The high endothermic enthalpies of solution indicate large changes in the temperature coefficients of the solubilities. Since auxiliary data are not available, ideal solution behavior is assumed, and utilizing only enthalpies and heat capacities of solution (*17*), the solubilities at 35 °C for both nucleosides in both solvents are estimated to be ca. 1.5 times those at 25 °C.

Glossary

$\Delta\bar{H}$	enthalpy of transfer from water to 1 *m* ethanol
$\Delta H°$	enthalpy of solution in pure water
ΔH	enthalpy of solution in 1 *m* ethanol
ΔC_p	heat capacity of solution
C_p	heat capacity of solids
$\bar{C}_p$	partial molar heat capacity
$\Delta\bar{C}_p$	heat capacity of transfer

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An Equilibrium Phase Diagram for the Glucose–Cellobiose–Water System at 30.5 °C

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The solid–liquid equilibrium phase diagram for the ternary system of water–glucose–cellobiose was studied at 30.5 °C. Samples of known composition were equilibrated at 30.5 °C over a period of 7 days. The resultant saturated liquid phase as well as a wet sample of the solid phase were analyzed by high-pressure liquid chromatography. Both graphical analysis as well as optical microscopy were used to identify the solid phases. The compositions of the saturated liquid phase and the identities of the accompanying solid phases are reported. Glucose monohydrate and anhydrous cellobiose were identified as the solid phases. For the three-phase region, the composition of the liquid at the invariant point was calculated to be 50.1 wt % glucose, 5.0 wt % cellobiose, and the remainder water. The two-phase region of solid anhydrous cellobiose and saturated liquid was found to dominate the ternary diagram. This suggests that it is possible to obtain cellobiose in sufficient purity and yield from aqueous solutions obtained by the enzymatic hydrolysis of cellulose.

Introduction

Glucose and cellobiose are the major products of the enzymatic saccharification (hydrolysis) of cellulose (*1*). Glucose is presently used extensively in the production of valuable pharmaceuticals. Cellobiose finds important use in bacteriology (*2*). In the synthesis of certain pharmaceuticals, the use of cellobiose as a substrate may prove more efficient than glucose. The limited availability and high cost of cellobiose have inhibited its use up to now. The enzymatic saccharification of cellulose yields cellobiose in significant quantities together with glucose (*1*). A separation scheme needs to be devised to separate the high-value cellobiose from the relatively cheaper glucose. This recovery of cellobiose could provide a new substrate for industry.

Although the binary, ternary and even quaternary systems of various combinations of water, glucose, fructose, and sucrose (*3–12*) have been studied, there is no report in the literature of any system involving cellobiose, one of the major sugars produced by most current hydrolysis processes.

Young (*8*) investigated the D-glucose–water phase diagram and obtained solubility curves for all four solid phases, viz., ice, α-D-glucose, β-D-glucose, and α-D-glucose monohydrate. Taylor (*11*, *12*) has measured the solubilities of cellobiose in water. It was noted that the solid phase in equilibrium with the solution at 20 °C was not hydrated.

Considering ternary and higher order phase diagrams, the work of Kelly is most extensive. Kelly's studies (*3–7*) at 30 °C have shown that glucose occurs most frequently as the monohydrate in the solid phase. Young (*8*) has shown that the transition (in the absence of other solutes) from the monohydrate to the anhydrous glucose solid phase occurs above 54.7 °C (i.e., the monohydrate is the stable solid phase below 54.71 °C). In all of Kelly's studies, no evidence of double compounds or solid solutions of sugars was ever found. Hence this possibility may be eliminated from this study.

Experimental Section

Materials. The D-glucose and D-cellobiose, as well as the acetonitrile used for liquid chromatography, were of certified ACS purity. Distilled water was used for the preparation of all samples.

Preparation of Synthetic Samples. In accordance with Hill and Ricci's "synthetic complex" method (*13*), it was decided to start the equilibration procedure with samples of known composition of water, glucose, and cellobiose (*14*). Since saturated solutions of sugars together with their precipitated crystals form very viscous slurries which are often difficult to pipet or filter, the synthetic compositions should preferably be chosen so as to give only small quantities of crystals; i.e., they should be close to the solubility curve. On the basis of the results

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of the pilot run, the actual runs were begun. Twenty-two compositions were selected so as to cover both the two- and three-phase regions. Duplicates of each of these compositions were prepared as an added precaution.

Equilibration of Samples. The samples were placed in 25-mL Erlenmeyer flasks, and the flasks were sealed tightly with rubber stoppers. the flask contents were agitated by placing them on a gyratory water bath shaker (Model G76, New Brunswick Scientific Co., New Brunswick, N.J.) and rotated at about 100 rpm. The temperature of the bath equilibrated at 30.5 ± 0.5 °C.

Kelly (*2*) confirmed that 2 or 3 days suffice for equilibration, and at most 15 days are necessary for the most viscous solutions (e.g., water–fructose–sucrose). Due to the limited solubility of cellobiose, solutions of water–glucose–cellobiose are not quite as viscous. Accordingly all solutions were agitated for at least 6 or 7 days before samples were withdrawn for analysis. Equilibrium was approached from the undersaturated condition only, due to difficulty in precipitating oversaturated sugar solutions. Extensive studies by Kelly (*3*, *4*, *8–10*) have confirmed that satisfactory results can be obtained. The achievement of equilibrium can be optically identified too, as described later.

Removal and Preparation of Samples for Analysis. At the end of the equilibration period, two types of samples were removed from each flask: (i) a wet sample of solids and saturated solution for use with Schreinemaker's (*15*, *16*) graphical analysis method for determining the solid phase; (ii) a clear saturated liquid phase free of any solid phase by pressure filtration of the slurry through a 0.45-μm filter.

The collected samples were then diluted to dissolve the crystals, as well as to provide a sufficiently dilute sample to prevent overloading the liquid-chromatography column.

Analysis by High-Pressure Liquid Chromatography. A Waters Associates ALC-200 liquid chromatograph equipped with a Model 6000 solvent delivery system and a Model U6K universal injector (Water Associates, Milford, Mass.) was used. The sugars were detected by a Waters Associates R401 differential refractometer detector having a sensitivity of 1×10^{-7} refractive index units. A prepacked Bondapak carbohydrate analysis column, 30 cm × 4 mm i.d. stainless steel, supplied by Waters Associates, Milford, Mass., was used at a room temperature of 22 °C.

The eluent was an aqueous solution of 80 vol % acetonitrile and the flow rate was 5 mL/min. The sample injection volume was 10 μL.

The calibration curves (peak areas vs. μg of sugar) for glucose and cellobiose were straight lines up to 1000 μg of each sugar per injection volume. The peak areas were measured by using a Hewlett-Packard HP 3380A integrator. Due to the unstable nature of the column packing, a slow drift in the slope of the calibration curves was noticed. This error was minimized by obtaining new calibration curves at regular intervals. Two to three injections were made for each sample and the peak areas were averaged. To obtain the weight percent of sugar in the sample, the total mass of the injection volume was obtained by weighing accurate to 0.1 mg. The weight percent of water was obtained by difference. The precision of this sugar analysis was estimated to be about 1 wt % (*14*).

Optical Examination of the Solid Phase. The solid-phase samples were examined by using a Leitz optical microscope. A small amount of the sample was placed on a regular glass slide and covered with a slip cover. The sample was then examined at medium light intensity for maximum contrast between the crystals and the surrounding liquid. The use of polarized light increased this contrast even further.

To identify an unknown crystal sample by this microscopic examination method, four "calibration" photomicrographs were first prepared by photographing a representative field of crystal

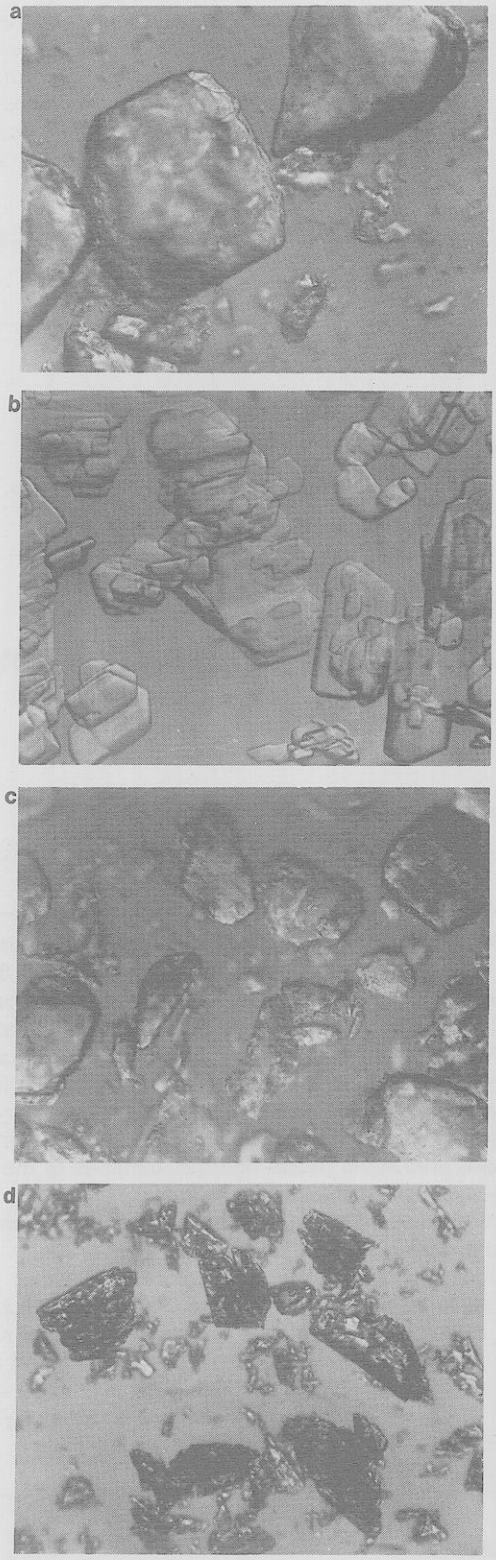

Figure 1. "Calibration" photomicrographs for optical analysis of the solid phase: (a) anhydrous glucose crystals in oil suspension; (b) glucose monohydrate crystals in their saturated aqueous solution at 30.5 °C; (c) anhydrous cellobiose crystals in oil suspension; (d) anhydrous cellobiose crystals in their saturated aqueous solution at 30.5 °C.

structures of known chemical composition. The comparison of a representative field of the unknown sample with these photomicrographs led to the identification of the solid phase (see Figure 1).

The crystals in Figure 1b were recognized to be the monohydrate of glucose, since they were obtained from a saturated solution of glucose at 30 °C. Young (*8*) had shown that the monohydrate is the stable solid phase for saturated glucose

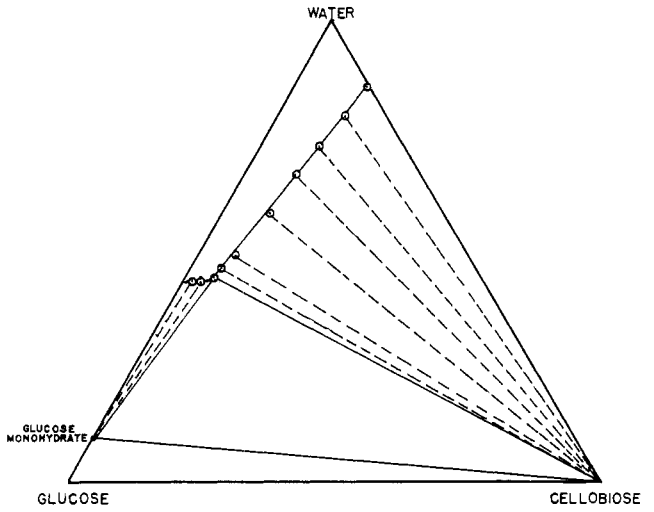

Figure 2. System water–glucose–cellobiose at 30.5 °C.

solutions below 54.71 °C. Further it was noticed that although this solution was prepared by using anhydrous glucose crystals (Figure 1a), rapid and complete recrystallization to the monohydrate (Figure 1b) occurred. This would not be the case if anhydrous glucose is the solid phase.

The crystals in Figure 1d were recognized to be the anhydrate of cellobiose since they appear to have the same structure as the anhydrous cellobiose (Figure 1c) from which the saturated solution of cellobiose was prepared. Thus no recrystallization is apparent in this case. This was also confirmed by X-ray diffraction studies of the same samples (*14*) and matched Taylor's (*11*) observation that anhydrous cellobiose forms the solid phase.

The method of microscopic examination provided a reliable method of identifying the solid phase. This method also helped in the recognition of samples having more than one solid phase in equilibrium with the saturated solution.

The microscopic inspection of the crystals also helped in determining whether a sugar solution had reached equilibrium with its solid phase. Kelly (*4*) noted that although his synthetic samples were prepared from anhydrous glucose, the undissolved glucose recrystallized quite rapidly to the monohydrate at 30 °C. The completeness of this recrystallization as observed from microscopic examination was seen as contributory evidence that the solution was saturated with glucose. The process of recrystallization could be followed very readily by microscopic examination as the original particles of anhydrous glucose were rounded solvent-eroded grains and the monohydrate appeared as well-defined needlelike crystals.

X-ray Diffraction. The solid phase was also examined by X-ray diffraction using Ni-filtered Cu Kα radiation. The observed d spacings matched those reported in ref 17. The dry sugars used for preparing the synthetic samples were confirmed to be anhydrous glucose and anhydrous cellobiose. The solid phase, shown in Figure 1b, was confirmed to be glucose monohydrate, while the solid phase, shown in Figure 1d, was confirmed to be anhydrous cellobiose. The method of microscopic examination was thus seen to provide reliable results. Finally the solid phases from a few of the equilibration flasks were also examined by X-ray diffraction, and the results were again found to be in agreement with those obtained by microscopic examination.

Results

The 22 synthetic samples were equilibrated at 30.5 ± 0.5 °C for 7 days. The compositions of the saturated liquid phase from each of the duplicate flasks for each synthetic composition were averaged. These values have been tabulated in Table I and are also plotted in Figure 2. The tie lines were obtained

Table I. Liquid-Phase Solubilities

synth sample compn, wt %		av liquid-phase compn, wt %		
% glucose	% cellobiose	% glucose	% cellobiose	solid phase(s)[a]
0.0	18.0	0.0	13.5	CA
7.0	18.0	7.3	12.8	CA
15.0	18.0	15.8	11.0	CA
22.0	16.0	23.1	9.5	CA
30.0	14.0	32.2	8.5	CA
40.0	12.0	43.3	6.7	CA
40.0	20.0	47.3	5.1	CA
42.0	8.0	41.1	7.6	none
45.0	10.0	48.2	5.5	CA
48.0	12.0	51.2	4.8	GM + CA
50.0	7.0	49.5	6.8	none
50.0	15.0	49.7	4.8	GM + CA
51.0	10.0	49.7	5.1	GM + CA
52.7	9.5	50.8	5.3	GM + CA
53.0	9.0	48.9	5.1	GM + CA
56.0	5.0	50.1	5.6	GM
56.0	4.0	51.3	4.3	GM
56.0	3.0	52.8	3.2	GM
56.0	2.0	54.0	2.1	GM
56.0	1.0	54.4	1.2	GM
56.0	0.5	55.1	0.8	GM
60.0	0.0	52.1	0.0	GM

[a] Key: GM = glucose monohydrate; CA = cellobiose anhydrate; none = undersaturated solution.

by joining the average saturated liquid phase compositions with the corresponding solid phase, as determined by the graphical and optical methods described later.

Since the weight percent of water was always obtained by the difference between 100 and the sum of the weight percents for glucose and cellobiose, the percentage of water is not reported in any of the tabulated results.

The average error in the analysis of each sugar was estimated to be 1% for the higher sugar concentration and proportionally lower for the lower concentrations.

Identification of Solid Phases by Graphical Analysis. The use of the tie-line method in three-component systems as a means of determining the solid phase is a common practice. One of the points on the tie line is the composition of the liquid phase as obtained by analysis. The second point on the tie line may be chosen by using either (i) Hill and Ricci's (*13*) "synthetic complex" method or (ii) Schreinemaker's (*15*, *16*) "residue" method.

The wet "residue" method involves the removal of a wet sample of solid and solution from the vessels after equilibrium has been attained. The "synthetic complex" method consists of placing in the equilibration vessel a synthetic composition made by weight from dry solids and solvent.

Graphical Analysis of the Solid Phase. A combination of both Hill and Ricci's (*13*) and Schreinemaker's (*16*) graphical analysis methods was used. Hence for each synthetic composition, the averaged saturated liquid phase composition, the synthetic sample composition, and the two "wet" sample compositions (from duplicate flasks) were plotted. It should be noted that the "wet" samples removed from duplicate flasks will, in general, not have the same composition since the weight fraction of the solid phase in each sample may not be the same. A straight line was then drawn through them and extrapolated to intersect the sides of the ternary diagram. These tie lines were found to intersect in the vicinity of thhe cellobiose apex of the ternary diagram. For the sake of comparison, the composition of a hypothetical monohydrate of cellobiose is also plotted on the same diagram.

Within limits of analytical error, the graphical analysis of Figure 3 indicated that anhydrous cellobiose is one of the solid phases. However this same method yielded inconclusive results as to

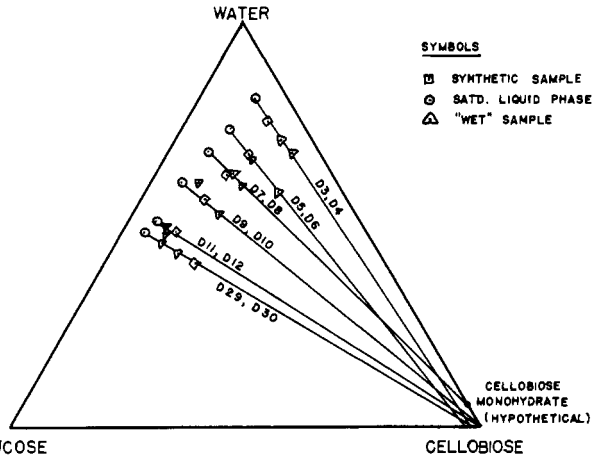

Figure 3. Determination of mode of precipitation of cellobiose by graphical analysis.

whether glucose was precipitated as the anhydrate or the monohydrate.

Optical Analysis of the Solid Phase. A "wet" sample was removed from each flask and examined under the optical microscope. A representative field was compared with the "calibration" photomicrographs (see Figure 1) to identify the solid phase.

Anhydrous cellobiose was confirmed as the solid phase in the cellobiose region of the ternary diagram, whereas glucose monohydrate was the apparent mode of precipitation of glucose.

The Invariant Point Composition. Microscopic examination of the solids from each flask also identified those having three phases (the saturated liquid and two solid phases) in equilibrium. The liquid phase in these flasks was thus known to be of invariant composition.

The saturated liquid phase compositions of these flasks were averaged to give the invariant point composition as 50.1 wt % glucose, 5.0 wt % cellobiose, and the remainder water.

Discussion

Graphical analysis allowed the determination of anhydrous cellobiose as one of the solid phases. This method gave inconclusive results with regard to glucose. This may be attributed to the failure of the liquid analysis for compositions in that region of the phase diagram. This region is characterized by saturated liquid phase samples as well as "wet" samples which contain high concentrations of glucose (approximately 50 wt %) and low concentrations of cellobiose (approximately 5 wt %).

The problem is further aggravated by the fact that the tie lines in this region lie almost parallel (10–20°) to the water–glucose side of the phase diagram. This means that even small errors in the sample analysis result in large errors in the solid-phase composition as obtained by the intersection of the tie line with the side of the ternary diagram.

For the same reasons outlined above, the application of Hill and Ricci's (*13*) algebraic extrapolation method yielded inconclusive results. Comparison of a representative field of a sample from a given flask with the previously obtained "calibration" photomicrographs was found to be a reliable way of identifying the solid-phase compound. This was confirmed by the fact that samples from flasks, for which the tie lines extrapolated in the direction of the glucose corner of the phase diagram, showed crystal structures which were identical with that shown by crystals in equilibrium with a saturated solution of pure glucose at 30 °C (Figure 1b). Since the latter were identified by Young (*8*) to be the monohydrate of glucose, it may be inferred that in our system too, glucose monohydrate is the solid phase.

Optical analysis also confirmed anhydrous cellobiose as the other solid phase. Samples, for which the tie lines extrapolated in the direction of the cellobiose corner of the phase diagram, were found to have a crystal structure identical with the anhydrous crystal structures in oil suspension or in a saturated solution of pure cellobiose (Figure 1c,d).

Another indication that glucose monohydrate is indeed the solid phase can be seen from the fact that although all solutions were prepared with solvent-eroded rounded grains of anhydrous glucose, upon equilibration, only fine needlelike crystals of glucose monohydrate are found with not even a single crystal of the original anhydrate in evidence.

Conclusions

The study of the ternary system of water–glucose–cellobiose at 30.5 °C resulted in the solid–liquid equilibrium phase diagram shown in Figure 2. It was noticed that the cellobiose-insoluble region dominated the ternary-phase diagram. Further the invariant composition was located at 50.1 wt % glucose and 5.0 wt % cellobiose. This implies that if we were to start out with a dilute solution of glucose and cellobiose with a weight ratio of glucose to cellobiose less than 9:1, then upon evaporation of the solution at 30.5 °C, pure anhydrous cellobiose will be initially precipitated as the solid phase. Since enzymatic hydrolysis of cellulose by *T. Reesei* (*1*) generally yields glucose and cellobiose in the ratio of 2:1, respectively, the separation of pure cellobiose from such solutions seems very favorable in terms of both yield and purity.

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