Materials. — All the aqueous solutions were prepared with double distilled water (second distillation performed in an all-glass apparatus). D-Fructose was obtained from Calbiochem (La Jolla, Calif. 92037), raffinose pentahydrate, (A grade) from Calbiochem; sucrose, AR, from Mallinckrodt Chem. Works, Science Prod. Div. (St. Louis, Mo. 63160). Dry nitrogen was C.P. and water-pumped grade; potassium acetate, potassium dihydrogen phosphate, and dipotassium hydrogen phosphate were reagent grade. Glucosyltransferase enzyme: The enzyme was obtained from *Streptococcus sanguis*, strain 804. The purification of the enzymes was achieved in two steps by adsorption on hydroxylapatite gel, followed by isoelectric focusing and fractionation as described previously[1,2]. The fraction focusing at pH 7.9 was employed.

Invertase was grade X(pfs) derived from *Candida utilis* (Sigma Chemical Company, St. Louis, MO).

Methods. — For maximum precision the values of the λ_{max} and ε for the various sugars listed in Tables II–IV were determined with a Cary Model 16 spectrophotometer, which was purged overnight with nitrogen. For routine kinetic measurements, a Perkin–Elmer spectrophotometer (Model DBG, Coleman 124) with a jacketed cuvette chamber maintained at 37° and a Beckman chart recorder were employed. In the u.v. method, the monochromator and cell compartment were purged with dry nitrogen for 1 h at 0.15–0.2 atm prior to the measurements. To a sucrose solution (1.5 ml) of known concentration in both the reference and the sample cells, was added 1.3 and 1.5 ml of the phosphate buffer (0.2mM, pH 6.8), respectively. After being mixed, the contents were allowed to attain thermal equilibrium. When a steady absorbance was observed, an aliquot (0.2 ml) of glucosyltransferase (equivalent to 5–7 DSU[8]) or invertase (100 units, where 1 unit hydrolyses 1 mmole of sucrose per min) was added to the sample cell. The reaction mixture was stirred quickly with a plastic rod and the enzymic reaction recorded at 195 or 207 nm for glucosyltransferase or invertase, respectively Initial slopes were measured by the tangent drawn to the recording. Kinetic measurements on invertase were conducted in a similar way with the exception that the phosphate buffer was replaced by an acetate buffer (0.5mM, pH 4.6) to obtain the optimal pH.

In the polarimetric method used for invertase, the standard sucrose (4.5 ml) and acetate buffer (3.6 ml) solutions (0.5mM, pH 4.6) were equilibrated to 37° and mixed with the invertase solution (0.9 ml, 225 μg protein). The reaction mixture was

transferred to a polarimetric tube (1 dm) and the rotation values determined every 0.5 min for the first 10–15 min with a Perkin–Elmer Model 141 polarimeter, maintained at 37°.

RESULTS

Selection of buffers. — Ions mask the absorption transitions. Buffers such as piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) and *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES)[9] were tried, but found to be unsatisfactory even at a low concentration. Dilute phosphate (0.1mM, pH 6.8) and acetate (0.5mM, pH 4.6) buffers caused minimal interference. Increased concentrations of the buffering agents exerted a consistent bathochromic shift accompanied by a larger hypochromic effect (Table I).

TABLE I

EFFECT OF CONCENTRATION OF PHOSPHATE BUFFER (pH 6.8) ON λ_{max} AND ε VALUES OF D-FRUCTOSE (25mM) AT 37°[a]

Molarity of phosphate buffer (mM)	λ_{max} *(nm)*	ε
50	199.5	14.56
10	194.5	40.56
5	193.5	49.20
1	193 0	54.80
0.1	192.5	64 00
0	192.5	64.20

[a]Determined with a Perkin–Elmer spectrometer.

Selection of absorption wavelength. — Sucrose solution (0.125M) exhibits an absorption peak at 191 nm, close to but much less intense than that of a D-fructose solution. The difference between the absorption of both solutions is maximal above their λ_{max}. Precise measurements in aqueous solution using the Cary 16 Spectrophotometer indicated that the maximum difference is at 194 nm (Fig. 1). However, under the experimental conditions (0.1mM phosphate buffer, pH 6.8, 37°) the Perkin–Elmer spectrophotometer was used and a wavelength of 195 nm was found optimal for kinetic measurements. The absorbance at increasing D-fructose concentrations was found to exhibit linearity and to follow the Beer–Lambert law over a usable range of product formation (up to 5 mmoles of D-fructose). The interference by sucrose absorption was further minimized by employing a sucrose solution of identical molarity in the reference cell, as described under "Methods".

Absorption by the enzyme–substrate complex is insignificant. Usually, the enzyme was used in small concentrations (20 μg protein per ml) and there was a large excess of the substrate. However, when the substrate concentration was low, such as

in the determination of the Michaelis–Menten constant, a substantial contribution to the absorption by the uncomplexed form of the enzyme was observed. This was compensated by addition of a similar amount of the denatured enzyme in the reference cell, since the native and the denatured enzyme show identical spectra.

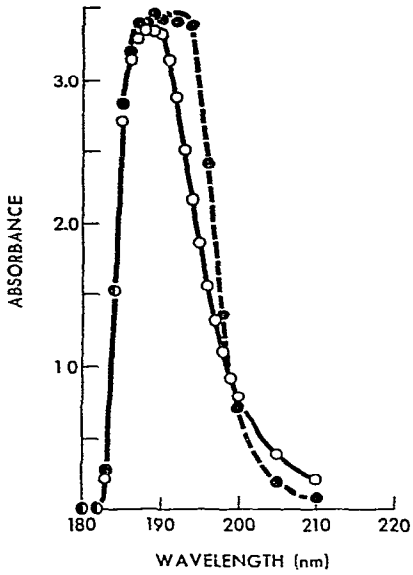
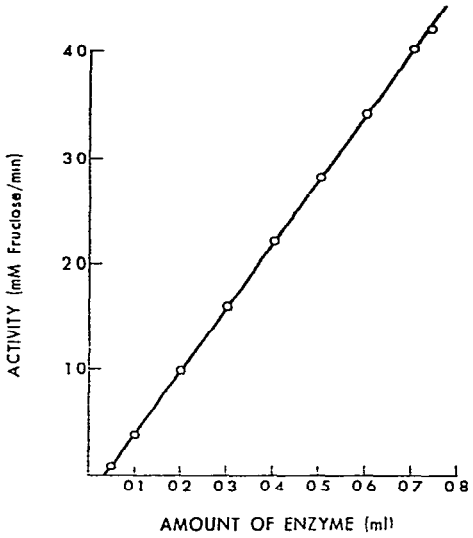


Fig 1. Absorption spectra of D-fructose (25 mM, O——O) and sucrose (0.125M, ●----●) in distilled water at 37° obtained with a Cary Model 16 spectrophotometer and a cell purged with nitrogen. Note the maximal absorption difference between the two spectra occurs at 194 nm.

The optimal wavelength for measuring the kinetics of invertase–sucrose (and invertase–raffinose) reaction was selected by scanning the reaction mixture over the u.v. range at intervals of time, and the rate of change of absorbance was found to be maximal at 207 nm (202 nm for raffinose).

Linearity of enzyme activity. — Glucosyltransferase activity, measured as change in absorption at 195 nm per min, was expressed in mmoles of D-fructose released per min, and these values were found to be linearly proportional to the enzyme concentrations, although the line of best fit does not pass precisely through the origin (Fig. 2). Similarly, the assay of invertase activity in the u.v. range is linearly proportional to the amount of enzyme present (Fig. 3). Here, the values of the ordinate are derived from the changes in slopes of absorbance at 207 nm per min during the initial phase of the reaction.

Determination of Michaelis–Menten constants. — The u.v. method was applied to the determination of the K_m value for glucosyltransferase and for invertase at substrate concentrations of 100, 10, 8, 4, and 2mM sucrose. A K_m value for glucosyltransferase of 2.05 ± 0.03mM sucrose ($\pm$ standard error) was obtained, as compared with 2.00 ± 0.05mM sucrose for the Somogyi–Nelson method[5,6].



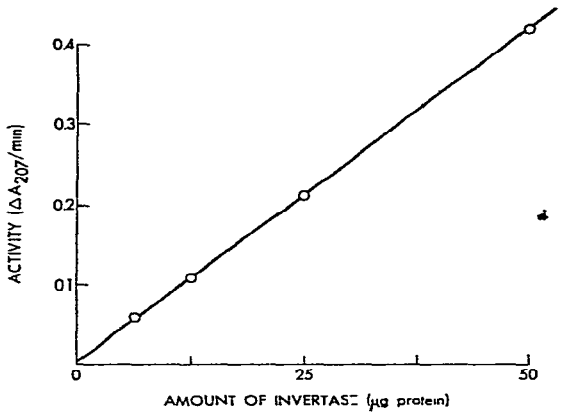


Fig. 2. Rate of glucosyltransferase (*Strep. sanguis* 804, Ip7.9) activity measured at 195 nm in phosphate buffer (pH 6 8, 0.1 mM) at 37° in presence of 0.125M sucrose. One ml of purified enzyme solution contains 383 μg of protein.

Fig. 3. Plot of amounts of invertase (μg protein/ml)*vs* activity, measured at 207 nm in acetate buffer (0.5mM, pH 4.6) at 37° in the presence of 0.125M sucrose.

Similarly, the K_m value for invertase of 13.5mM sucrose, determined by the u.v. method, is in agreement with values of 14.6 and 12.3mM sucrose obtained by polarimetric and colorimetric methods, respectively.

The method was extended for the determination of K_m values for the invertase–raffinose reaction. Comparable values of 176.5 and 163.7mM raffinose were obtained with the u.v. and polarimetric methods.

DISCUSSION

The method proposed here makes use of the absorption peaks of D-fructose (λ_{max} 188.5 nm, ε 133.6) or D-fructose and D-glucose (λ_{max} 186.0 nm, ε 123.0) in aqueous solution for the kinetic measurement of glucosyltransferase or invertase activity. In the far u.v. range, absorption interference by air is minimized by a continuous purging of the spectrophotometer with dry nitrogen. The hexoses exhibit two or three absorption peaks in the u.v. region (Table II).

The absorption characteristics of the sugars studied at extremely short wavelength, *i.e.* shorter than their apparent maximum, no longer appeared linear with concentration. The non-Gaussian nature of the peaks in the far u.v. region could be attributed either to the contributions by the vibrational modes or the lowered sensitivity of the detectors in the shorter region of wavelength. However, in all experiments described here, readings were taken at wavelengths where linearity with concentration was observed.

TABLE II

ULTRAVIOLET SPECTRA OF SOME HEXOSES IN WATER AND ETHANOL SOLUTION

Hexose	*Solvent*			
	Water		*Ethanol*[a]	
	λ_{max} (*nm*)	ε	λ_{max} (*nm*)	ε
D-Fructose	188.5	133.60	198	22.84
	278	1.12	284	0.76
D-Glucose	186.0	123.00	197.0	4.6
	228	3.40		
	280	1.04	282	0.90
D-Galactose	187.5	131.20	197.0	6 2
	228	4.00		
	278	1.68	282	0.94
D-Mannose[b]	187.5	131.20		
	225	2.40		
	278	1.52		

[a]The solutions (25mM) of D-fructose were prepared in 95% ethanol, and those of D-glucose and D-galactose in 90% ethanol because of differences in solubility. The bathochromic shift and hypochromic effect were apparent in a less polar solvent.[b] Very slightly soluble in ethanol.

The absorption peak in the far u.v. region was stronger and sharper than the other absorption peaks, which were weak and broad. Accordingly, in the latter case λ_{max} and ε could not be determined precisely, but they are in agreement with other reports. Avigad *et al.*[10] have reported absorption maxima at 275 and 280 nm for D-fructose and D-glucose, respectively, and have attributed this to the $n \rightarrow \pi^*$ transitions due to the carbonyl form of the sugars. We have observed a similar absorption with other sugars (Table II) capable of existing in a carbonyl (acyclic) form in solution.

In the study of u.v. absorption of D-fructose and some related compounds, such deoxyhexoses as 2-deoxy-D-galactose and 6-deoxy-L-galactose (L-fucose) were included to aid in the assignment of the oxygen atom responsible for the transition (Table III). Since both these compounds exhibited absorption peaks, absorption due to the oxygen atom at C-2 and C-6, and presumably also at C-3 and C-4, is ruled out. It is, therefore, likely that the glycosidic or the heterocyclic oxygen, because of its different linkage and location, is the source of the transition. This is supported by a comparison of the spectral data (Table III) of sugars with that of tetrahydrofuran and *p*-dioxane. All these compounds, in aqueous solution, have transitions around 188 nm. This compares with the spectral data reported for tetrahydrofuran in the vapor state where transitions at 172 and 187 nm were observed and for *p*-dioxane with a transition at 180 nm[11].

Both the $n \rightarrow \sigma^*$ and $n \rightarrow \pi^*$ transitions are known to exhibit a bathochromic shift in less polar solvents[12], as shown by the spectral data recorded for ethanol solutions (Table II) which indicate an oxygen $n \rightarrow \sigma^*$ transition. An $n \rightarrow \pi^*$ transition is

ruled out, since the transition occurs at comparatively longer wavelength, as pointed out by Avigad *et al.*[10].

TABLE III

EFFECT OF CARBONYL OXYGEN ON ABSORBANCE[a]

Compound	λ_{max} (*nm*)	ε
D-Fructose	188.5	133.6
1,3-Dihydroxy-2-propanone (Dihydroxyacetone)	192.5	136.0
Tetrahydrofuran	186.0	120.0
P-Dioxane	189.5	136.0
2-Deoxy-D-galactose	186.8	127.2
6-Deoxy-L-galactose	188.0	130.0
D-Glucitol	187.0	128.8
Inositol	185.5	110.4

[a]The molar absorbances were determined on 25 mM aqueous solutions at 37°.

Dilution of the sugar solutions in water exerted an apparent hypsochromic shift (Table IV). This effect was found for D-fructose at various dilutions and similarly for D-glucose and sucrose.

TABLE IV

ABSORBANCE OF D-FRUCTOSE SOLUTIONS AT VARIOUS CONCENTRATIONS[a]

Molarity (*mM*)	λ_{max} (*nm*)	ε
125	193.0	27.2
62.5	190 0	54.7
25	188.5	133.6
12.5	186.0	254.4
1	184.0	400.0

[a]Measured at 37° in aqueous solution.

Since the reactions involved in these studies do not effect the pH of the solution (both the reactants and products are neutral), a high buffering capacity and, therefore, a high ionic-strength is not essential. In order to minimize the hypochromic effect of the buffer and, thus, enhance the spectrophotometric sensitivity, a reasonably low ionic-strength was maintained.

In the u.v. method, wavelengths of 195 and 207 nm were selected for measuring the activity of glucosyltransferase and invertase, respectively. A wavelength different from the λ_{max} values of D-fructose (188.5 nm) and D-glucose (186.0 nm) was employed because the enzyme–substrate interaction shifted the absorption peaks of the reactants and products. Thus, under the reaction conditions, an optimal wavelength was selected at which change in absorption per min was maximal.

The kinetic data, in the case of glucosyltransferase, are expressed in terms of mmoles of D-fructose. The increase in absorbance observed is the difference between the absorbance due to the release of D-fructose and that of sucrose consumed in the course of the reaction. However, during the initial reaction, the increase in absorbance due to the release of D-fructose is so large that the decrease due to consumption of sucrose was ignored. The plot of glucosyltransferase activity *vs.* enzyme concentration does not pass through the origin (Fig. 2). This could be due to the inactivation or dissociation into subunits of the glucosyltransferase at low protein concentration. The absorption data on invertase could not conveniently be expressed in mmoles of the products, D-fructose and D-glucose, because both have their λ_{max} at different wavelengths.

The main advantages offered by the u.v. method over the chemical and polarimetric methods are: (a) The reaction rates can be measured continuously. Thus, initial reaction rates are conveniently determined. (b) Since the measurement of the reaction rate is based on the release of D-fructose, interference by other acceptor molecules (in studies with glucosyltransferase) having reducing groups, such as maltose, isomaltose, etc., is eliminated. (c) Finally, the method could be extended to enzyme systems in which hexoses appear as a product or disappear as a reactant.

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