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A SIMPLE AND RAPID DETERMINATION OF KETOSES BY CIRCULAR DICHROISM

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ABSTRACT

A simple and rapid determination of D-fructose is possible by circular dichroism measurement. The proportionality of the ellipticity to the concentration of D-fructose extends up to a concentration of 4.5M (81%, w/v). A constant value of the ellipticity was observed within 15 minutes after preparation of the solution. Such carbohydrates as aldoses, sucrose, and inulin, and several conventional inorganic salts, do not affect the determination. The ellipticity was found to depend on the temperature of measurement. This assay method was successfully applied to some reactions: *i.e.*, α-D-glucosidase-catalyzed hydrolysis of sucrose, glucose isomerase-catalyzed isomerization, and acid hydrolysis of inulin. The method was also found applicable to such other ketoses as D-tagatose, L-sorbose, and turanose.

INTRODUCTION

Avigad *et al*[1]. noted that D-fructose exhibits a dichroic band near 275 nm that is probably associated with the carbonyl absorption of the acyclic form of D-fructose. This suggested the possibility that ketoses might be quantitatively determined by circular dichroism measurement. We have examined the applicability of the phenomenon to the quantitative determination of D-fructose. Some reactions involving D-fructose as a reactant or product could be successfully monitored by this method.

EXPERIMENTAL

Materials. — D-Glucose was purchased from Wako Pure Chemical Ind., Ltd.; D-fructose and turanose (3-*O*-α-D-glucopyranosyl-D-fructose) from Sigma Co., Ltd.; sucrose, maltose, D-tagatose (D-*lyxo*-2-hexulose), and inulin from Nakarai Chemicals, Ltd. Before use, maltose and sucrose were purified by repeated recrystallization.

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Honey-bee α-D-glucosidase I (EC 3.2.1.20) was prepared as reported previously[2]. One unit of α-D-glucosidase was defined as the amount of enzyme that hydrolyzed 1 μmol of maltose per min at 35° in a reaction mixture containing 1 mL of a 0.5% solution of maltose, 1.45 mL of 0.1M sodium acetate buffer (pH 5.0), and 0.05 mL of the enzyme solution. Glucose isomerase (xylose isomerase, EC 5.3.1.15) was purchased from Nagase Ind. Co., Ltd. One unit of glucose isomerase was defined as the amount of enzyme that converted 1 μmol of D-fructose into D-glucose per min at 60° in a reaction mixture containing 1 mL of 2M D-fructose, 0.5 mL of 0.4M 1-(2-hydroxyethyl)-4-piperazine-(2-ethanesulfonic acid) (HEPES)–NaOH buffer (pH 7.0), 0.2M $CaCl_2$, and 0.5 mL of the enzyme solution.

Circular dichroism (c.d.). — C.d. spectra were obtained with a JASCO J-20 automatic recording spectropolarimeter, calibrated with androsterone[3] purchased from Tokyo Kasei Kogyo Co., Ltd. The measurements were conducted at temperatures of 20 to 23° for various times commencing no sooner than 15 min after preparation of the sample solution. The magnitudes of the observed ellipticity, $\theta \cdot (\text{cell length})^{-1}$, and molar ellipticity, $[\theta]$, are respectively given in units of deg.cm^{-1} and $\text{deg.cm}^2\text{.dmol}^{-1}$.

High performance liquid chromatography (h.p.l.c.). — Shodex Ionopak S-801, a column (0.8 × 30 cm) packed with a cation-exchange resin of the sodium form of sulfonated polystyrene cross-linked with divinylbenzene, Showa Denko Ind. Co., was incubated at 60°, and a Refractomonitor III (Milton Roy Co.) was used for the detector. The sample was eluted from the column with de-ionized water at a flow rate of 1.0 mL.min^{-1}. Sulfosalicyclic acid was used as the internal standard for determination of D-fructose.

Enzymic reaction. — *(a) Honey-bee α-D-glucosidase I.* A reaction mixture consisting of 20 mL of M sucrose, 19 mL of 0.1M sodium acetate buffer (pH 5.0), and 1 mL of the enzyme solution (12.8 U) was incubated at 35°. Aliquots (4 mL) were taken from the reaction mixture at appropriate intervals, and placed in a boiling-water bath for 3 min for termination of the reaction. Concentrations of D-fructose were determined by c.d. measurement and by h.p.l.c.

(b) Glucose isomerase. To 16 mL of 2M D-fructose (or D-glucose) and 8 mL of 0.4M HEPES–NaOH buffer (pH 7.0) was added 8 mL of the enzyme solution (59.0 U) containing 0.2M $CaCl_2$ at 60°. Aliquots (4 mL) were taken from the reaction mixture, and placed in a boiling-water bath for 3 min. D-Fructose and D-glucose were respectively determined by c.d. measurement and by the Tris–glucose oxidase–peroxidase method[4,5] (Wako Pure Chemical Ind., Ltd., Glucose AR-Test).

Acid hydrolysis of inulin. — Inulin solution (10%; 20 mL) was mixed with 20 mL of 0.02M HCl at 100°. Aliquots (4 mL) were taken from the reaction mixture at intervals. A solution (0.5 mL) of 0.08M NaOH containing 0.9M sodium acetate buffer (pH 5.0) was used for termination of the reaction, and the mixture was rapidly cooled. Measurement of the reducing power of the solution was carried out according to the Somogyi–Nelson method[6,7].

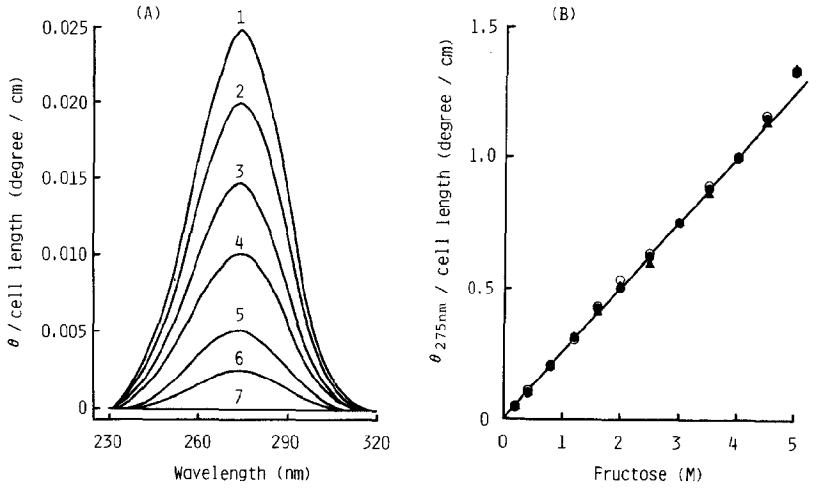

Fig. 1. C.d. spectra (A) and calibration curves (B) of D-fructose. In (A), the numbers 1–7 respectively indicate concentrations of D-fructose of 100, 80, 60, 40, 20, 10, and 0 mM. In (B), the observed ellipticity of D-fructose at 275 nm was measured in H_2O (●), 0.1M sodium acetate buffer (pH 5.0, ○), and 0.1M sodium phosphate buffer (pH 7.0, ▲).

RESULTS AND DISCUSSION

Determination of the concentration of D-*fructose.* — The c.d. spectrum of D-fructose shows a maximum ellipticity at ~275 nm, reportedly due to the n-π^* transition of the carbonyl group of acyclic D-fructose[1]. In Figs. 1A and 1B, the observed ellipticity of D-fructose at 275 nm is shown to be proportional to the concentration of D-fructose. The proportionality was observed for a wide range of concentration from 0.010 to 4.5M (81%, w/v). Thus, this method seemed to be effective for the determination of D-fructose at rather high concentrations. The same results were obtained with different solvents, *i.e.*, water, 0.1M sodium acetate buffer (pH 5.0), and 0.1M sodium phosphate buffer (pH 7.0).

Various factors influencing the molar ellipticity of D-*fructose. — (a) Effect of pH.* Dependence of the molar ellipticity of D-fructose on pH at values <7 was not observed in buffers of either 0.1M sodium acetate (pH 4.0 to 5.9) or 0.1M sodium phosphate (pH 5.4 to 7.0). Fig. 2A shows the time dependence of the molar ellipticity after solid D-fructose was dissolved in 0.1M sodium acetate buffer of various pH values. It was observed that, in the lower pH range, it takes more time to reach a constant value. The same phenomenon was observed in 0.1M sodium phosphate buffer. The equilibration rate between cyclic and acyclic forms of D-fructose is probably low when the pH is low. This situation is summarized, in terms of the dependence, upon pH, of the time required to reach a constant value, in Fig. 2B. Judging from these observations, in the pH range observed, it is desirable to measure the ellipticity at times more than 15 min after dissolution of the D-fructose

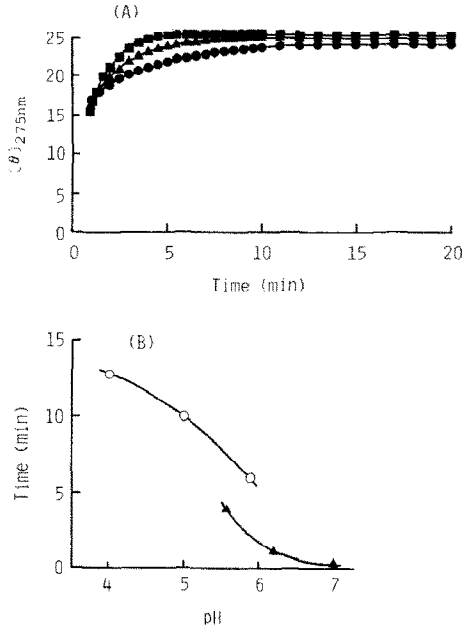

Fig. 2. Effect of pH on molar ellipticity of D-fructose at 275 nm. (A) Time dependence of molar ellipticity after dissolution of solid D-fructose in 0.1M sodium acetate buffer (pH 5.9, ■; pH 5.0, ▲; pH 4.0, ●). (B) Dependence on pH of the time required to reach a constant value of molar ellipticity in 0.1M sodium acetate buffer (○) and sodium phosphate buffer (▲).

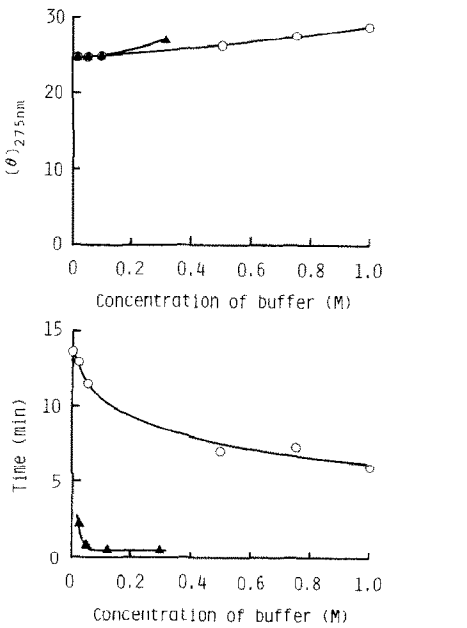

Fig. 3. Effect of buffer concentration on molar ellipticity of D-fructose at 275 nm. (A) Molar ellipticity in sodium acetate buffer (pH 5.0, ○) and sodium phosphate buffer (pH 7.0, ▲). (B) Dependence of the time required to reach a constant value of molar ellipticity on concentration of sodium acetate buffer (pH 5.0, ○) and sodium phosphate buffer (pH 7.0, ▲).

at room temperature. The time lag seemed to be shorter in the phosphate buffer than in the acetate buffer.

(b) Effect of the concentration of the buffer solution. Fig. 3A shows that the molar ellipticity of D-fructose increases slightly with increase in buffer concentration. However, the effect of buffer concentration is negligible when buffer concentrations <0.1M are used, as in the case of the enzyme reactions. The time lag of the molar ellipticity after the dissolution of D-fructose was found to be influenced by buffer concentration. It was observed that, at low concentration, it took more time to reach a constant value. However, the equilibrium was attained within 15 min in any concentration of the acetate buffer, phosphate buffer, or water (see Fig. 3B). The buffer concentration probably increases the rate of equilibration between cyclic and acyclic D-fructose.

(c) Effect of various carbohydrates and salts. The effect of various carbohydrates and salts on the molar ellipticity of D-fructose was examined. As summarized in Table I, the carbohydrates examined do not show any effect at the concentration used, which means that the concentration of D-fructose can be determined specifically in the presence of these carbohydrates. Among the salts examined, ammonium, magnesium, and manganese chlorides showed no effect, whereas sodium, potassium, calcium, and zinc chlorides caused a slight increase (~10% or less) in the ellipticity.

(d) Effect of temperature. The molar ellipticity of D-fructose was measured at various temperatures. The magnitude of the molar ellipticity of D-fructose at 275 nm, $[\theta]_{275}$, increased from 25.0 to 32.4 with an increase in temperature from 22.5 to

TABLE I

EFFECT OF CARBOHYDRATES AND SALTS ON THE MOLAR ELLIPTICITY OF D-FRUCTOSE AT 275 nm

Additives	*Relative* $[\theta]_{275}$ *(%)*		
	H_2O	*0.1M Sodium acetate buffer (pH 5.0)*	*0.1M Sodium phosphate buffer (pH 7.0)*
None	100	100	100
M D-Glucose	100	100	100
M D-Galactose	103	100	100
M D-Mannose	97.3	98.3	98.4
M Sucrose	102	103	102
M Maltose	103	100	102
5% Inulin	101	98.6	99.0
0.3M NH_4Cl	100		
0.3M NaCl	108		
0.3M KCl	107		
0.3M $CaCl_2$	113		
0.3M $MgCl_2$	103		
0.3M $MnCl_2$	101		
0.3M $ZnCl_2$		106	

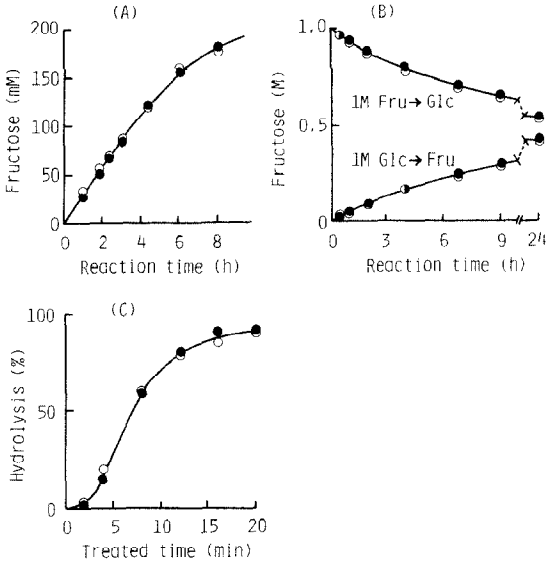

Fig. 4. Applications of the c.d. method. (A) α-D-Glucosidase-catalyzed hydrolysis of sucrose. [D-Fructose was determined by the c.d. method (●) and by h.p.l.c. (○).] (B) Glucose isomerase-catalyzed isomerizations. [The concentration of D-fructose was measured by the c.d. method (●), and calculated from that of D-glucose assayed by the Tris–glucose oxidase–peroxidase method (○).] (C) Acid hydrolysis of 5% solution of inulin. [The products from inulin were examined by the c.d. method (●) and the Somogyi–Nelson method (○).]

27.5°. The increase in molar ellipticity at high temperature is probably due to increase in the carbonyl form of D-fructose. From the data in Figs. 1A and 1B, $[\theta]_{275}$ of D-fructose was calculated to be 25.0 at 20–23°. Avigad *et al.*[1] reported that $[\theta]_{275}$ of D-fructose is 33.0 at 25–27°. Our result does not contradict theirs because the temperatures of the measurements differed. Clearly, however, it is important to specify the temperature of the measurement when molar ellipticity data are presented.

Application of the c.d. method. — (a) α-D-Glucosidase-catalyzed hydrolysis of sucrose. Because D-glucose and sucrose do not affect the determination of D-fructose, the present method allows the determination of D-fructose in their presence. Thus, the hydrolysis of sucrose with honey-bee α-D-glucosidase I[2] to D-fructose and D-glucose can be monitored by the c.d. method. For comparison, the reaction was also examined by the h.p.l.c. method. As shown in Fig. 4A, the two methods give comparable results.

(b) Glucose isomerase-catalyzed isomerization. The reactions of glucose isomerase were checked by the present method and by the Tris–glucose oxidase–peroxidase method[4,5] for comparison. In the latter method, the concentration of D-fructose was calculated indirectly from the D-glucose assay. As for assaying the conversion of D-fructose into D-glucose, and *vice versa*, measurements by the two methods gave concordant results (see Fig. 4B).

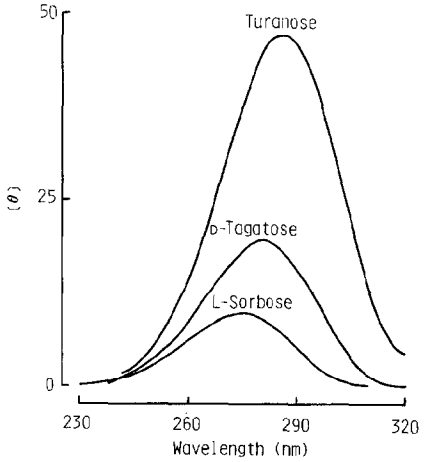

Fig. 5. C.d. spectra of D-tagatose, L-sorbose, and turanose. C.d. spectra were measured in 0.1M sodium acetate buffer (pH 5.0) and 0.1M sodium phosphate buffer (pH 7.0).

(c) Acid hydrolysis of inulin. The hydrolysis of inulin could be monitored by c.d. measurement, because the reducing-end units of D-fructo-oligosaccharides produced during hydrolysis could be assumed to be an acyclic form. The hydrolysis of inulin was conducted with M hydrochloric acid at 100°, and the products were examined by the c.d. method and by the Somogyi–Nelson method[6,7]. The results are presented in terms of the percentage of inulin hydrolyzed (see Fig. 4C). As may be seen from Fig. 4C, the values obtained by the two methods agree well.

Determination of other ketoses. — Fig. 5 shows the c.d. spectra of D-tagatose, L-sorbose, and turanose. The ellipticity maxima range[1] from 270 to 290 nm. As in the case of D-fructose, the observed ellipticity of these ketoses was found to be proportional to their concentration, making quantitative determination of these ketoses possible by the c.d. method. In principle, this simple and rapid method could be applied to other ketoses.

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