malonate, 156-80-9; DL-tartrate, 133-37-9; oxalate, 338-70-5.

LITERATURE CITED

(1) Small, H.; Miller, T. E., Jr. *Anal. Chem.* **1982**, *54*, 462–469.
(2) Scott, R. P. W.; Scott, C. G.; Kucera, P. *Anal. Chem.* **1972**, *44*, 100–104.
(3) Cochrane, R. A.; Hillman, D. E. *J. Chromatogr.* **1982**, *241*, 392–394.
(4) Larson, J. R.; Pfeiffer, C. D. *Anal. Chem.* **1983**, *55*, 393–396.
(5) Larson, J. R.; Pfeiffer, C. D. *J. Chromatogr.* **1983**, *259*, 519–521.
(6) Wheals, B. B. *J. Chromatogr.* **1983**, *262*, 61–76.
(7) Cortes, H. J.; Stevens, T. S. *J. Chromatogr.* **1984**, *259*, 269–275.
(8) Haddad, P. R.; Cowie, C. E. *J. Chromatogr.* **1984**, *303*, 321–330.
(9) Naish, P. J. *Analyst (London)* **1984**, *109*, 809–812.
(10) Jenke, D. R. *Anal. Chem.* **1984**, *56*, 2468–2470.
(11) Jenke, D. R. *Anal. Chem.* **1984**, *56*, 2674–2681.
(12) Iskandarani, Z.; Miller, T. E., Jr. *Anal. Chem.* **1985**, *57*, 1591–1594.
(13) Mho, S.; Yeung, E. S. *Anal. Chem.* **1985**, *57*, 2253–2256.
(14) Brown, D.; Payton, R.; Jenke, D. R. *Anal. Chem.* **1985**, *57*, 2264–2267.
(15) Hayakawa, K.; Hiraki, H.; Miyazaki, M. *Bunseki Kagaku* **1983**, *32*, 504–505.
(16) Miyazaki, M.; Hayakawa, K.; Choi, S. *J. Chromatogr.* **1985**, *323*, 443–446.
(17) Rigas, P. G.; Pietrzyk, D. J. *Anal. Chem.* **1986**, *58*, 2226–2233.
(18) Hayakawa, K.; Hiraki, H.; Miyazaki, M. *Bunsek Kagaku* **1985**, *34*, T71–76.
(19) Yoshida, I.; Hayakawa, K.; Miyazaki, M. *Eisei Kagaku* **1985**, *31*, 317–323.
(20) Gjerde, D. T.; Schmuckler, G.; Fritz, J. S. *J. Chromatogr.* **1980**, *187*, 35–45.
(21) Yamamoto, M.; Yamamoto, H.; Yamamoto, Y.; Matsushita, S.; Baba, N.; Ikushige, T. *Anal. Chem.* **1984**, *56*, 832–833.
(22) Matsushita, S. *J. Chromatogr.* **1984**, *312*, 327–336.
(23) MacDonald, J. C. *Ion Chromatographic Analysis*; Wiley: New York, 1985; Chapter 6.
(24) Muto, G.; Oikawa, K. *Ion Chromatography*; Kodansha: Tokyo, 1983; Chapter 2.
(25) IUPAC *Stability Constants of Metal-Ion Complexes: Part B—Organic Ligands*; Pergamon: New York, 1979.

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Immobilized Enzyme Kinetic Study of D-Glucose Mutarotation by Flow Injection Analysis

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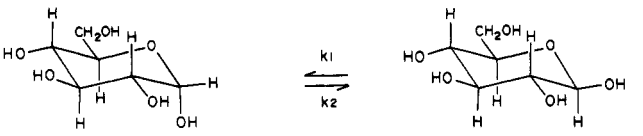
A novel analytical application of immobilized enzymes is presented in which the mutarotation kinetics of D-glucose are studied. A glucose oxidase single bead string reactor is combined with the Trinder reaction in a flow injection system. The apparatus is used to monitor the mutarotation of D-glucose in a batch reactor. The mutarotation coefficients obtained are in good agreement with the literature values. Phosphate ion is shown to catalyze the reaction and the mutarotation coefficient is directly proportional to its concentration. The selectivity of the enzyme reactor is also demonstrated.

Numerous reviews (*1–5*) and monographs (*6–9*) have appeared in recent years extolling the merits of immobilized enzymes as reusable reagents; analytical systems which employ immobilized enzymes have become commonplace. The major use of such systems has been for the determination of a specific substrate in various types of samples (*6*). Chibata (*7*) gives an excellent review of other ways in which immobilized enzymes can be used (e.g. structural analysis of biopolymers and the elucidation of enzyme mechanisms).

In this paper a novel application of these immobilized reagents is described. An immobilized enzyme reactor is used to follow the kinetics of a batch reaction which produces a substrate for which the enzyme is specific. This is accomplished by injecting a sample from the solution in the batch reactor onto the FIA manifold at prescribed time intervals. The method requires minimal operator intervention. Since the enzyme is specific for a single sugar, this method facilitates the study of the mutarotation process for that sugar in a matrix of other optically active species (e.g. a mixture of sugars, several of which mutarotate). The mutarotation of some sugars is complex since, when in solution, furanose as well as pyranose structures are present (*10*). It may be possible to investigate separately the slow reactions of the pyranose form of other sugars with an apparatus similar to that described here.

The mutarotation of D-glucose is a well-characterized reaction (*10, 11*) and therefore can be easily used as a model since the kinetic parameters are well-known. The reaction



α-D-glucose β-D-glucose

proceeds by a first-order reversible process until equilibrium is reached. The integrated form of the rate equation for this process is

$$-\ln \frac{\beta_t - \beta_{eq}}{\beta_0 - \beta_{eq}} = (k_1 + k_2)t \qquad (1)$$

where β_0, β_t, and β_{eq} are the concentrations of the β anomer initially, at time t, and at equilibrium, respectively. The term $k_1 + k_2$ is known as the mutarotation coefficient.

Previously described kinetic studies by FIA are predominantly those of reactions that occur in the manifold. In one method the flow is stopped and the absorbance of a species monitored as it reacts within the flow cell (*12*). In another, the sample plug circulates continuously in a loop that contains the detector (*13*). Painton and Mottola recently showed that the rate constant for a reaction in a single sample plug can be determined by comparison of the signal profile with those from numerical simulations (*14*). FIA has only recently been used to study the kinetics of relatively slow batch reactions (*15, 16*). One unique feature of the work reported here is the use of an immobilized enzyme single bead string reactor (SBSR) to follow the kinetics of a slow reaction that occurs in a batch reactor.

In this work an FIA manifold containing immobilized glucose oxidase (GO) was used to continuously monitor the mutarotation of D-glucose. Glucose oxidase is specific for the β anomer of D-glucose. The enzyme reaction, when coupled

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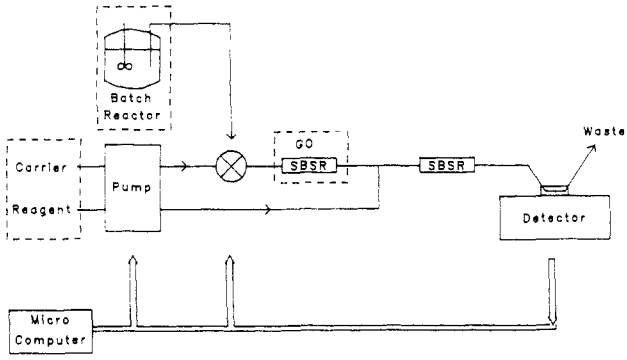

Figure 1. FIA manifold for investigation of mutarotation kinetics. Hatched lines indicate thermostated components.

with the Trinder reaction (*17*), yields a colored product whose concentration is directly proportional to the original β-D-glucose concentration. In the Trinder reaction, peroxidase (PO) is used to catalyze the coupling of 4-aminoantipyrine (AAP) with 3,5-dichloro-2-hydroxyphenylsulfonate (DCPS). The reaction sequence is as follows:

$$H_2O + O_2 + \beta\text{-D-glucose} \xrightarrow{GO} \text{gluconic acid} + H_2O_2$$

$$H_2O_2 + AAP + DCPS \xrightarrow{PO} \text{red dye} + H_2SO_4$$

In this study the absorbance of the dye generated from β-D-glucose was used as a measure of its concentration. It is more common to use the optical rotation of the solution in place of the concentration of the β anomer (*18*).

The focus of this work was to use the apparatus to follow the mutarotation kinetics of D-glucose in the presence of various concentrations of phosphate. The mutarotation process was also followed for D-glucose in the presence of D-galactose. Since D-galactose also mutarotates, the conventional method for this type of study (polarimetry) would be more complex than the method presented here.

EXPERIMENTAL SECTION

Apparatus. The apparatus is shown schematically in Figure 1. All solutions and the glucose oxidase SBSR were maintained at 21 °C by a thermostated circulating water bath. The carrier stream was pumped by a 12-channel peristaltic pump (Ismatec). The flow rate of the sample carrier stream was made 10 times that of the reagent stream by appropriate choice of pump tubing and the overall flow rate was 1.0 mL min^{-1}. Flow-rated pump tubing (Technicon) was used. Sample solution flowed continuously to waste through a 30-μL sample loop on a pneumatically actuated six-port sample injection valve (Rheodyne). The sample was injected at preprogrammed intervals into a flowing stream of phosphate buffer which entered a glucose oxidase SBSR. The single bead string reactor was composed of chemically modified 0.6 mm diameter nonporous glass beads (Proper Manufacturing, New York) in 10 cm of poly(tetrafluoroethylene) tubing (0.86 mm i.d., Benton-Dickinson) as reported elsewhere (*19*). The reacted sample was then merged with a reagent stream that contained horseradish peroxidase, 4-aminoantipyrine, and 3,5-dichloro-2-hydroxyphenylsulfonate. Formation of the colored product followed within a subsequent plain SBSR (ca. 40 cm in length). The product absorbance was then detected in a miniaturized flow-through colorimeter designed by Patton et al. (*20*). An in-house designed Intel 8088 based microcomputer (*21*) controlled the pump speed, sample injection, and data acquisition. Software was written in FORTH (Forth, Inc., Hermosa Beach, CA) (*22*). The data acquired were sent across a network to a DEC LSI 11/23 for postexperiment processing. A simplex routine, developed in our laboratory and based on the method of Nelder and Mead (*23*), was used to find the best fit of the experimental data to the kinetic model. From this, the mutarotation coefficients were obtained.

Reagents. All chemicals, unless otherwise noted, were reagent grade and obtained from Sigma Chemical Co. (St. Louis, MO). They were used without further purification. All solutions were prepared with distilled water (DW) and filtered as necessary.

A 0.2 M phosphate buffer stock solution was prepared by adding 13.609 g of KH_2PO_4 (Mallinckrodt) and 14.196 g of Na_2HPO_4 (Fisher Scientific Co.) to a 1-L flask and diluting to the mark with DW. Additional buffer solutions were made by dilution of the stock solution. In each case the pH was adjusted to 6.84 by addition of 0.2 M NaOH (made with pellets from EM Science).

The reagent solution for the Trinder reaction was prepared in a volumetric flask immediately before use. It was composed of 0.8 mg mL^{-1} PO (EC 1.11.1.7, type II, specific acitivity ≈200 U mg^{-1} at 20 °C), 1 mM AAP, and 1 mM DCPS, diluted to the mark with 0.05 M phosphate buffer.

Glucose solutions were prepared at the time of use by measuring 15.0 mg of the appropriate anomer into a 25-mL volumetric flask and diluting to the mark with the appropriate solution. For the experiments in which galactose was also in solution, 15.0 mg of D-galactose was added to the 15.0 mg of glucose before transfer to the volumetric flask.

Procedures. Glucose oxidase (EC 1.1.3.4, Sigma type II from Aspergillus Niger, specific activity ≈ 17 800 U g^{-1} at 35 °C) was immobilized on nonporous glass beads in the manner described previously (*19*). The beads were stored in 0.05 M phosphate buffer at 4 °C.

Four different phosphate concentrations were used: 0.00, 0.05, 0.10, and 0.20 M. Only the 0.05 M phosphate buffer was used in the case where galactose was added. The mutarotation process was followed in both the forward and reverse directions, starting with the pure α and β forms, respectively. For each anomer in each concentration of phosphate, the following experiment was repeated two to four times. The anomer was transferred to the volumetric flask and the clock was started. The sample was diluted as described above and allowed to dissolve completely. The tube leading to the sample injection valve was inserted into the flask (see Figure 1) and the pump started. After 90 s the solution had had sufficient time to occupy the sample loop and the first injection was made. Subsequent injections were 60 s apart. Each injection was followed by monitoring the output voltage from the detector. This signal was digitized by the microcomputer's 12-bit analog to digital converter (ADC) and stored as ADC counts in a file on a floppy disk. The counts were later converted to absorbance units. The peak height in absorbance units was taken as a measure of the β anomer concentration. The time and peak absorbance values were stored in a data file for later application of the fitting routine.

The following model was used with the simplex fitting routine to find the best values of the three parameters—($\beta_0 - \beta_{eq}$, $k_1 + k_2$, and β_{eq}):

$$\beta_{calcd} = \beta_{eq} + (\beta_0 - \beta_{eq}) \exp(-t(k_1 + k_2)) \quad (2)$$

This is simply a different version of eq 1 in which all of the β values represent peak absorbance rather than concentration of the β anomer. The calculated β value, β_{calcd}, was obtained during the fit. All points were weighted equally. Initial values of the three parameters were supplied by the user. In the case of the α anomer, β_0 was assumed to be zero. For the β anomer, β_0 could not be measured directly, and therefore the peak absorbance vs. time data were extrapolated back to $t = 0$. Initial β_{eq} values were calculated from the average of the last two peak absorbance values, except in the case of the water solution. It is known from polarimetric measurements (*18*) that at room temperature 64% of the D-glucose is in the β form at equilibrium and thus β_{eq} for the water solution was estimated by appropriate multiplication of the β_0 value. Initial estimates of $k_1 + k_2$ were obtained by calculating the initial slope of the curve for each set of data points.

The initial simplex consisted of four vertices and was calculated from initial estimates and an appropriately chosen step size for each of the three parameters. At each vertex the following procedure was performed. For each value of t in the data set a value of β_{calcd} was computed. The squares of the differences between this value and the experimentally obtained value (β) were summed for all values of t using the function: $R = \sum(\beta - \beta_{calcd})^2$. The magnitude of this sum of the squares of the residuals, R, was a measure of how closely the current estimates for the three parameters matched the experimental behavior. The simplex moved through successive iterations as it tried to minimize R (i.e. to obtain the closest match possible). Convergence was assumed when the

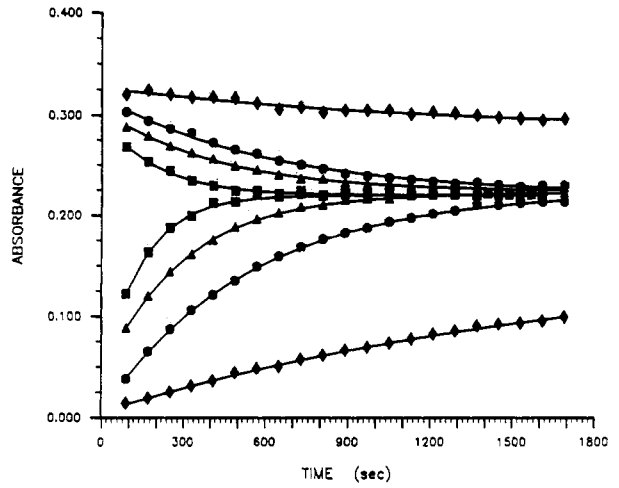

Figure 2. Effect of phosphate concentration on rate of mutarotation of D-glucose. Lines represent the curves of best fit. Upper curves were obtained with only β-D-glucose initially present. Lower curves were obtained with only α-D-glucose initially present. Phosphate buffer concentrations were (♦) 0.00 M, (●) 0.05 M, (▲) 0.10 M, and (■) 0.20 M.

Table I. Mutarotation Coefficients for D-Glucose at 21 °C

	coefficient × 10^3, s^{-1}	
phosphate buffer concn, M	only α initially	only β initially
0.00	0.3	0.3
0.05	1.9	1.5
0.10	3.3	2.6
0.20	6.9	5.0
slope, M^{-1} s^{-1}	0.033	0.024
intercept, s^{-1}	0.00016	0.00027
corr coeff	0.998	0.999

sum of the squares of the residuals did not decrease after further iterations.

RESULTS AND DISCUSSION

Determination of Mutarotation Coefficients. Convergence of the simplex was usually obtained in less than 100 iterations and was not more than 140 in any instance. Complete separation of β_0 and β_{eq} values in eq 2 was investigated. This did not decrease the number of iterations necessary nor did it improve the final R value obtained. The experimental points (shown in Figure 2) are connected by the corresponding fitted curves. The fitted curves show good agreement with the experimental data. The standard error of the estimate was less than 10% and the between-run deviation was at most 5%. The mutarotation coefficients obtained with α and β anomers in various phosphate concentrations are listed in Table I.

The generally accepted value for the mutarotation coefficient of D-glucose in water at 20 °C is 0.015 min^{-1} (*24*, *25*). The value of 0.018 min^{-1} at 21 °C obtained here is in good agreement with the literature value. The small deviation can be accounted for by the slight temperature difference and the standard error of the measurement.

Effect of Phosphate Concentration. It is well-known that phosphate ion catalyzes the mutarotation of D-glucose (*10*). Preliminary experiments indicated that the phosphate concentration had no affect on the GO/Trinder reaction for an equilibrium mixture of α- and β-D-glucose. Therefore the increase in exponential character of the curves (Figure 2) is attributed to the catalytic effect of the phosphate ion on the batch reaction alone. The results, shown in Table I, indicate the mutarotation coefficient to be linear with phosphate concentration. This same trend was observed in the polarimetric studies of Murschhauser (*26*). The slopes, intercepts, and correlation coefficients are given in Table I. The mutarotation coefficient obtained when the D-glucose is initially in the α conformation is larger than that for the β conformation by approximately 30%. The values for the y intercepts in the equations relating phosphate concentration to the mutarotation coefficient indicate that the coefficient in water ought to be 0.009–0.018 min^{-1}, which compares favorably with literature values.

Effect of Galactose. Since the apparatus employed here is to be used to investigate the mutarotation of D-glucose in the presence of other sugars and mutarotase, it was desirable to demonstrate the selectivity of the GO reactor. The main interference envisioned was that of galactose since its mutarotation is catalyzed by mutarotase at a rate similar to that for glucose. Since there is a small amount of galactose oxidase impurity in the glucose oxidase preparation, a positive error would result. The mutarotation coefficients for D-glucose in the presence of an equal amount of galactose were found to be 1.9 × 10^{-3} s^{-1} and 1.7 × 10^{-3} s^{-1} for the α and β forms, respectively. These values agree with those obtained for the solutions containing glucose alone and thus demonstrate the selectivity of the glucose oxidase reactor.

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LITERATURE CITED

(1) Bowers, L. D.; Carr, P. W. *Anal. Chem.* **1976**, *48*, 544A.
(2) Gray, D. N.; Keyes, M. H.; Watson, B. *Anal. Chem.* **1977**, *49*, 1067A.
(3) Bowers, L. D.; Carr, P. W. *Adv. Biochem. Eng.* **1980**, *15*, 89.
(4) Bowers, L. D. *Trends Anal. Chem.* **1982**, *1*, 191.
(5) Bowers, L. D. *Anal. Chem.* **1986**, *58*, 513A.
(6) Guilbault, G. G. *Analytical Uses of Immobilized Enzymes*; Marcel Dekker: New York, 1984.
(7) Chibata, I. *Immobilized Enzymes*; Wiley: New York, 1978; Chapter 4.
(8) Weetall, H. H. Ed. *Immobilized Enzymes, Antigens, Antibodies, and Peptides*; Marcel Dekker: New York, 1975.
(9) Bowers, L. D.; Carr, P. W. *Immobilized Enzymes in Analytical and Clinical Chemistry*; Wiley: New York, 1980.
(10) Pigman, W.; Isbell, H. S. *Adv. Carbohyd. Chem.* **1968**, *23*, 11–57.
(11) Isbell, H. S.; Pigman, W. *Adv. Carbohyd. Chem. Biochem.* **1969**, *24*, 14–65.
(12) Ruzicka, J.; Hansen, E. H. *Flow Injection Analysis*; Wiley: New York, 1981; Chapter 8.
(13) Rios, A.; Luque de Castro, M. D.; Valcarcel, M. *Anal. Chim. Acta* **1986**, *179*, 463–468.
(14) Painton, C. C.; Mottola, H. A. *Anal. Chim. Acta* **1984**, *158*, 67–84.
(15) Koupparis, M. A. 2nd International Symposium on Kinetics in Analytical Chemistry, Preveza, Greece, Sept 1986.
(16) Hirano, H.; Baba, Y.; Yoza, N.; Ohashi, S. *Anal. Chim. Acta* **1986**, *179*, 209–216.
(17) Trinder, P. *Ann. Clin. Biochem.* **1969**, *6*, 24.
(18) Hudson, C. S.; Dale, J. K. *J. Am. Chem. Soc.* **1917**, *39*, 320–328.
(19) Stults, C. L. M.; Wade, A. P.; Crouch, S. R. *Anal. Chim. Acta* **1987**, *192*, 155–163.
(20) Patton, C. J.; Crouch, S. R. *Anal. Chim. Acta* **1986**, *179*, 189–201.
(21) Newcome, B. H.; Enke, C. G. *Rev. Sci. Instrum.* **1984**, *55*, 2017–2022.
(22) Ratzlaff, E. H. Ph.D. Dissertation, Michigan State University, East Lansing, MI, 1982.
(23) Nelder, J. A.; Mead, R. *Comput. J.* **1965**, *7*, 308.
(24) Kellin, D.; Hartree, E. F. *Biochem. J.* **1952**, *50*, 331–341.
(25) Livingston, G.; Franks, F.; Aspinall, L. J. *J. Solution Chem.* **1977**, *6*, 203–216.
(26) Murschhauser, H. *Biochem. Z.* **1920**, *110*, 181–192.

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