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Kinetic Studies on the Oxidation of Glucose by Immobilized Glucose Oxidase[1)]

TOYOHISA TSUKAMOTO, HIDEKI NOMURA, SHUSHI MORITA, and JUTARO OKADA*

Faculty of Pharmaceutical Sciencies, Kyoto University, Yoshida-Shimoadachi-cho, Sakyo-ku, Kyoto 606, Japan

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The kinetics of oxidation of D-glucose in the presence of glucose oxidase and catalase was studied in a stirred-tank reactor. Experiments were performed using free or immobilized enzyme in 0.1 M acetate buffer solution (pH = 5.5) at atmospheric pressure and 25 °C. The immobilized enzyme catalyst, which consisted of both glucose oxidase and catalase supported on activated carbon, was prepared by the carbodiimide method. The initial rate of reaction was determined from the amount of oxygen consumed in the absence of external mass transfer resistances.

Using free enzyme, the existence of competitive product inhibition by δ-D-gluconolactone was confirmed from Lineweaver–Burk plots, and our results could be well explained by the single substrate mechanism. The effect of mutarotation on the rate of reaction was also taken into account.

The activity of immobilized enzyme catalyst was reduced to about 30% of that of the free enzyme. However, the Michaelis and product inhibition constants were not appreciably changed by immobilization. The time-course data were well explained by the proposed kinetic model.

Keywords——kinetics; oxidation; D-glucose; inhibition; δ-D-gluconolactone; mutarotation; intraparticle mass transfer; glucose oxidase; catalase; immobilized enzyme

It is well known that the immobilization of enzymes on insoluble supports offers several advantages for their use in enzyme reactors. In the previous paper,[2)] we discussed the effects of immobilization on the global rate of enzymatic reaction, for the oxidation of D-glucose by oxygen using a catalyst consisting of glucose oxidase and catalase both immobilized on activated carbon. The most significant factor was the intraparticle mass transfer of oxygen, which is inherent in heterogeneous operation. Also, the maximum velocity was reduced to some extent, due to enzyme deactivation during the immobilization process and to steric hindrance of the immobilized enzyme. On the other hand, the Michaelis constant for glucose was found to be nearly the same as that in the soluble system.

In this paper, our attention is focused on formulation of the rate of glucose oxidation on the immobilized enzyme up to high conversion. For this purpose, the mutarotation between α- and β-forms of glucose and the product inhibition in the immobilized enzyme system were considered to be additional factors of importance.

Oxidation of D-glucose (G) in the presence of both glucose oxidase and catalase proceeds according to the following reaction scheme:[3)]

$$\alpha G \rightleftharpoons \beta G \tag{1}$$

$$\beta G + O_2 \xrightarrow{\text{glucose oxidase}} P + H_2O_2 \tag{2}$$

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + \frac{1}{2} O_2 \tag{3}$$

The glucose oxidase is highly specific for β-D-glucose (βG); the α anomer (αG) is not affected.[4] In solution, reaction (1) is reversible.

Several mechanisms have been proposed for reaction (2).[5-7] There are two representative mechanisms, one being the Michaelis–Menten equation for the case of a single substrate[7] and the other being the two-substrate mechanism.[5,6] The product which most strongly retards the rate of reaction is hydrogen peroxide, which is produced in reaction (2).[8-11] When excess catalase is used to promote the decomposition of hydrogen peroxide, this problem becomes negligible.[2] Inhibition by the other product, δ-D-gluconolactone (P), has been proposed by Nakamura *et al.*[5]

Experiments were performed using free or immobilized enzyme in 0.1 M acetate buffer solution (pH = 5.5) at atmospheric pressure and 25 °C. Hereafter, experiments with the free or immobilized enzyme are referred to as the soluble or insoluble system, respectively. The initial rates of reaction were determined from measurements of oxygen absorption in a stirred-tank reactor under conditions of no external mass transfer resistances. The cumulative amounts of oxygen were also measured in the insoluble system to check the applicability of the kinetic model, which was established from the initial rate data, up to high conversion.

Experimental

Chemicals——Glucose oxidase (EC 1.1.3.4, from *Aspergillus niger*, 24300 units/g stated activity) and catalase (EC 1.11.1.6, from bovine liver, 2000 units/mg stated activity) were both purchased from Sigma Chemical Co. δ-D-Gluconolactone was purchased from Wako Pure Chemical Industries Ltd. All other reagents used were the same as described in the previous paper.[2] The buffered solution of D-glucose was allowed to stand overnight, and was pre-equilibrated before use.

Preparation of Immobilized Enzyme Catalyst——The immobilized enzyme catalyst was prepared in the same manner as described in the previous paper.[2] In this study, two average particle sizes (d_p) of activated carbon were chosen for the support: $d_p = 0.0069$ cm (200 to 250 mesh) and $d_p = 0.0163$ cm (80 to 100 mesh). For one gram of activated carbon, 70 mg of glucose oxidase and 20 mg of catalase were used. The physical properties of the immobilized enzyme catalyst are given in Table I of the previous paper.[2]

Apparatus and Operating Procedure——The glass reactor was 7.0 cm in diameter, and four baffles (0.78 cm in width, stainless steel) were fitted to the wall of the reactor. The stirrer was a six-bladed turbine impeller (2.5 cm in diameter, stainless steel) located at 2.3 cm above the bottom of the reactor. The liquid height was 5.2 cm. Two hundred ml of buffered glucose solution (δ-D-gluconolactone was dissolved as well when necessary) was saturated with oxygen or air by continuous bubbling. The temperature was kept constant at 25 °C, and the stirring speed was 1500 rpm. In the previous paper,[2] external mass transfer resistances (gas-to-liquid and liquid-to-solid) were found to be negligible at stirring speeds above 1300 rpm. Unless otherwise noted, the reaction was initiated by adding 1 g of immobilized enzyme catalyst or, in the soluble system, 20 mg of glucose oxidase and 15 mg of catalase. The volume of oxygen gas consumed during the reaction was followed at regular intervals by means of a gas burette.

The rate of reaction was evaluated from the oxygen consumption rate. In the presence of excess catalase, one mol of oxygen consumed corresponds to 2 mol of β-D-glucose reacted.

Results and Discussion

Soluble System

In the soluble system, the initial rate of reaction was measured by changing the concentrations of D-glucose, δ-D-gluconolactone and oxygen in the liquid. The Lineweaver–Burk plots are shown in Fig. 1. Since the reaction was initiated in the pre-equilibrated glucose solution, the concentration of β-D-glucose, $C_{\beta G}$ [mol/cm^3], is calculated by assuming that the equilibrium constant, K_e [−], is 1.7.[12,13]

$$\alpha G \underset{k_\beta}{\overset{k_\alpha}{\rightleftharpoons}} \beta G \qquad (4) \qquad \text{and} \qquad K_e = k_\alpha / k_\beta \qquad (5)$$

where k_α [1/s] and k_β [1/s] are first-order rate constants. When the liquid is saturated with

oxygen, three lines for different δ-D-gluconolactone concentrations, C_P [mol/cm³], meet at the same intercept on the ordinate as shown in Fig. 1, indicating typical competitive product inhibition.[14]

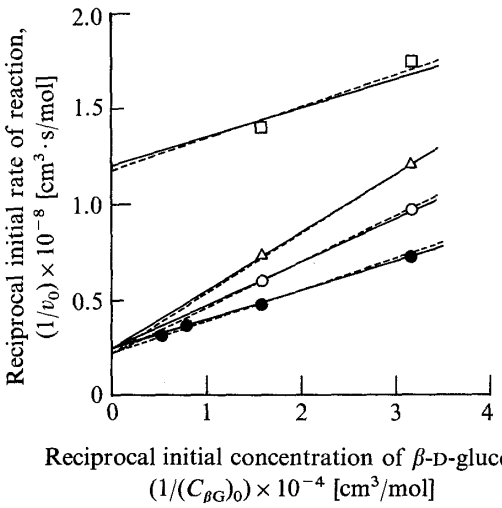

Fig. 1. Lineweaver–Burk Plot for the Oxidation of D-Glucose by Glucose Oxidase in the Soluble System

Reaction temperature, 25 °C; pH, 5.5 (in 0.1 M acetate buffer). Concentration of oxygen in the liquid [mol/cm³], concentration of δ-D-gluconolactone [mol/cm³]: ●, 1.12×10^{-6}, 0; ○, 1.12×10^{-6}, 5.0×10^{-5}; △, 1.12×10^{-6}, 1.0×10^{-4}; □, 0.234×10^{-6}, 0. Single substrate mechanism, ——; two-substrate mechanism, ------.

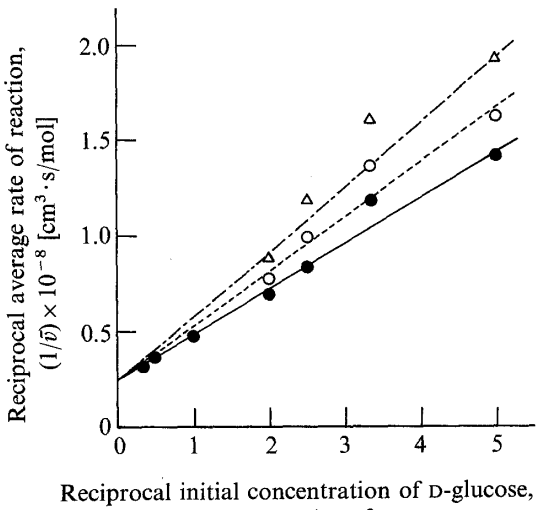

Fig. 2. Dependence of the Average Rate of Reaction on the Mutarotation of D-Glucose

Reaction temperature, 25 °C; pH, 5.5 (in 0.1 M acetate buffer); concentration of oxygen in the liquid [mol/cm³], 1.12×10^{-6}. Initial rate of reaction as observed and as calculated from Eq. (6): ●, ——. Average rate of reaction during the initial 5 min as observed and as calculated from Eqs. (8)—(10): ○, ------. Average rate of reaction during the initial 10 min as observed and as calculated from Eqs. (8)—(10): △, – – – –.

TABLE I. The Estimated Values of Kinetic Parameters[a]

	Soluble system		Insoluble system	
	Previous work[b]	This work[b]	Previous work[b,c]	This work[b,c]
k, cm³/(unit·s)	1.3×10^{-2}	1.5×10^{-2}	4.1×10^{-3} [d]	4.0×10^{-3} [d]
k_C, cm³/(g·s)	—	—	7.4	6.8
K, –	41[e]	54	53[f]	46
K_P, mol/cm³	—	9.8×10^{-5}	—	6.2×10^{-5}
D_e, cm²/s	—	—	7.8×10^{-6}	6.3×10^{-6}
τ[g], –	—	—	2.0	2.5

a) At 25 °C and in 0.1 M acetate buffer (pH = 5.5).
b) Activity of glucose oxidase used: previous work, 25.6 unit/mg; this work, 24.3 unit/mg.
c) 70 mg of glucose oxidase was immobilized per 1 g of activated carbon.
d) Calculated by dividing the estimated k_C by the number of units of glucose oxidase used for 1 g of catalyst.
e) Calculated from the originally presented value of $K_m = 7.21\times10^{-5}$ [mol/cm³][2] which had been obtained on the basis of the total glucose (α- plus β-form), with the use of the equilibrium constant ($K_e = 1.7$ [−][3]) and the concentration of oxygen in the liquid saturated with oxygen ($C^*_{O_2} = 1.12\times10^{-6}$ [mol/cm³][2]).
f) Calculated from the originally presented value of $K_m = 9.46\times10^{-5}$ [mol/cm³][2] in the same manner as described in footnote *e*).
g) Calculated by $\tau = D\varepsilon_P/D_e$, where D [cm²/s] is the molecular diffusivity of oxygen and ε_P [−] is the porosity of particles. The value of D was assumed to be 2.3×10^{-5} [cm²/s][15] and the value of ε_P was 0.684 [−] (measured).

In the previous work,[2] the influence of oxygen concentration in the liquid, C_{O_2} [mol/cm^3], on the intrinsic rate of reaction was examined in the absence of δ-D-gluconolactone. The rate data were well described by the Michaelis–Menten equation for a single substrate, in which the maximum velocity, V_{max} [mol/(unit·s)], and the Michaelis constant, K_m [mol/cm^3], were both proportional to the concentration of oxygen in the liquid. Taking into account the competitive inhibition by δ-D-gluconolactone, the rate of reaction, v [mol/(cm^3·s)], can be expressed by

$$v=\frac{V_{max}C_E C_{\beta G}}{K_m\left(1+\frac{C_P}{K_P}\right)+C_{\beta G}}=\frac{kC_{O_2}C_E C_{\beta G}}{KC_{O_2}\left(1+\frac{C_P}{K_P}\right)+C_{\beta G}} \tag{6}$$

where K_P [mol/cm^3] is the competitive product inhibition constant, C_E [unit/cm^3] is the concentration of glucose oxidase, and k [cm^3/(unit·s)] and K [−] are the proportional coefficients of V_{max} and K_m, respectively.

The values of parameters in Eq. (6) estimated by means of multi-regression analysis are summarized in Table I together with those obtained in the previous work[2] (except K_P). The parameter values are in fair agreement with each other, even though different lots of glucose oxidase were used. With these parameter values, the calculated lines based on Eq. (6) are indicated by the solid lines in Fig. 1.

The Michaelis–Menten equation for two substrates, based on the so-called ping-pong mechanism, can be written as follows:

$$v=\frac{k'C_{O_2}C_E C_{\beta G}}{C_{O_2}C_{\beta G}+K_m^G C_{O_2}\left(1+\frac{C_P}{K_P}\right)+K_m^O C_{\beta G}} \tag{7}$$

The dotted lines in Fig. 1 were calculated from Eq. (7) using the values of constants k', K_m^G, K_m^O and K_P estimated by the multi-regression analysis. Since the solid and dotted lines seemed not to be significantly different, Eq. (6) was employed in the remaining parts of this paper.

In this study, the initial rate of reaction was evaluated from the slope at the beginning of the reaction, which was obtained by extrapolating the plots of the amount of oxygen consumed. When the initial concentration of β-D-glucose was high and the conversion was very low, the plots were nearly linear, so that the errors in the measurements were not large. However, at low concentrations, the errors inherent in this method become serious. For example, at the initial concentration of β-D-glucose ($(C_{\beta G})_0$) of 1.26×10^{-5} [mol/cm^3], about 14% of β-D-glucose was converted during the first 5 min and the plots were so curved that the rate could not be evaluated accurately. This implies that the mutarotation of glucose should be taken into account, especially at low concentration levels, since the rate of mutarotation is slow compared to the rate of oxidation.

Figure 2 shows the average rate of reaction during the initial 5 and 10 min. The solid line is calculated from Eq. (6) for $C_P=0$ and $C_{\beta G}=(C_{\beta G})_0$. It is clear that, in the region of low glucose concentration, the reciprocal rates are much higher than the solid line. Taking into account the mutarotation (Eq. (4)), the following differential equations can be developed.

$$v=\frac{1}{2}\frac{dC_P}{dt}=\frac{kC_{O_2}C_E C_{\beta G}}{KC_{O_2}\left(1+\frac{C_P}{K_P}\right)+C_{\beta G}} \tag{8}$$

$$\frac{dC_{\beta G}}{dt}=-\frac{dC_P}{dt}+k_m\{(C_{\beta G})_0-C_{\beta G}\}+k_\alpha\{(C_P)_0-C_P\} \tag{9}$$

where k_m [1/s] is the mutarotation constant ($=k_\alpha+k_\beta$), and $(C_{\beta G})_0$ [mol/cm^3] and $(C_P)_0$ [mol/cm^3] are the initial concentrations of β-D-glucose and δ-D-gluconolactone, respectively. The dotted and chained curves in Fig. 2 are the average rates during the initial 5 and 10 min, respectively, obtained by the numerical integration of Eqs. (8) and (9) with $k_m=4.0\times10^{-4}$ [1/s].[12] As shown in Fig. 2, the data agreed well with the calculated curves.

Insoluble System

Next, experiments were carried out in a slurry reactor, where the catalyst immobilized on activated carbon was suspended in the reaction mixture by stirring. The buffered solution of D-glucose (and δ-D-gluconolactone when necessary) pre-saturated with oxygen was kept at 25 °C. The measurements were initiated after adding the catalyst particles to the solution. After an induction period of 1—2 min, the rate of oxygen consumption appeared to be linear during the subsequent 5 min, even when the concentration of glucose was low. The rates of reaction in the insoluble system were considerably lower than those in the soluble system because of the intraparticle mass transfer resistance and the reduced catalyst activity owing to immobilization. In Fig. 3, the results are shown for both particle sizes.

Because of low oxygen solubility, the concentration of oxygen in the liquid was much smaller than that of β-D-glucose. Therefore, it could be considered that the limiting substance is oxygen and that the concentration of β-D-glucose (and δ-D-gluconolactone when added in advance) is uniform throughout the reaction system. In other words, the initial rate of reaction in the insoluble system can be determined by the intraparticle mass transfer of oxygen and the reaction of oxygen on the immobilized enzyme catalyst. The catalyst particle may be regarded as a sphere. Assuming the pseudo-steady state, the mass balance equation for oxygen inside the catalyst pores can be expressed as

$$\frac{d^2C_i}{dr^2}+\frac{2}{r}\frac{dC_i}{dr}-\frac{\rho_P}{D_e}\frac{k_C C_i C_{\beta G}}{KC_i\left(1+\frac{C_P}{K_P}\right)+C_{\beta G}}=0 \tag{10}$$

where C_i [mol/cm^3] is the concentration of oxygen inside the catalyst particle, r [cm] is the radial distance from the center of the catalyst particle, ρ_P [g/cm^3] is the particle density, D_e

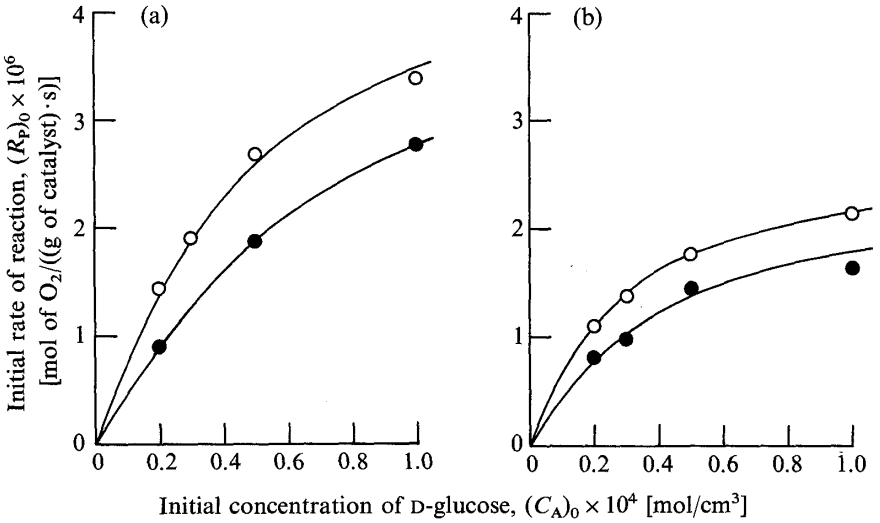

Fig. 3. Initial Rate of Reaction in the Insoluble System for $d_P=0.0069$ cm (a) and $d_P=0.0163$ cm (b)

Reaction temperature, 25 °C; pH, 5.5 (in 0.1 M acetate buffer); concentration of oxygen in the bulk liquid [mol/cm^3], 1.12×10^{-6}; concentration of catalyst [g/cm^3], 5.0×10^{-3}. Concentrations of δ-D-gluconolactone [mol/cm^3]: ○, 0; ●, 5.0×10^{-5}.

[cm^2/s] is the effective diffusivity of oxygen inside the catalyst particle and k_C [$cm^3/(g \cdot s)$] is the reaction rate constant based on the mass of catalyst. The boundary conditions for Eq. (10) can be written as

$$\text{at} \quad r=0; \quad \frac{dC_i}{dr}=0 \tag{11}$$

$$\text{at} \quad r=r_S; \quad C_i=C^*_{O_2} \tag{12}$$

where r_S [cm] is the radius of the catalyst particle and $C^*_{O_2}$ [mol/cm^3] is the concentration of oxygen in the liquid saturated with pure oxygen. The rate of reaction R_P, expressed in [mol of oxygen/((g of catalyst)·s)] can be written as

$$R_P=\frac{3D_e}{r_S\rho_P}\left(\frac{dC_i}{dr}\right)_{r=r_s} \tag{13}$$

Based on the initial rate data shown in Fig. 3, the values of k_C, K, K_P and D_e were estimated by nonlinear least-squares analysis by using Eqs. (10)—(13), where $\sum\{(R_P)_{obsd}-(R_P)_{calcd}\}^2$ was minimized. The confidence region for each estimated value was within $\pm 20\%$ of the converged value. The estimated values of different parameters are summarized in the table together with those obtained in the previous work[2] (except K_P). The best fit curves are presented in Fig. 3.

To compare the activity of the immobilized enzyme catalyst with that of the free enzyme, the value of k_C in [$cm^3/(g \cdot s)$] was converted into k in [$cm^3/(unit \cdot s)$] (see footnote *d*) in the table). Here, the "unit" should be taken as the unit number of glucose oxidase used for reaction. In this and previous studies, the value of k for the insoluble system was reduced to nearly the same extent, *i.e.*, to about 30% of that of the soluble system. This reduction may be mainly attributed to enzyme deactivation during the catalyst preparation stage and to steric hindrance of the immobilized enzyme.

In a number of kinetic studies in immobilized enzyme reactors, the apparent Michaelis constant, K_m (app), has been found to become much larger than that of the free enzyme with increasing mass transfer effect.[16] For example, in a kinetic study on the transformation of urea by urease, a more than 50 times larger value of K_m (app) than K_m was reported when the enzyme was entrapped in a hollow fiber reactor, where the rate-determining step was the permeation of the substrate across the membrane.[16a] The K in the table corresponds to the Michaelis constant for β-D-glucose since $K_m=KC_{O_2}$ (see Eq. (6)). In our work, the reaction was initiated by adding the immobilized catalyst to the buffered reaction mixture. Thus, it took a short time for β-D-glucose (and δ-D-gluconolactone when added) to take up a uniform concentration profile inside the catalyst pores. In the previous work, the value of K in the insoluble system was larger than that of the soluble system, but the opposite result was obtained in this work. These differences were too small to regard them as significant, indicating that the Michaelis constant did not change on immobilization and that the mass transfer effect of β-D-glucose inside the pores was negligible.

The product inhibition constant (K_P) in the insoluble system was about two-thirds of that in the soluble system. The changes in enzyme characteristics might be due to the immobilization, or the product concentration inside the pores might be different from that in the bulk liquid. However, taking into account the experimental errors and sample sizes, the difference is not large and both values can be considered to be essentially the same.

As for the effective diffusivity of oxygen inside the pores (D_e), the values in this and previous studies are in reasonable accord. The estimated value of D_e gives a tortuosity factor of 2.5 [−] for the activated carbon used. Tortuosity factors of the order of 2 to 5 have been reported for several kinds of porous particles,[17] so that the obtained value of D_e seems reasonable.

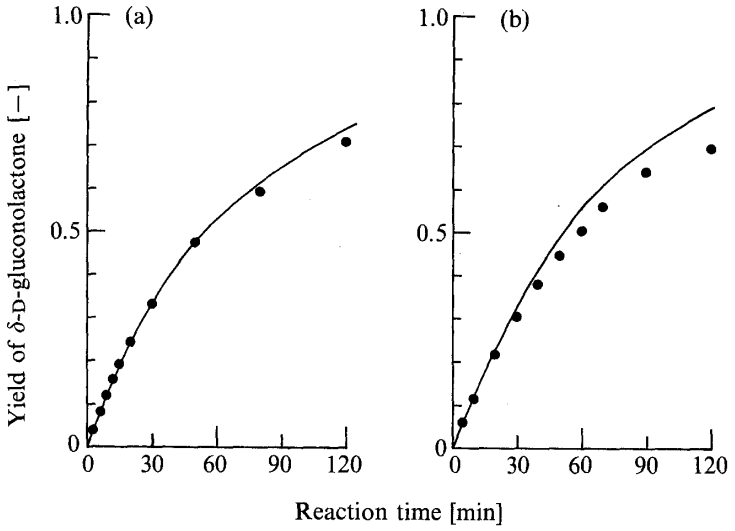

Fig. 4. Production of δ-D-Gluconolactone in the Insoluble System for d_P= 0.0069 cm (a) and d_P=0.0163 cm (b)

Reaction temperature, 25 °C; pH, 5.5 (in 0.1 M acetate buffer); concentration of oxygen in the bulk liquid [mol/cm^3], 1.12×10^{-6}; initial concentration of D-glucose [mol/cm^3], 1.0×10^{-4}. Concentrations of catalyst [g/cm^3]: (a), 3.3×10^{-3}; (b), 5.0×10^{-3}.

Thus, we conclude that, when no external mass transfer limitation exists, the rate of reaction in the insoluble system can be well expressed by Eqs. (10) to (12), where the concentrations of β-D-glucose and δ-D-gluconolactone are uniform throughout the reaction system.

Some experiments were performed for a period of 1—2 h to confirm the kinetic model presented above. In the stirred-tank reactor, the cumulative amount of oxygen gas absorbed was measured by using the catalyst of d_P=0.0069 and 0.0163 cm. The results are shown in Fig. 4.

As described in the previous section, mutarotation is an important process only when the reaction is continued for a long time, since the rate of mutarotation is very slow compared to that of oxidation. Therefore, to follow the time-course data, Eqs. (9) and (10) should be integrated simultaneously with the boundary conditions (11) and (12) and the initial conditions $(C_{\beta G})_0=0.063$ M and $(C_P)_0=0$, and with the following relationship between R_P and the production rate of δ-D-gluconolactone;

$$R_P=\frac{3D_e}{r_S\rho_P}\left(\frac{\partial C_i}{\partial r}\right)_{r=r_s}=\frac{2}{C_M}\frac{\partial C_P}{\partial t} \tag{14}$$

where C_M [g/cm^3] is the concentration of catalyst in the liquid. Note that the differential symbol d in Eqs. (9) to (11) should be replaced by the partial differential symbol ∂. The integration was done numerically assuming that at a fixed time the concentrations of β-D-glucose and δ-D-gluconolactone were uniform throughout the reaction system. The calculated curves are shown in Fig. 4. For the case of smaller particles, after 1 h the calculated curve gives a somewhat higher value than the data. For larger particles, the same tendency is observed after 30 min. At the initial stage, the β-D-glucose concentration of 0.063 M is much larger than that of oxygen (0.00112 M), but, for example, at 1 h the calculated value of β-D-glucose concentration was only 0.018 M for d_P=0.0163 cm. Since 1 mol of oxygen consumed corresponds to 2 mol of β-D-glucose reacted, only about an 8-fold excess of β-D-glucose is present. Therefore, when the concentration of β-D-glucose becomes low, the intraparticle mass transfer of β-D-glucose may become important, since the diffusivity of glucose in the liquid is expected to be considerably lower than that of oxygen.[18] This may be the main reason for the

deviation. As reported in the previous paper,[19] the deactivation of the immobilized enzyme catalyst was too slow to explain the deviation.

On the whole, the differential Eqs. (9) and (10) account for the time-course data fairly well. That is, the kinetics of the oxidation of D-glucose by the immobilized glucose oxidase and catalase on activated carbon can be well expressed by the Michaelis–Menten equation based on the single substrate mechanism with competitive inhibition, taking into account the mutarotation of D-glucose. In the subsequent paper,[20] the results in the present work will be used to analyze the performance of this reaction in a trickle-bed reactor packed with the immobilized enzyme catalyst.

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