A KINETIC COMPARISON OF *STREPTOMYCES* GLUCOSE ISOMERASE IN FREE SOLUTION AND ADSORBED ON DEAE-CELLULOSE[1]

N. E. LLOYD and K. KHALEELUDDIN, Clinton Corn Processing Company, a Division of Standard Brands, Inc., Clinton, Iowa

Cereal Chemistry 53(2): 270-282

ABSTRACT

Mycelium from a *Streptomyces* sp. was treated with a dilute solution of a cationic surfactant to prepare an extract containing glucose isomerase (D-xylose ketol isomerase, EC 5.3.1.5). The isomerase was partially purified and immobilized by adsorption on DEAE-cellulose. Recovery of activity in the immobilized form was complete. The velocities of glucose-fructose interconversions were examined using the integrated form of the Michaelis-Menten equation for reversible reactions. Both the free and immobilized forms of the enzyme gave the same maximum velocities and Michaelis constants in batch reactions. The immobilized form gave reaction velocities in shallow-packed bed reactors which were slightly lower than in stirred-batch reactors. The lower velocity was attributed principally to channeling. The equilibrium fructose content of reaction mixtures varied from 46.5% at 30° C to 52.4% at 70° C.

Immobilization of enzymes on inert carriers has generated much interest over the past ten years and several good reviews of methods for their preparation, their properties, and utilization have been published (1,2,3). Although the potential for use of enzymes in immobilized form is great, only a few are presently used commercially, chiefly because of factors stemming from the high cost of preparing suitable carriers, the inability of most carriers to bind large amounts of enzyme, poor recovery of enzymatic activity in immobilized form, and lack of high enough stability of most immobilized enzymes under conditions of storage and use.

Glucose isomerase (D-xylose ketol isomerase, EC 5.3.1.5) is an enzyme of microbial origin of great industrial importance which is presently being used in immobilized form on a large scale for the preparation of fructose-containing corn syrups (4). Examination of the literature reveals that this enzyme has been immobilized in a variety of ways. These include treatment of whole cells containing glucose isomerase with heat or glutaraldehyde to destroy the cell's lytic mechanism and stabilize the cell-wall structure (5,6). The whole cells may be entrapped or enmeshed in a polymeric matrix which makes them more amenable to handling and use in fixed-bed reactors (7,8). Other methods involve the extraction of isomerase from the cells followed by immobilization on or within a variety of carriers, such as polyacrylamide gel (9), cellulose acetate fibers (10), porous glass (11), cellulose-titanium complexes (12), DEAE-Sephadex (13), and DEAE-cellulose (14,15). Adsorption of isomerase on DEAE-cellulose is a method which yields all of the features necessary for a practical system.

In this paper, the kinetics of the interconversion of glucose and fructose is described for the reactions catalyzed by soluble glucose isomerase from a *Streptomyces* sp. and for reactions catalyzed by the same isomerase adsorbed on DEAE-cellulose used in stirred-batch and fixed-bed reactors.

[1]Presented at the 59th Annual Meeting, Montreal, Canada, Oct. 1974.

MATERIALS AND METHODS

DEAE-cellulose was Whatman DE-23 from H. Reeve Angel, Inc. It was thoroughly washed with 1*M* hydrochloric acid, neutralized, and then washed with water before use. D-glucose was crystalline anhydrous α-D-glucose ($[\alpha]^{25}_{546.1}$ = 63.1, water, c = 2.5 g/100 ml). D-fructose was C.P. special grade from Pfanstiehl Laboratories ($[\alpha]^{25}_{546.1} = -106.2$, water, c = 2.5 g/100 ml). Both sugars were at least 99.8% pure as established by paper chromatography and high pressure liquid chromatography (described below). All other chemicals were of reagent grade quality.

Analytical Procedures

The contents of glucose and fructose in isomerization reaction mixtures were established from specific rotations determined at 25° C on solutions containing from 2 to 3 g sugar/100 ml in a Bendix automatic polarimeter, Series 1100, equipped with a digital readout device. The ratios of glucose to fructose in reaction mixtures from the study of the effects of temperature on the glucose-fructose equilibrium were also determined by high pressure liquid chromatography on a 2 ft × 3/8 in. o.d. stainless-steel column packed with Aminex AG50W × 4 (BioRad, 20–30 nm). Elution was with water at 0.4 ml/min and 75° C. Waters Associates' equipment was used, including a Model 201 chromatograph, Model R401 differential refractometer-detector, and Model M6000 pump. Output of the detector was evaluated with an Autolab Systems I computing integrator. Standard error was ±0.5% relative when samples containing 4 mg total sugars were injected. Activity of isomerase preparations was determined as described previously (16). One unit of activity is defined as that amount of isomerase which catalyzes the conversion of glucose to fructose at the rate of 1 μmol/min at 60° C and pH 7.0.

Partially Purified Isomerase Preparation.

Streptomyces sp. ATCC 21175 was cultured as described previously (17) and the fermented broth containing the *Streptomyces* mycelium treated with 0.02% $CoCl_2 \cdot 6H_2O$ and 0.15% dimethylbenzylalkylammonium chloride at pH 6.5 and 40° C for 6 hr to extract the isomerase. The mixture was filtered to remove the cell debris and 7.5 g of DEAE-cellulose added per liter of extract to adsorb the isomerase. The mixture was filtered, the filter cake washed with water and suspended in 10 ml of pH 7, 0.6*M* NaCl solution per g of cellulose, and stirred for 30 min to desorb the isomerase. The DEAE-cellulose was removed by filtration, 100 g of filter aid added per liter of filtrate, and the isomerase precipitated on the filter aid by addition of 2 vol of ethanol at 4° C. The precipitate was recovered by filtration, washed with cold ethanol and acetone, and dried at room temperature. This yielded an isomerase preparation having a specific activity of 4.5 units per mg protein (N × 6.25).

Determination of Equilibrium Constants

Equilibrium mixtures of glucose and fructose were prepared by isomerizing 2*M* glucose-fructose solutions initially having fructose-glucose ratios slightly higher or slightly lower than the equilibrium ratio so that equilibrium was approached from both directions. Isomerizations were conducted at the desired temperature and samples taken at intervals, until constant specific rotation was

attained. The samples were immediately acidified (pH <4) to stop enzymatic action. Glucose-fructose ratios were determined by liquid chromatography as described above and also from specific rotation.

Preparation of Batch Reactors

Batch reactions were run in 16-oz jars fitted with closures, propeller stirrers, and nitrogen inlets. The headspace of the reactor was filled with nitrogen during the reaction to avoid oxidation of the bisulfite buffer used for pH control. The jar was immersed in a water bath to maintain the contents at 65° ± 0.1° C. For reactions with the soluble isomerase, an isomerase solution was prepared by dissolving an appropriate amount of the partially purified isomerase preparation in water to provide a solution containing 20 units of activity per ml. An aliquot of the solution was heated rapidly to 65° C and was transferred quantitatively to the reactor containing substrate solution previously attemperated to 65° C. The substrate solution contained enough salts so that the final reaction mixture was 20 m*M* in pH 7.4 $NaHSO_3$–Na_2SO_3 buffer and 5 m*M* in $MgSO_4$. The reaction mixture was sampled periodically and the samples acidified and analyzed for glucose-fructose ratio polarimetrically as described above.

The batch reactions catalyzed by isomerase in adsorbed form were run in the same manner as just described except that 2.5 g of DEAE-cellulose per 1000 units of activity was added to the aliquot of isomerase solution and the mixture stirred 10 min before combining it with substrate solution in the reactor. Adsorption of the isomerase on the cellulose was complete. Stirring was maintained during the reaction period to ensure that the immobilized isomerase was uniformly suspended throughout the reaction mixture.

Fixed-Bed Reactors

For fixed-bed reactions, immobilized isomerase was prepared by complete adsorption of a known quantity of isomerase on DEAE-cellulose as described above. Portions containing from 250 to 1000 units of isomerase were packed into 1-in. i.d. glass columns which were 12 in. long and fitted with water jackets for temperature control (Ace Glass, Inc., Adjustachrom). The lower part of the column was packed with 0.3 g of filter aid, then with the immobilized isomerase, and finally with another layer of filter aid. The layer of immobilized isomerase was 0.5–2.0 cm thick. A stainless-steel perforated plate was placed on top of the packing and the upper portion of the column filled with 65° C substrate solution. Water was circulated through the jacket to maintain the column contents at 65° ± 0.2° C. After attemperation for 20 min, the column was pressurized with nitrogen at 4 psi and effluent withdrawn at the bottom through a peristaltic pump (Technicon, Inc.) which acted as a flow regulator. The column attained steady-state operation (effluent composition was constant) after three bed vol of substrate solution had passed, and samples were then collected for flow rate determination and analysis of glucose and fructose content. The column was then refilled with substrate solution and the degree of isomerization determined at a different flow rate. Isomerizations were carried out at from six to ten different flow rates using the same column packing within an 8-hr period. Flow rates were controlled so that conversions in the range of about 10 to 46% were obtained.

Theory

Reversible Michaelis-Menten kinetics can be generalized as follows:

$$S + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E + P$$

in which S, E, ES, and P represent substrate, enzyme, enzyme substrate complex, and product, respectively, and the k's are pseudo first-order rate constants. Haldane (18) showed that the steady-state velocity for this mechanism is given by

$$\frac{d(P)}{dt} = \frac{(E)\,[k_3\,(S)\,/K_s - k_2(P)\,/K_p]}{1 + (S)\,/K_s + (P)\,/K_p} \tag{1}$$

where t is time, (S) and (P) are substrate and product concentrations, (E) is the total concentration of enzyme, K_s is the Michaelis constant for substrate ($(k_2 + k_3)/k_1$), and K_p is the Michaelis constant for product ($(k_2 + k_3)/k_4$).

The equilibrium constant, K, for the overall reaction is given by

$$K = (P)_{eq}/(S)_{eq} = \frac{k_3\,k_p}{k_2\,k_s} \tag{2}$$

where $(P)_{eq}$ and $(S)_{eq}$ are product and substrate concentrations at equilibrium.

The following can be derived from equations 1 and 2:

$$\frac{dI}{dt} = \frac{k_3(E)\,(1 - I/I_e)}{(C)\,[1 + K_s/(C) + I(K_s/K_p - 1)]} \tag{3}$$

in which (C) is the combined concentration of substrate and product ((S)+(P)), I is the fractional conversion of substrate to product ((P)/(C)), and I_e is the fractional conversion at equilibrium ($(P)_{eq}/(C)$). Integration of 3 with initial conditions $I = I_o$ when $t = t_o$ gives

$$N = k_3(E)\,(t - t_o)/(C) \tag{4}$$

where

$$N = [I_e(K_s/(C) + 1) + I_e^2(K_s/K_p - 1)\,]\ln\left[\frac{I_e - I_o}{I_e - I}\right] - I_e(K_s/K_p - 1)\,(I - I_o)$$

Three types of reaction systems will be considered: 1) a homogenous reaction using soluble isomerase in a batch reactor, 2) a heterogenous reaction using immobilized isomerase in a stirred batch reactor, and 3) a heterogenous reaction with immobilized isomerase in a packed bed in which it is assumed that the packing is perfectly uniform, plug flow is attained, and the steady-state condition prevails, *i.e.*, at constant flow rate through the bed, composition of the effluent is constant when composition of the feed is constant.

An average specific production rate, $\bar{r}$, is defined as the amount of product produced in unit time with unit amount of enzyme. For batch reactions as in systems 1 and 2,

$$\bar{r} = \frac{V(C)(I - I_o)}{E_u(t - t_o)} \tag{5}$$

where E_u is the total units of enzyme present (in either soluble or immobilized form), $t - t_o$ is the contact time of enzyme with substrate solution, and V is the volume of reaction mixture for the homogenous reaction. For the reaction catalyzed by immobilized isomerase, V is the volume of the liquid phase only.

For a packed bed reactor (system 3), average contact time between the immobilized isomerase and substrate solution is also taken as $t - t_o$, where t_o is the time at which the solution enters the packing and t the time it leaves the packing. If the void volume of the packing is taken as V, then flow rate, R, through the packing is

$$R = \frac{V}{t - t_o} \tag{6}$$

Therefore, the average specific rate for the steady-state fixed-bed reactor is obtained by combining equations 5 and 6 to give

$$\bar{r} = \frac{R(C)(I - I_o)}{E_u} \tag{7}$$

where I_o is the fractional conversion of the feed and I the fractional conversion of the effluent.

Setting $(E) = E_u / V$ in equation 4, multiplying by $(I - I_o)$, and rearranging gives

$$\bar{r} = \frac{V(C)(I - I_o)}{E_u(t - t_o)} = \frac{R(C)(I - I_o)}{E_u} = \frac{k_3(I - I_o)}{N} \tag{8}$$

The above equations relate average specific rate in batch reactions to that of a packed bed in terms of the basic-rate constants and the fractional conversion. This allows determination of the rate constants from the easily measured or controlled variables, V, (C), I, I_o, t, t_o, E_u, and R.

To evaluate average specific rate and fractional conversion data, the reciprocal form of equation 8 is used, giving

$$\frac{1}{\bar{r}} = A \cdot f(I) + B \tag{9}$$

where

$$A = \frac{I_e(K_s/(C) + 1) + I_e^2(K_s/K_p - 1)}{k_3}$$

$$B = \frac{I_e(1 - K_s/K_p)}{k_3}$$

$$f(I) = \left[\frac{1}{I - I_o}\right] \ln \left[\frac{I_e - I_o}{I_e - I}\right]$$

A plot of $1/\bar{r}$ *vs.* f(I) should therefore yield a straight line with slope equal to A and intercept equal to B. When $K_s = K_p$, the intercept, B, is equal to zero. When K_s and K_p are not equal, B for the forward reaction (glucose as substrate) is equal in absolute magnitude to that for the reverse reaction (fructose as substrate) but is opposite in sign. The slope, A, is dependent on the combined substrate and product concentrations, (C), and decreases as the latter increases.

The specific rate, $\bar{r}$, is at a maximum for any given value of (C) when I = O. Thus, average specific rate has a limiting maximum value (here termed r) which is the specific velocity at the beginning of batch reactions when conversion is zero (I = O) and which is approached in fixed-bed reactions as flow rate approaches infinity (I→O). In the right-hand side of equation 9, when $I_o = 0$

$$\lim_{I \to 0} f(I) = \frac{1}{I_e} \tag{10}$$

Thus, a plot of $1/\bar{r}$ *vs.* f(I) extrapolated to $f(I) = 1/I_e$ will give the reciprocal of the limiting rate, $1/r$. Substituting the limiting values for $1/\bar{r}$ and f (I) in equation 9 and simplifying yields

$$\frac{1}{r} = \frac{K_s}{k_3} \cdot \frac{1}{(C)} + \frac{1}{k_3} \tag{11}$$

This is equivalent to the familiar double reciprocal form of the Michaelis-Menten equation and gives the basis for evaluation of the Michaelis constant and first-order rate constant, k_3, from either batch reactions or from fixed-bed reactions under steady-state conditions.

RESULTS AND DISCUSSION

Data Reduction

Figure 1 shows typical conversion curves for the isomerization of glucose and fructose in batch reactions. The velocities of the reactions are at their maxima at the beginning of the conversions and approach zero near the equilibrium point. Usually plots of I *vs.* t did not extrapolate to the origin. This was due to slight errors in timing and difficulty in bringing the reaction mixtures to the exact desired temperature in the first few minutes of the reaction. Therefore, the value of t_o used to calculate $\bar{r}$ according to equation 5 was established by multiple linear regression using a second-order model as follows:

$$I = \beta_o + \beta_1 t + \beta_2 t^2 + \epsilon \tag{12}$$

where I and t are defined as above, β_o, β_1, and β_2 are regression coefficients, and ϵ is error. Values of I predicted by the equation usually were within ± 0.002 of the actual values for data within the interval I = 0 to 0.4. The value of t_o was found by solving the regression equation for t when I = 0.

The value of I_e for each individual conversion curve determined in batch reactions was found using the following function:

$$\frac{1}{I} = \frac{1}{I}\left(e^{-0.693t/\tau}\right) + \frac{1}{I_e}$$

wherein τ is the time to reach one-half maximum conversion ($I = I_e/2$) as found from visual inspection of the plots of I *vs.* t as illustrated in Fig. 1. In the above function, I approaches I_e as t approaches infinity. Figure 2 shows a typical plot wherein data points in the range of $I = 0.4$ and higher were used. The reciprocal of the intercept on the vertical axis gives the value of I_e. The average value of the equilibrium fructose content at 65° C calculated from all batch conversions was 51.9%, in good agreement with the data shown in the table at the end of this paper. In calculating the kinetic parameters from data obtained with the packed-bed reactors, an equilibrium fructose of 51.4% was used.

Data in the approximate range of $I = 0.1$ to 0.45 were the most useful for determining the kinetic parameters according to equation 9. This is because a small error in I results in a large error in the calculated value of $\bar{\tau}$ when I is small. When $I > 0.45$, small errors in I (or I_e) give large deviations in f(I). These situations are illustrated in Fig. 3.

Reactions Catalyzed by Soluble Isomerase

Conversion data obtained from isomerization of glucose at five different initial concentrations and plotted according to equation 9 are shown in Fig. 4. All plots tend toward a common intercept as predicted by theory. Data on the conversion of fructose to glucose gave results very similar to those shown for glucose conversion. The limiting values of $1/\bar{\tau}$ obtained by extrapolation of the plots to $f(I) = I/I_e$ yielded straight lines when plotted *vs.* the reciprocal of the combined

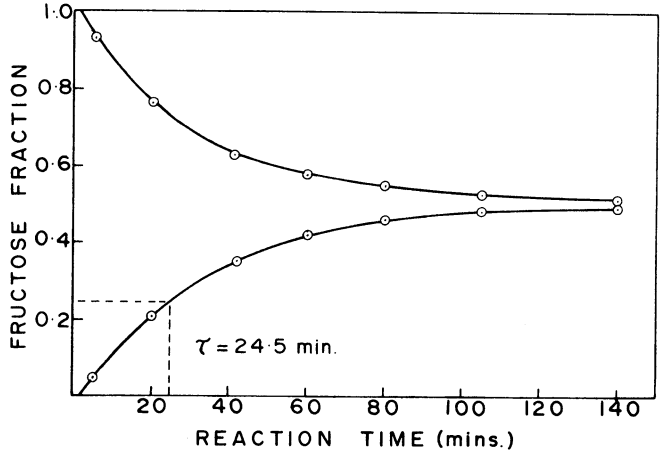

Fig. 1. Typical results for the conversion of glucose (lower curve) and fructose (upper curve) with soluble glucose isomerase at 65° C, pH 7.3. Substrate concentration was 0.8*M*, enzyme concentration 6.7 units/ml. Estimation of the time of half-maximum conversion (τ) is shown by the dotted line.

concentration of substrate and product as predicted by equation 11. The reversible Michaelis-Menten mechanism holds over the entire course of the reaction and for a wide range of substrate concentrations.

The turnover number of the isomerase (for glucose as substrate) is calculated to be 7300 min^{-1} based on the value for k_3 shown in Table I, with a molecular

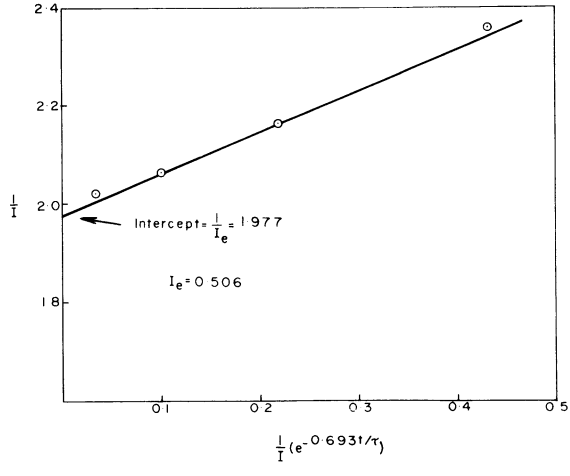

Fig. 2. Estimation of equilibrium fractional conversion, I_e, according to equation 13 in the text, using data from the lower curve in Fig. 1. The value of the intercept on the vertical axis gives the reciprocal of I_e.

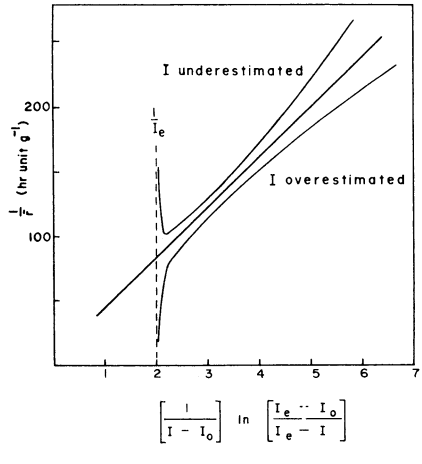

Fig. 3. Influence of small errors in the determination of I on plots of $1/\bar{\tau}$ *vs.* f(I) according to equation 9 in the text. The central line was plotted for $I_e = 0.5$, $k_3 = 0.025$ g hr^{-1} $unit^{-1}$, $K_s = K_p = 0.25M$, and (C) $= 0.25M$. The curved lines correspond to consistent underestimation or overestimation of I by 0.01.

weight of 172,000 and a specific activity of 21.8 units per mg protein for the pure enzyme.[2] Thus, glucose isomerase is a relatively fast-acting enzyme since most enzymes have turnover numbers of 10 to 10^4 min^{-1} (19).

Batch Reactions with Immobilized Isomerase

Isomerizations were run in the exact same manner as for the reactions with soluble enzyme above, with the exception that the isomerase was completely adsorbed on DEAE-cellulose just before initiation of the reactions. The results for the isomerization of glucose are given in Fig. 5, and are practically identical to those for the soluble form of the isomerase at substrate concentrations above about 0.2*M*. At low concentration, reaction velocity was slightly higher for the reaction catalyzed by the adsorbed isomerase. Fructose conversion with the adsorbed form of isomerase gave results indistinguishable from the conversions with the soluble form in batch reactions.

The kinetic parameters, summarized in Table I, show that the maximum velocities attainable with the free and adsorbed forms of isomerase are equivalent.

The equilibrium constant calculated from the kinetic data according to equation 2 is in only fair agreement with the value obtained by direct analysis of equilibrium mixtures. The lack of better agreement reflects inaccuracies in the determination of the Michaelis constants.

The fact that recovery of activity in the adsorbed form is complete indicates that the isomerase molecule is bound to the support with the active site(s) fully exposed. There is no rate limitation due to mass transfer within the pores of the immobilized isomerase particles since, by direct measurement, the effectiveness factor is 1 (effectiveness factor is defined as average reaction rate within the pores of a solid catalyst divided by the rate if not slowed by diffusion resistance) (20). An effectiveness factor near 1 is in accord with what would be expected based on the high surface area and open structure of cellulose fibers (21).

Column Reactions

Figure 6 gives results of isomerizations of glucose run in packed beds of the immobilized isomerase. Fructose conversions run in the same manner yielded similar plots. The plots are linear as expected but do not converge to a common intercept near the origin. Thus, equation 9 is not general for all substrate concentrations. The lack of agreement with theory could be due to three factors, channeling, film diffusion resistance, and mutarotation effects. The packed beds were relatively shallow (0.5–2.0 cm) and it is probable that channeling was an important factor. Isomerase converts only the α-anomer of glucose to fructose (22) so that very rapid isomerizations could be limited by mutarotation. Mutarotation effects would be most rate-limiting at substrate concentrations lower than the Michaelis constant where reaction velocity is substrate-concentration-dependent. Also, contact times between catalyst and substrate at the lower concentrations were short, ranging from 0.13 to 2.0 min for the reactions run on the 0.1 and 0.2*M* substrate solutions. The influence of all three factors should disappear as flow rate is increased and the value of I approaches

[2]Molecular weight and specific activity of the isomerase are unpublished data from this laboratory supplied by R. A. Johnson.

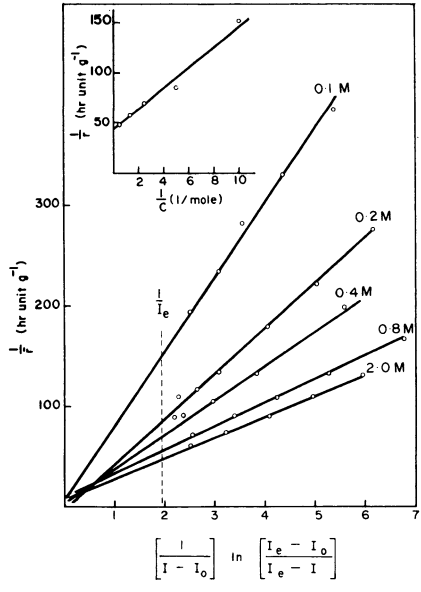

Fig. 4. Isomerization of glucose with soluble glucose isomerase at pH 7.3, 65° C, at five different initial glucose concentrations as shown (lower figures). Solutions were 20 m*M* in sodium bisulfite buffer and 5 m*M* in magnesium sulfate. Data are plotted according to equation 9 in the text. Points on the lower portion of the lines are from data obtained during the first part of the conversions. All lines were fitted by least squares and were extrapolated to $f(I) = 1/I_e$ to find the reciprocal of the initial rate, $1/r$, which is plotted *vs.* the reciprocal of the substrate concentration, $1/(C)$, in the upper left-hand corner according to equation 11.

TABLE I
Kinetic Parameters Obtained for Glucose-Fructose Interconversion[a]

Form of Isomerase	Reactor Type	Kinetic Parameters[b] for Glucose k_3 (g hr^{-1} $unit^{-1}$)	for Glucose K_G[c] (mol/l.)	for Fructose k_2 (g hr^{-1} $unit^{-1}$)	for Fructose K_F[c] (mol/l.)	Equilibrium Constant K[d]
Free	Batch	0.024	0.25	0.028	0.26	0.89
Immobilized	Stirred batch	0.025	0.17	0.027	0.23	1.25
Immobilized	Packed bed	0.026	0.21	0.037	0.32	1.07

[a]Reactions were run at 65° C and pH 7.3 in the batch mode with free and immobilized isomerase, and in the packed-bed mode with the immobilized form. Substrate solutions were 20 m*M* in sodium bisulfite buffer and 5 m*M* in magnesium sulfate.
[b]See text for definition of symbols.
[c]K_G and K_F are Michaelis constants for glucose and fructose, respectively.
[d]Equilibrium constant calculated according to equation 2 in the text. The equilibrium constant at 65° C calculated from the data in Table II is 1.05.

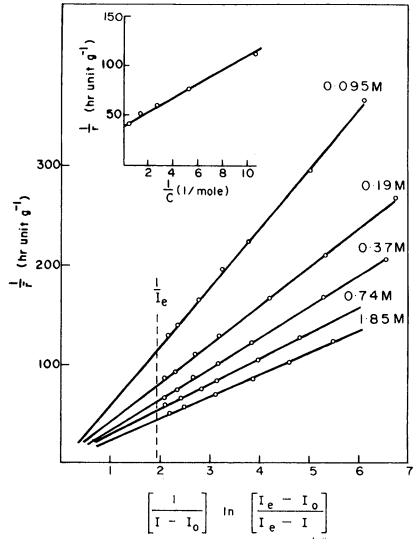

Fig. 5. Isomerizations of glucose with immobilized glucose isomerase in stirred-batch reactors at five different initial glucose concentrations as shown. See Fig. 4 for conditions and explanation.

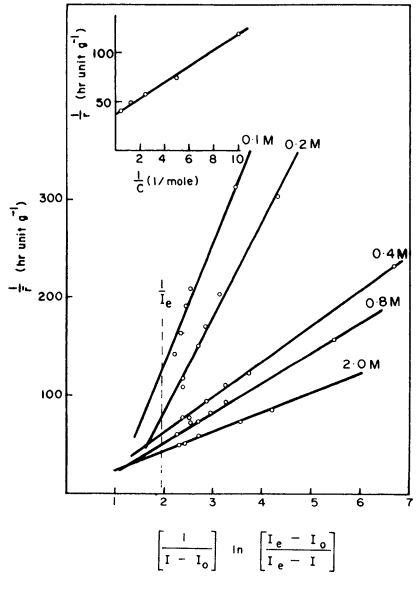

Fig. 6. Isomerization of glucose in packed beds of immobilized glucose isomerase at five different initial glucose concentrations. Each point on the plots in the lower section represents isomerizations carried out at a different flow rate under steady-state operation with the higher flow rates giving lower conversion and, hence, lower values of f(I) defined in equation 9 in the text. See Fig. 4 for conditions and further explanation.

zero. Consequently the plots tend to give the correct values for the limiting rate, r, and for the kinetic parameters calculated therefrom (see Table I). However, the data are less accurate than those based on batch reactions.

Glucose-Fructose Equilibria

The conversion of glucose to fructose is slightly endothermic. The equilibrium sets the upper practical limit to which glucose can be converted to fructose and so is of especial importance in the commercial production of fructose containing syrups by isomerization. Equilibrium fructose contents at different temperatures are given below.

Temperature °C	*Fructose at Equilibrium*
30	46.5
45	48.2
60	49.9
70	52.4

Isomerizations were conducted at pH 7.3 on 2*M* solutions of glucose and fructose which were 20 m*M* in sodium bisulfite buffer, 5 m*M* in magnesium sulfate and contained 15 to 35 units of isomerase per g sugar. Equilibrium was approached from both directions and the results shown are the average of at least four determinations. The equilibrium point is about the same as reported by Takasaki (23) at the low temperature, but is lower at 70° C by about 5% fructose. The heat of reaction (ΔH) calculated from the data reported here is 1080 cal/mol.

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[Received January 13, 1975. Accepted June 30, 1975]