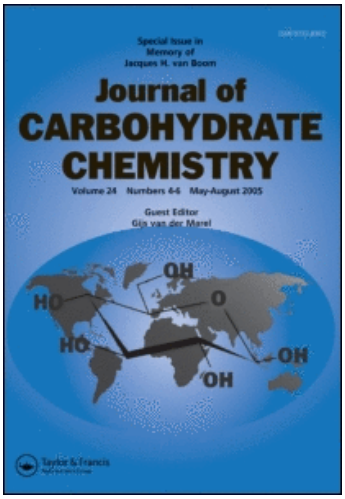

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The Mutarotation of Fructose and the Invertase Hydrolysis of Sucrose

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THE MUTAROTATION OF FRUCTOSE

AND

THE INVERTASE HYDROLYSIS OF SUCROSE

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ABSTRACT

Studies by GLC and GLC/MS of the mutarotation of fructose in water have been made to determine equilibrium composition as a function of temperature. The major components are β-fructopyranose, β-fructofuranose and α-fructofuranose in agreement with studies in the literature. There are small amounts of the α-fructopyranose and the open-chain ketone form. The major change during mutarotation is the ring size change for β-fructopyranose $\rightleftharpoons$ β-fructofuranose, but other changes contribute. For this reason, polarimetric rate studies of this system as a simple first-order equilibrium process are not valid. Hydrolysis of sucrose catalyzed by invertase has been accomplished to the extent of 99% in 1 min. The mutarotation of glucose and fructose were then studied without the complication of further production of the products from sucrose. The enzymatic cleavage is stereospecific to provide retained configuration in α-glucopyranose and β-fructofuranose which each mutarotate to equilibrium. The mutarotation lag was demonstrated and its importance to sucrose hydrolysis discussed.

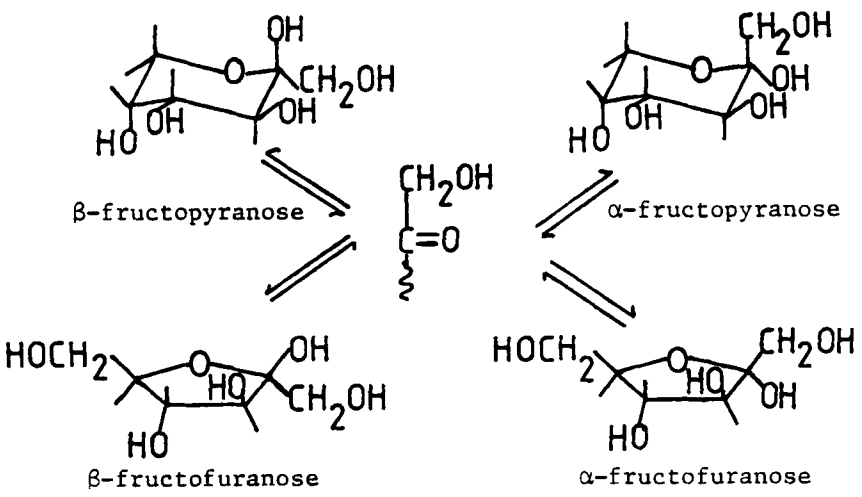
INTRODUCTION

The hydrolysis of sucrose has been of central importance in the development of the subject of chemical kinetics with active work on the subject from 1854 up to the present time.[4,5,9] In

spite of these extensive studies, there is still substantial interest in this system because of unresolved questions such as those related to reaction mechanisms[6] and kinetic salt effects.[7,8] Recent studies from our laboratory resolved the long standing question about the reported change in the Arrhenius energy of activation (Ea) with temperature.[4,9,10] The value of Ea for the hydrolysis of sucrose does not change with temperature over the range of 10 to 40 °C and probably not from 0 to 60 °C or higher. We presented evidence which explained that previously reported dEa/dt as not being zero was due to the mutarotation lag for polarimetric measurements. The mutarotation lag is caused by the rapid mutarotation of fructose and its large net levoratory contribution and the slower mutarotation of glucose and its modest dextrorotatory contribution to the observed polarimetric readings with time. Thus polarimetric measurements, in contrast to several other methods, are not proportional to the amount of sucrose changed with time.

The mutarotation of glucose has been studied in substantial detail, and the mechanism for that relatively simple process is well understood. On the other hand the more complex process of fructose mutarotation is not as well understood, and, in spite of the many studies on this system, the definitive kinetic study has yet to be made.[11,12] Spectroscopic studies of the past fifteen years, particularly by ^{13}C NMR,[13] by Raman,[14] and by infrared[15] and chromatographic studies by GLC[11,16] and by GC/MS[16,17] have cleared up a number of questions as fundamental as which of the tautomers of fructose exist in solution and approximately how much of each is present. There are three major tautomeric forms, α-fructofuranose, β-fructofuranose and β-fructopyranose, and two minor forms, α-fructopyranose and the acylic keto isomer. It is this last form that all of the other forms are presumed to pass through on mutarotation. The overall equilibrium system for fructose mutarotation is shown in Scheme I.

The β-fructopyranose is the normal crystalline form of fructose, and it is the only tautomer which has been isolated.[11,18] The non-availability of the other tautomeric



SCHEME I

forms, except in derivative form, has caused considerable difficulty in attempts to interpret the data for both kinetic and equilibrium studies of this system. This lack creates a special problem for the polarimetric studies since there are five tautomers and the specific rotation for the β-fructopyranose is the only one known with assurance.[19] The specific rotations for the α-fructofuranose and the β-fructofuranose have been estimated indirectly but without much assurance of their reliability.[20] The specific rotations for the α-fructofuranose and the acyclic ketone form have not been estimated. Both the α- and β-fructofuranoses have been isolated as their glycosidic ethers, but Shallenberger has demonstrated that one cannot use the specific rotations of these ethers to estimate the rotations for the free sugars.[19]

The reverse of the mutarotation of the β-fructopyranose has been studied by following the mutarotation of β-fructofuranose. The β-fructofuranose was produced in solution by the rapid enzymatic hydrolysis of both methyl β-fructofuranoside[19] and of sucrose.[19,21,22] The early studies by Hudson[21] and the more recent studies by Andersen and Degn,[22] by Shallenberger,[19] by Hyvönen[23,24] among others [13,14,15] all are in agreement about the

existence of several tautomeric forms for fructose in solution. There is, however, disagreement about the detectability and the amounts of these five tautomers. The results for the distribution of fructose tautomers in water are summarized in Table 1. As is readily apparent all workers agree that the two major species for mutarotated fructose are β-fructofuranose and β-fructopyranose, with the latter being the most stable of the two.

The amounts of each of the other three reportedly vary from none to a few percent.[11,13,16,17] There is one report claiming as much as 20% of the acyclic ketone tautomer, but this result is surely in error.[16] There is also some question about the initial products other than glucose and fructose formed as a trisaccharide during the enzymatic hydrolysis of sucrose. Most workers consider these two products to be the only ones formed at all stages of the reaction. However, Bacon and Edelman, and Blanchard and Albon have demonstrated that a trisaccharide is formed when invertase is used to catalyze the hydrolysis of sucrose.[25] If a trisaccharide is formed in other than trace amounts, its presence would adversely influence measurements made for the mutarotation of glucose and fructose. For these reasons, as well as to better understand the sucrose hydrolysis process, we have used a combination of gas chromatography and gas chromatography-mass spectrometry to reinvestigate the mutarotation of fructose and the enzymatic hydrolysis of sucrose.

RESULTS AND DISCUSSION

Tautomer Identification - A problem associated with most of the past studies of the analyses of fructose tautomers has been the failure to obtain baseline separation of silylated forms in GC/MS analyses which causes uncertainty in product identification. This lack of resolution also contributes to uncertainty in the results for the composition of fructose tautomers in solution because most workers have reported only two or three major components (Table 1). The α-fructopyranose and the open-chain form of fructose usually are not reported, which means they were either not present in detectable amounts or the analytical procedure failed to distinguish them from the major components.

TABLE 1. Composition of Mutarotated Fructose in Water by various Workers

	Temp. (°C)	α-Fructo-Furanose	β-Fructo-Furanose	α-Fructo-Pyranose	β-Fructo-Pyranose	Open-Chain	Method
1. Hyvönen[24]	22		31		69		Polarimetry[24]
2. Hyvönen[16]	22		31		69	<20%>	GLC[16]
3. Hyvönen[23]	22	6	21		73		^{13}C NMR[23]
4. Andersen & Degen[22]	25		32		68		Polarimetry[22]
5. Angyal & Bethell[13d]	27	4	21	Trace	75		^{13}C NMR[13d]
6. Que & Gray[13c]	30	5	23	Trace	72		^{13}C NMR[13c]
7. Shallenberger[11]	30	6	22		72		GLC[11]
8. Doddrell & Allerhand[13a]	36	9	31	3	57		^{13}C NMR[13a]
9. Funcke et al.[13e]	80	10	32	2	53	3	^{13}C NMR[13e]

Most of the GLC and GC/MS studies of aqueous solutions of sugars have used the silylation procedure of Bently and Botlock[27] which has been amply validated. Our initial attempts to use this procedure provided inconsistent results due to an error of technique on our part. Before we discovered our error, we resorted to a number of variations in the silylation procedure. The most reliable procedure was a very fast vacuum dehydration of a 10-mL sample of the fructose solution. This dehydration freezes the equilibrium concentrations into a glass, and this residue can be silylated with trimethylsilylimidazole and trimethylchlorosilane in pyridine in a 2:1:10 ratio. We applied this procedure in all of our GLC and GC/MS studies, including the identification of the silylated tautomers. A typical chromatograph (FID) is shown in Figure 1 for the Hewlett-Packard gas chromatographs. The peaks are identified in sequence of appearance as the silylated tautomers of (a) α-fructofuranose, (b) β-fructofuranose, (c) β-fructopyranose, (d) α-fructopyranose, and (e) the open-chain form of fructose. The major peak (c) was identified as β-fructopyranose by the silylation of crystalline fructose. The second largest peak (b) is that of β-fructofuranose which we obtained by the silylation of a solution of sucrose hydrolyzed to the extent of 99% in one minute by invertase.

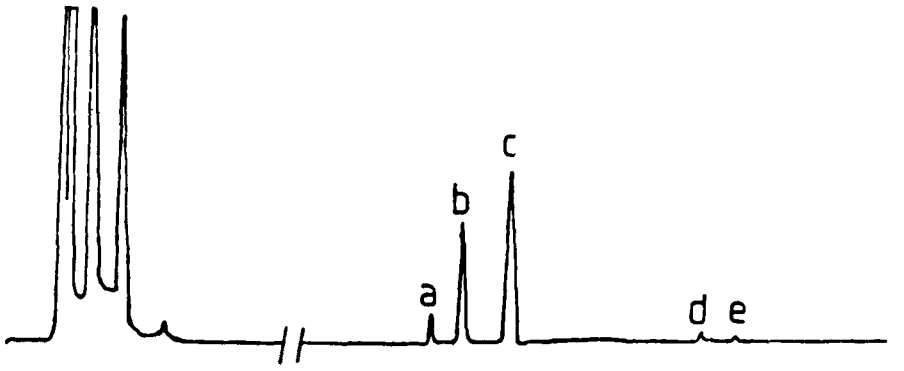


Fig. 1: Gas chromatogram for mutarotated fructose, 25 °C, pH 4.4; (a) α-fructofuranose; (b) β-fructofuranose; (c) β-fructopyranose; (d) α-fructopyranose; (e) open-chain keto form of fructose.

The hydrolysis of sucrose by invertase also provided the silylated α-glucopyranose which we had independently identified by using the known sugar.

Curtis[17] has shown that the silylated furanohexoses provide a large 217 mass fragment and a small 205 mass fragment, whereas silylated pyranohexoses show a large 205 mass fragment and a small 217 mass fragment. The mass spectral results for these five silylated tautomers are shown in Table 2. As may be readily seen, the Curtius rule applies to our results leading to the identity of all five tautomers. The number 5 peak is the open-chain compound as shown by essentially equivalent intensities of the 147, 205, 217, 306 and 319 peaks. We also note that the silylated fructose tautomers show a significant 437 peak and that this peak is smaller for the furanose forms (20% and 17%) than those for the pyranose forms (48% and 28%). The ion chromatograms were scanned for mass homogeneity, and each of the five peaks were found to be a single component.

Mutarotation of Fructose - With identification having been made of all of the fructose tautomers, we studied the effect of temperature on the equilibrium composition and on the optical rotation of fructose in water. It is well established that glucose shows only a small compositional change for mutarotation

TABLE 2. Mass Spectral Data for Silylated Fructose forms

Compound	73	147	205	217	306	319	437
α-Fructo-furanose[a]	100	25	-	64	-	-	20
β-Fructo-furanose[a]	100	25	-	72	-	-	17
α-Fructo-pyranose[a]	100	44	108	35	-	12	48
β-Fructo-pyranose[b]	100	30	98	25	-	6	28
Open-chains[b]	100	33	31	30	43	19	1

[a] 50 m Capillary

[b] 25 m Capillary

with temperature (i.e., $\Delta H \sim 0$ for mutarotation), and as a consequence the change in the specific rotation is slight as the temperature is changed.[28] In contrast to glucose, fructose shows a large change in the specific rotation with temperature and becomes more positive by 0.6° for each 1 °C increase in the temperature (589 nm). Shallenberger has studied this change for fructose over the temperature range of 20 to 90 °C by gas chromatography and found that the large rotational change with temperature was due to the mutarotation composition change.[20] The major change was ascribed to the increase in the concentrations of the fructofuranoses at the expense of the fructopyranoses as the temperature is increased. We would note that this temperature effect on the optical rotation of mutarotated fructose causes an added complication for the measurement of the energy of activation for sucrose.[9] This complication occurs because the fructose mutarotates to equilibrium rapidly, but the equilibrium optical rotation decreases markedly as the temperature is raised. On the

other hand, glucose only slowly mutarotates to equilibrium thus causing the mutarotation lag, but its equilibrium optical rotation changes only very slightly with temperature.

We repeated the study of Shallenberger, because he found no evidence for the α-fructopyranose or the open-chain compound, while others had reported the measure of both of these compounds, and Hyvonen reported up to 20% of the open-chain compound.[16,29] Our studies were made with a 0.50 M fructose solution of KHP (pH 4.4) over the temperature range of 10 to 55 °C. The results are summarized in Table 3.

As is readily seen from these results, the amounts of the α-fructopyranose and the open-chain form are small, both totaling about 1% and within experimental error, remained at constant concentration over the entire temperature range. These small amounts do not interfere with conclusions based upon analyses for the three major tautomers and validate those results found by Shallenberger.[20] These results are also very close to those reported by Schneider et al. who used ^{13}C NMR to study the fructose tautomers (excepting the open-chain form) in water from 0 to 80 °C.[13g]

The major transformation for the fructose thermal mutarotation is the decrease in the amount of the β-fructopyranose and the increase in the amounts of the two furanose forms with an increase in temperature. The ratio of the β-fructofuranose to the α-fructofuranose was found to remain essentially constant at about 4:1 over the temperature range of 10 to 55 °C although the amounts of both were rapidly increasing. In contrast, Shallenberger found that this ratio decreased from 4.8:1 at 20 °C to 1.87 at 90 °C. Obviously these different composition ratios with temperatures are reflected in the different slope and intercept values of the linear expressions and we offer no reason for the descrepancy. The slope and intercept terms for the specific rotation - temperature equations for our results and Shallenberger's results are remarkably close when account is taken for the constant ratio of $[\alpha]_{578}/[\alpha]_{589} = 1.040$ for measurements for fructose, glucose and sucrose and at various temperatures.

TABLE 3. Mole Per Cent for Fructose Tautomers Equilibrated in Water with Temperature Change

Temperature	α-Fructofuranose	β-Fructofuranose	β-Fructopyranose	α-Fructopyranose	Open-Chain
10 °C	3.67	19.39	76.31	0.41	0.22
15 °C	5.67	20.45	72.71	0.82	0.35
25 °C	5.49	22.25	71.38	0.52	0.36
35 °C	5.73	23.65	69.36	0.96	0.30
45 °C	5.86	24.54	68.83	0.55	0.22
55 °C	6.88	25.84	66.43	0.49	0.36

Mutarotation Rates - When β-fructopyranose is dissolved in aqueous buffer (KHP, pH 4.4) at room temperature there is a rapid change in the tautomer composition for about 10 to 12 minutes, followed by a much slower change over the next 10 to 15 minutes to the equilibrium composition. The results for a typical experiment for composition changes with time are shown in Table 4.

This experiment was done in triplicate to provide composition-time data. For the change β-fructopyranose $\underset{k_2}{\overset{k_1}{\rightleftharpoons}}$ β-fructofuranose, one may use the equilibrium rate expression $k_1 + k_2 = 1/t \ln (X_e/X_e-X)$, where k_1 and k_2 are the forward and reverse constants, X_e is the equilibrium concentration of the fructofuranose, and X is its concentration at any time in t seconds. This usage assumes that all of the changes are for β-pyranose - β-furanose interconversion, a fact which is not strictly true. However, the concentration of these two tautomers totals to 95% or more so that the results are correct to a first approximation. For these three mutarotation experiments with GLC analyses, we calculate by linear least squares an average $k_1 + k_2 = 5.08 \times 10^{-3}$ sec^{-1} which were reasonably well correlated (r = 0.95, 0.999 and 0.99). We have also determined the mutarotation rate constants for the reverse process of β-fructofuranose $\underset{k_1}{\overset{k_2}{\rightleftharpoons}}$

TABLE 4. Mutarotation of Fructose in Aqueous KHP (pH 4.4) at 25 °C[a]

Time, Mins.	α-Fructo-furanose, %	β-Fructo-furanose, %	β-Fructo-pyranose, %	α-Fructo-pyranose, %	Open-Chain
0.5	1.49	5.86	91.27	0.34	1.04
2.5	2.74	11.86	85.22	0.29	0.08
4.0	3.23	14.79	81.54	0.37	0.07
5.0	4.53	17.28	77.49	0.67	0.03
6.0	4.65	18.02	76.66	0.65	0.03
7.0	4.44	19.17	75.87	0.48	0.15
8.0	4.07	20.01	75.60	0.29	0.02
9.0	4.78	20.86	72.81	0.40	0.15
11.0	4.92	22.13	72.15	0.59	0.21
12.0	5.00	21.90	71.99	0.91	0.19
15.0	5.11	22.40	72.40	-	-
20.0	5.08	22.30	71.97	-	-
Equilibrium (24 hrs)	5.15	22.37	72.30	0.28	0.66

[a] All analyses were made of silylated samples on the Varian GC.

β-fructopyranose. We were able to do this experiment by using the Hudson techniques of rapidly hydrolyzing sucrose by invertase.[21] Inasmuch as the hydrolysis was 99% complete in one minute, we could follow the mutarotation after that time without the complication of further production of fructose (and glucose) by hydrolysis. For two experiments we obtained $k_1 + k_2 = 4.45 \times 10^{-3}$ sec ($r = 0.98$ and 0.99) thus giving good duplication of the values obtained for the reaction in both directions. These values are somewhat different from the consistent values obtained by Hudson[21], by Purves and Hudson,[30] but which are calculated by Pigmann and Isbell,[31] and by Bak and Andersen.[32] The values calculated by Pigmann and Isbell were for the 0 °C and 20 °C data

of Purves and Hudson and are calculated on log base 10 in min^{-1}. Using E_a = 14,900 cal mol^{-1} we calculate $k_1 + k_2 = 3.24 \times 10^{-3}$ sec^{-1} for Pigmann and Isbell's values at 25 °C and pH 4.6. This agreement with our values to within a factor of 2 is reasonable since all of the above workers used polarimetric measurements which include changes for all five optically active species, whereas our values were calculated for only two species measured by GLC.

It appears to us that at 25 °C the rate of fructose mutarotation is predominately a β-pyranose $\rightleftharpoons$ β-furanose interconversion for the first phase since these two species constitute 95% of the observed change. After the first and rapid phase, the concentration adjustments are small and are probably due to the α-β interconversions for the two ring forms. In following the mutarotation by GLC analysis the process is essentially over in 12-15 minutes, but for the same process followed polarimetrically, changes are easily discernable after 30 minutes. In any case one cannot truly treat this system as a simple first-order process. It is possible that by the procedure used by Anderson for galactose that one could unravel all of the rate constants for the competing processes.[33] At this point we do not believe that the available rate data are of sufficient quality to merit the calculation. It is also obvious that the measurement of a single physical property such as optical rotation or density cannot be used as an adequate probe to determine the kinetics for the mutarotation of fructose. The definitive kinetic study for the mutarotation of fructose remains to be made.

Hydrolysis of Sucrose by Invertase - Previous workers who have used the Hudson procedure of the rapid hydrolysis of sucrose catalyzed by invertase have found that the sucrose was incompletely hydrolyzed in the first few min. Yet their interests were in following the subsequent processes after 100% hydrolysis. For example Hudson[21] estimated he had obtained 52% hydrolysis of sucrose after 4 min, Purves and Hudson[30] had less than 90% hydrolysis in three min and Shallenberger[19] obtained only 50% hydrolysis in 3 min. Clearly this fact of incomplete sucrose

hydrolysis is a serious complication for the study of the mutarotation of glucose and particularly of fructose. If the sucrose reaction is 50% complete in 3 min, it is only 75% complete in 6 min, and yet in this same time at 25 °C, the mutarotation of fructose is about 50% complete and both reactions are continuing. Even if the data provide straight line plots, they cannot even approximately represent the first-order change of β-furanose $\rightleftharpoons$ β-pyranose since the β-furanose is being produced in significant amounts while disappearing to the mutarotational equilibrium. In contrast to the results of these other workers, Andersen and Degn[22] were able to obtain 99% hydrolysis of sucrose in 3 min at 25 °C with a refined invertase. From polarimetric measurements they were able to calculate the rate constants for both fructose and glucose mutarotations.

We have been able to improve on this invertase hydrolysis rate by using an increase in the concentration of invertase (2 mg invertase, 65 mg sucrose in 0.40 mL KHP solution at 25 °C. In this way the sucrose was 99% hydrolyzed in 1 min, and none was detectable in 1.5 min. A typical set of chromatograms for this system is shown in Figure 2 for 1, 6, and 75 min with the peaks identified for the 1-min run.

In a duplicate experiment for which a sample was taken in 0.5 min, the amounts of the α-fructofuranose, the α-fructopyranose and of the β-glucopyranose were less than for the one-minute sample, and the relative amounts of the β-fructofuranose, the α-glucopyranose and the sucrose (98% hydrolyzed) were greater. Clearly these early analytical results with nearly 100% hydrolysis permit the following conclusions: (1) fructose is cleaved with retention of configuration from sucrose to provide β-fructofuranose which mutarotates at equilibrium to the five-component mixture, (2) glucose is cleaved with retention of configuration to provide α-glucopyranose which mutarotates to the two-component mixture, and (3) the hydrolysis reaction is essentially 100% complete after 1 min of reaction so that changes after that time are due to the mutarotation of fructose and of glucose.

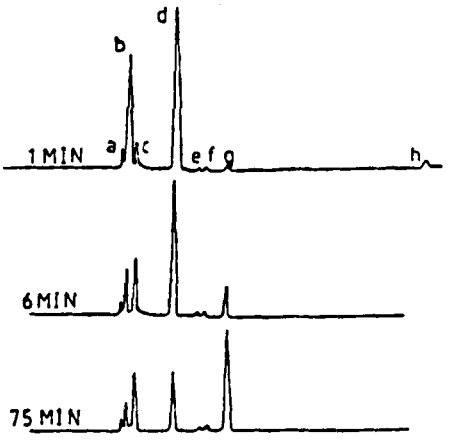


Fig. 2: Gas chromatograms of mutarotating fructose and glucose from invertase treatment of sucrose, 25 °C. pH 4.4.
a. α-fructofuranose; b. β-fructofuranose; c. β-fructopyranose; d. α-glucopyranose; e. α-fructopyranose; f. Open-chain keto form of fructose; g. β-glucopyranose; h. sucrose.

For these experiments samples were removed each min for the first 15 min and less frequently to 75 min and analyzed by GLC. The results are plotted in Figure 3 for the four major components. The α-fructopyranose increases in direct relationship to the disappearance of the β-fructofuranose, and that process is complete in 10 to 12 min. Similarly, the β-glucopyranose increases directly as the α-glucopyranose disappears, but this process is not complete after 75 min. Not only does this plot represent the mutarotation reactions with time for both fructose and glucose, but in addition demonstrates vividly the mutarotation lag for sucrose hydrolysis.

When the 25 °C data for the change with time for α-glucose $\underset{k_2}{\overset{k_1}{\rightleftharpoons}}$ β-glucose were treated by linear least squares as a first-order equilibrium rate process, the values of $k_1 + k_2 = 5.12 \times 10^{-4}$ sec^{-1} ($r = 0.997$) and 5.09×10^{-4} sec^{-1} ($r = 0.996$). These values compare quite favorably with the value of Isbell and Pigmann[31] of $k_1 + k_2 = 4.07 \times 10^{-4}$ sec^{-1} at 25 °C and pH 4.6 (our calculation using their 20 °C data and $E_a = 17.0$ Kcal mole^{-1}) and

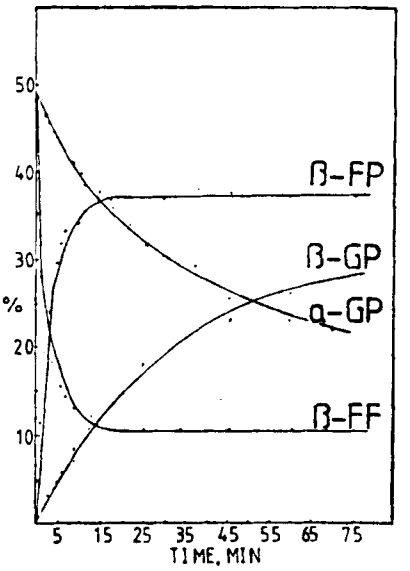


Fig. 3: Mutarotation composition vs. time for the rapid hydrolysis of sucrose by invertase; 25°C; pH 4.4; GLC analysis.

the value of Bak and Andersen[32] of $k_1 + k_2 = 4.13 \times 10^{-4}$ sec^{-1}. Treatment of the fructose mutarotation rates obtained from the rapid hydrolysis of sucrose were discussed earlier in comparison to the reverse mutarotation for crystalline fructose.

Even though it has been shown that sucrose hydrolysis undergoes a transglycosylation process to form a trisaccharide on catalysis by invertase,[25] that fact does not change our conclusions. By whatever processes the fructose and glucose moieties are cleaved from sucrose, when they first appear in the gas chromatogram it is as the β-fructofuranose and the α-glucopyranose, respectively. If higher saccharides are formed, we were unable to find them by GLC. No persilylated species of higher molecular weight than sucrose were found by GLC at up to 2.5 h elution time at 270 °C (sucrose is eluted in 41.5 min).

It is unfortunate that the method used for the invertase process cannot be applied to the acid-catalyzed hydrolysis of sucrose. When gas chromatographic studies were made for sucrose

hydrolysis catalyzed by 0.5M HCl, all of the fructose was found to be completely mutarotated from 1 min reaction time on, although the glucose mutarotation had not reached equilibrium. The reasons for this fact is that, in order to have a reasonable rate of hydrolysis of the sucrose, the concentration of the HCl needs to be at least 0.1M to 0.5M. Under these conditions the rate of mutarotation of fructose is fast and, in fact, is faster than the hydrolysis of sucrose, and thus the former reaction cannot be observed. Using the data of Isbell and Pigmann[31] for the rate of mutarotation of fructose and glucose as a function of acid concentration, one calculates $t_{1/2}$ = 0.0812 min for fructose mutarotation and $t_{1/2}$ = 3.36 min for glucose mutarotation in 0.5M HCl. This half-life ratio of 1/41 is further verification of the mutarotation lag for sucrose hydrolysis even under strongly acidic conditions. It is quite reasonable to expect that the mechanism for sucrose hydrolysis by acid-catalysis involves protonation of the sucrose. Cleavage then must occur between the protonated oxygen joining the rings and the fructosyl carbon leading to α-glucopyranose and the fructosyl carbocation. The α-glucopyranose then mutarotates slowly and the fructosyl carbocation rapidly hydrates and mutarotates.

EXPERIMENTAL

Reagents - Deionized, distilled water was used to prepare a 0.050M buffer solution of potassium biphthalate (Fisher primary standard) having pH 4.4. This buffer solution was used to prepare all other aqueous solutions.

Silylation Reagent - The silylating reagent was prepared from 40 mL of anhydrous pyridine, 8 mL of trimethylsilylimidazole and 4 mL of trimethylchlorosilane (reagents from Pierce Chemical Co.). This solution was refrigerated and dispensed with a syringe for the silylation of the sugar solutions.

Sucrose - Granulated sugar from a grocery store was vacuum dried in an oven (50 °C) overnight and stored in a desiccator over phosphorus pentoxide. Sucrose dried in this manner had $[\alpha]^{25}_{589}$ = 66.033° and $[\alpha]^{25}_{578}$ = 69.495°.

Glucose - Crystalline forms of α-glucose and β-glucose (Sigma Chemical Co.) were stored in a desiccator over phosphorus pentoxide until used.

Fructose - β-Fructopyranose (Sigma Chemical Co.) was purified by the procedure of Jackson and Matthews.[26] The fructose (300 g) was dissolved by warming to 50 °C in 300 mL of water in a 1-L Erlenmeyer flask. This solution was decolorized with Norite (Fisher Scientific Co.) at 50 °C for 30 minutes and filtered. To the solution at room temperature was added 200 mL of absolute ethanol, after the addition of which the solution stood at room temperature overnight. The crystals of fructose were removed by filtration and vacuum dried at room temperature for 6 h and then for 48 h at 70 °C under vacuum. The dried fructose was stored in a desiccator over phosphorus pentoxide. Preparation of 0.5M solutions in KHP buffer and following the optical rotation to equilibrium provided the following specific rotations; $[\alpha]^{20}$ = -92.50°, $[\alpha]^{20}_{578}$ = -96.20.° Extrapolation of the time-rotation values to zero time provided $[\alpha]^{20}_{589}$ = -133.20° and $[\alpha]^{20}_{578}$ = -138.53°.

Invertase - Invertase (Sigma Chemical Co., Grade VII from bakers' yeast) had an activity of 400 units per mg of solid where one unit will hydrolyze 1.0 μmol of sucrose per min at pH 4.5 at 55 °C. At a concentration of 80 mg invertase in 10.0 mL of KHP buffer solution, the observed optical rotation was +0.210 at 25 °C and 578 nm.

Silylation Procedure - The aqueous sugar solutions used in these studies were placed in 13 x 100-mm disposable culture tubes. The open end of the tube was attached to a piece of vacuum tubing which in turn was attached through a stopcock to a T-tube. The T-tube also was connected to the vacuum pump and to a 1-L flask serving as a vacuum reservoir. A full vacuum (0.1 torr) was pulled on the reservoir, and then the system was opened to the culture tube containing the sugar solution. The formation of a glass was almost instantaneous, but vacuum was applied for a min. The culture tube was removed, placed on a Fisher Mini-Shaker and agitated as 1.0-mL (syringe) of the silylating agent was added.

The silylated samples were transferred to a 3.5-mL septum-capped vial for storage in a refrigerator until analyzed.

Polarimetric Measurements - All optical rotations were made on a Perkin-Elmer 241 MC polarimeter using a sodium lamp (589 nm) for reference work and a mercury lamp (578 nm line) for all measurements. The cell was a Perkin-Elmer water-jacketed 1-dm cell. Temperature-regulated water was circulated to the cell with the temperatures measured in the cell by means of a calibrated thermistor thermometer. Temperatures were reproducible to $\pm$0.05 ^{o}C.

Gas Chromatography - A Varian 6000 gas chromatograph was used along with a Varian CDS 401 data system. The chromatograph was equipped with a Supelco SPB-1 wide bore capillary column of 0.75mm x 60 m with a 1-μ bonded phase of Sp 2100. The operating conditions were: 10 mL per minute helium, injection temperature 250 ^{o}C, FID 290 ^{o}C and a temperature program of an initial temperature of 140 ^{o}C for 2 min, 8 ^{o}C min to 160 ^{o}C, 2 ^{o}C/min to 210 ^{o}C and 15 ^{o}C/min to 270 ^{o}C and hold for 20 min. The injection samples were 2 μL with integration inhibited for 12 min until all solvent and reagent peaks had eluted. The retention times for the silylated sugars were: α-fructofuranose 22.8 min, β-fructofuranose 23.2 min, β-fructopyranose 23.6 min, α-glucopyranose 26.0 min, α-fructopyranose 27.6 min, open-chain form of fructose 28.0 min, β-glucopyranose 29.3 min, and sucrose 41.5 min.

A Hewlett-Packard 5890 gas chromatograph was used with an FID detector and with a 5970 mass selective detector. With the FID the data was recorded and integrated on a Hewlett-Packard 3392A integrator. With the mass-selective detector the data was stored and manipulated on the HP9133 data station. The column used with the FID was an 0.2 mm x 50 m fused silica capillary having a 0.3 μ phenylmethylsilicone bonded phase. The fructose peaks and glucose peaks were baseline resolved by isothermal operation at 195 ^{o}C but to elute the sucrose the system was programmed to 275 ^{o}C at 25 ^{o}C/min and held for 27 min. The helium carrier gas was used at a flow rate of 30 mL/min with a 100:2 split ratio. The injector and

detector temperatures were 250 °C and 2 μL samples of the silylated sugars were injected. For the mass-selective detector, the temperature program was an initial temperature of 210 °C for 25 min and then heated to 270 °C at 25 /min and held for 25 min. The transfer line was maintained at 210 °C, and the injection samples were 0.5 μL with a solvent delay of 15 min. The helium flow was 1.0 mL/min with a splitter ratio of 100:1. A solvent delay of 15 min was used.

Temperature-Composition Studies for Fructose - A solution of 4.5046 g of fructose (0.500 M) in 50 mL of KHP solution (volumetric flask), pH 4.4, was prepared and equilibrated for 24 h at room temperature. The polarimeter cell was filled, and the balance of the solution was maintained in the constant temperature bath used to circulate the temperature regulated water to the cell. The solution was held at various lengths of time depending upon the temperature before recording the optical rotations and analyzing the solution for tautomeric composition. The times for the different temperatures were 10 °C, overnight; 15 °C, 4 h; 25 °C and up, 2 h. Rotations were recorded every 5 min until found to be constant for one h. For the silylation studies, 10 μL(GC syringe) of the equilibrated solution was transferred to a 13 x 100 mm culture tube which had been placed in an insulated beaker maintained at the same temperature as the bath (± 1 °C). The solutions were immediately evacuated on the vacuum system and the residues silylated and analyzed on the Varian system.

Mutarotation of Fructose - A sample of 0.9005g of fructose was weighed into a clear dry 25.0-mL vial at 25 °C, 10 mL of KHP buffer of pH 4.4 was added rapidly and the mixture shaken vigorously for 10 sec and then maintained at 25 °C in the constant temperature bath. Samples (10 μL) were removed, transferred to the vacuum system, evacuated and silylated. Samples were removed at 1-min intervals for 9 min and then less frequently up to 75 min and at equilibrium after 24 h. Analyses were made with the Varian system. The experiment was run in triplicate.

Hydrolysis of Sucrose by Invertase - A solution of 10 mg of invertase was made in 1.25 mL of KHP buffer. Separately 0.135 g

of sucrose was dissolved in 0.315 mL of KHP buffer. A mixture of 0.25 mL of the invertase solution was made with 0.15 mL of the sucrose solution and maintained in the temperature bath at 25 °C. Samples (10-mL) were removed and silylated each min for the first 10 min and then less frequently up to 75 min and at equilibrium after 24 h. The analyses were made on the Varian system, and the experiment was run in duplicate.

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