Effects of Immobilization on the Kinetics of Enzyme-Catalyzed Reactions. I. Glucose Oxidase in a Recirculation Reactor System

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Summary

Glucose oxidase from *Aspergillus niger* was immobilized on nonporous glass beads by covalent bonding and its kinetics were studied in a packed-column recycle reactor. The optimum pH of the immobilized enzyme was the same as that of soluble enzyme; however, immobilized glucose oxidase showed a sharper pH-activity profile than that of the soluble enzyme. The kinetic behavior of immobilized glucose oxidase at optimum pH and 25°C was similar to that of the soluble enzyme, but the immobilized material showed increased temperature sensitivity. Immobilized glucose oxidase showed no loss in activity on storage at 4°C for nearly ten weeks. On continuous use for 60 hr, the immobilized enzyme showed about a 40% loss in activity but no change in the kinetic constant.
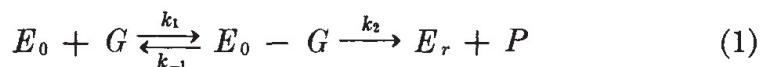
INTRODUCTION

With the recent development of immobilization techniques, interesting potential applications of enzymes as catalysts have been proposed in fields as diverse as medicine, sewage treatment, and industrial processing. Since enzymes depend upon specific three-dimensional conformation of their molecules for activity, any physical influence of the matrix or chemical modification of the enzyme might alter its properties. Indeed, a number of recent publications[1–6] have reported altered properties of enzymes after immobilization. However, it has not always been taken into consideration that the apparent change in the chemical properties are not entirely due to the physical influence of the matrix or chemical modification of the enzyme. External and internal diffusion effects can considerably alter the Michaelis-Menten constant, the activity, and the thermal sensitivity. For engineering purposes, better understanding is needed of immobilized enzyme kinetics and the factors that influence

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the rate of the reaction. In this study glucose oxidase was immobilized on nonporous glass beads by covalent bonding and its kinetics studied under well-defined reactor geometry and flow conditions. Glass was chosen as the support material because of its strength and incompressibility.

BACKGROUND

The kinetics of the homogeneous glucose oxidase reaction have been widely studied[7-12] with β-D glucose, at 25°C, and pH = 5.5. The mechanism is generally given as

$$E_0 + G \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_0 - G \overset{k_2}{\longrightarrow} E_r + P \tag{1}$$

$$E_r + O_2 \overset{k_4}{\longrightarrow} E_0 + H_2O_2 \tag{2}$$

where E_0, E_r, and $E_0 - G$ stand for the oxidized and reduced forms of the enzyme and the enzyme complex, respectively. Based upon this mechanism the reaction rate at steady state can be expressed as

$$v = \frac{k_{cat}E_T[O_2][G]}{[O_2][G] + \dfrac{k_{cat}}{k_{red}}[O_2] + \dfrac{k_{cat}}{k_{ox}}[G]} \tag{3}$$

where E_T is the enzyme concentration, $k_{cat} = k_2$, $k_{ox} = k_4$ and $k_{red} = (k_1k_2/k_{-1} + k_2)$ or (k_1k_2/k_{-1}), if the assumption is made of rapid equilibrium.

For a given amount of enzyme and at fixed glucose concentration eq. (3) can be reduced to

$$v = \frac{V_{max}[O_2]}{[O_2] + K_M} \tag{4}$$

where

$$K_M = \frac{k_{cat}k_{red}[G]}{k_{ox}k_{red}[G] + k_{cat}k_{ox}} \tag{5}$$

and

$$V_{max} = \frac{k_{cat}k_{red}E_T[G]}{k_{red}[G] + k_{cat}} \tag{6}$$

Previous kinetic studies of glucose oxidase, immobilized by various techniques, have indicated that immobilization can affect the properties of the enzyme. For example, Weibel and Bright[13] immobilized
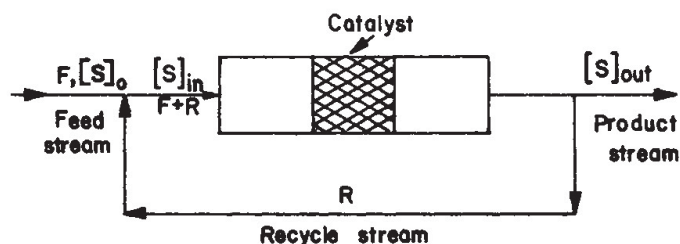
Fig. 1. Flowsheet for a recycle reactor system.

glucose oxidase on porous glass beads by covalent bonding and found that the apparent bimolecular constant was increased by a factor of 14. For gel entrapped glucose oxidase, Hinberg et al.[14] observed an increase in the value of the bimolecular constant by a factor of 2, but Miyamura and Suzuki[15] found that the values of the kinetic constants approached those of soluble enzyme as the particle size was decreased. Glucose oxidase crosslinked on a cellophane membrane[16] and covalently coupled on a nickel oxide screen[17] showed no change in the value of kinetic constants. In the same studies the optimum pH of the enzyme also did not change, however, in both cases the sensitivity of the enzyme to changes in pH was increased. Immobilized glucose oxidase showed decreased temperature sensitivity[17] and increased storage stability.[18]

The chemical engineering literature[19] describes the advantages of a packed-bed reactor linked to an external recirculation system. In such a continuous flow recirculation reactor system part of the effluent stream is returned and mixed with the feed stream, as schematically shown in Figure 1. A mass balance on the substrate at the mixing point gives:

$$[S]_{in} = \frac{F[S]_0 + R[S]_{out}}{F + R} \tag{7}$$

As the (R/F) ratio is increased sufficiently, the concentration changes within the reactor decrease to the point where the reactor is called "differential," i.e., the reaction may be considered to occur at a constant average concentration level. The overall conversion must however be significant enough to be detectable by the available measurement techniques. Under these circumstances the reaction rate can be calculated from

$$v = \frac{F([S]_0 - [S]_{out})}{W} \tag{8}$$

where W is the catalyst weight. Equation (8) can also be written in the modified form

$$v = \frac{F[O_2]_S(1 - X)}{W} \tag{9}$$

where $[O_2]_S$ is the saturated oxygen concentration. Values for different temperatures are reported in the literature.[20]

Since the glucose concentrations used in this study were very much higher than those of oxygen, they can be assumed to be constant throughout the reactor and equal to the inlet value. From eq. (7) the inlet oxygen concentration to the reactor can be written as

$$[O_2]_{in} = \frac{[O_2](F + RX)}{F + R} \tag{10}$$

and the average oxygen concentration is given by

$$[O_2] = \frac{[O_2]_{in} + X[O_2]_S}{2} \tag{11}$$

Equations (9)–(11) provide the numerical values needed in the evaluation of the rate expression eq. (3). Kinetic constants in this rate expression were calculated by using Rosenbrock's search technique.[21]

EXPERIMENTAL

Materials

Nonporous glass beads (40–60 mesh) used for covalent coupling of glucose oxidase were obtained from Ana Laboratories Incorporated, New Haven, Connecticut. The enzyme preparation (analytical grade from *Aspergillus niger*) used for immobilization was obtained from Sigma Chemical Company, St. Louis, Missouri and further purified. D-Glucose solutions of different concentrations were prepared by using "Baker analyzed" reagents purchased from J. J. Baker Chemical Company, Phillipsburg, N. J. The buffer solution used was $0.1M$ sodium acetate and the pH was adjusted to the desired level by adding acetic acid. Since the enzyme preparation contained trace amounts of catalase, $0.1mM$ KCN was added to suppress its activity. EDTA in the amount of $0.5mM$ was also added to the glucose solution to protect the enzyme from metal ions which may deactivate it. Sodium acetate, KCN and EDTA used were analytical grade materials (J. J. Baker). Compressed air used

for saturating the glucose solution was obtained from Linde Gas Company. All solutions were prepared from distilled–deionized water.

Methods

The nonporous glass beads were prepared by first adding 100 ml of water to 50 g of beads and then slowly adding 100 ml of 50% hydrofluoric acid, allowing the contents of the beaker to cool between additions. The mixture was allowed to react for 1 hr and then $10N$ NaOH was added, enough to cover the beads. The slurry was heated to 80°C for 1 hr, washed with distilled water, and dried overnight in an oven at 80°C. The dry beads were immersed in a 2% solution of 3-aminopropyltriethoxy silane in acetone. Excess liquid was decanted and the beads were allowed to stand in an oven at 45°C for 24 hr. The alkylamine glass was refluxed for 24 hr in 200 ml of chloroform containing 10 ml of triethylamine and 20 g of nitro-benzoyl chloride. The beads were washed with chloroform and ethyl alcohol and dried in an oven at 60°C for 12 hr. The arylamine glass was reduced by refluxing in 200 ml of 5% (w/v) sodium dithionite in water for 1 hr. The beads were washed with water and benzene and dried at 60°C.

For diazotization and coupling the glass was slurried in 50 ml of $2N$ HCl and placed in an ice bath in a dessicator connected to a vacuum source. When cool, 2.5 g of sodium nitrate was added to the slurry; the reaction was allowed to proceed under vacuum for 20 min. The beads were then quickly but thoroughly washed with ice-cold 1% (w/v) sulfamic acid, until no more bubbling was seen. A $0.1M$ Tris–Cl solution (pH $= 8.7$) was used for a last rinse. Excess liquid from the top of the beads was removed by decantation to prevent dilution of the enzyme solution. Glucose oxidase that had been column purified and concentrated was diluted by 1:10 and 10 ml was added to the glass beads. The reaction was allowed to proceed for 1 hr. The beads were then washed with Tris–Cl buffer thoroughly to remove the loosely bound enzyme and the supernatant containing unreacted enzyme was saved. Glucose oxidase beads were stored at pH $= 6.5$ in the cold.

Recycle Reactor

The essential features of the recycle system consist of the reactor, the feed preparation and product analysis parts, and the measurement and control devices. Glucose feed solution is maintained within ±0.2°C by a Blue M Electric Co. Model MR-3240A-1 con-

stant temperature bath. It is saturated with air by bubbling through four gas dispersion rods. A positive pressure Micro Pump, model 12-00-316 supplies the feed to the reactor, at rates measured by rotameters. The heat added to the solution by the agitation of the pump is removed by passing the reactants through stainless steel coils maintained inside the constant temperature bath.

The reactor consists of a glass tube of 2.5 cm diameter and 30 cm length, surrounded by a Plexiglas water jacket. Spacers on both sides are used to adjust the bed height to any desirable level. The glass beads are supported inside the column by nylon screens, one of which also serves as a fluid distributor. Water from the constant temperature bath is circulated through the water jacket to maintain uniform reactor temperature.

A part of the reactor effluent stream is metered and recycled. The rest of the stream is passed through a specially built holder for the dissolved oxygen electrode. The holder is provided with magnet-driven agitation to maintain a minimal velocity past the faces of the electrodes and the oxygen level in the reactor product is measured by using a polarographic electrode Model YSI 5331 Yellow Spring oxygen analyzer. The oxygen probe is connected to a Model YSI 53 biological oxygen monitor, which measures the dissolved oxygen level as a percentage of saturation value and supplies a signal for a continuous record. The reactor is also equipped with a bypass line for calibrating the oxygen probe.

The temperatures in the water bath, and at the entrance and exit of the reactor are monitored by thermocouples. All tubings and tube fittings are made of either stainless steel or polyethylene. Further details of equipment and procedure are recorded elsewhere.[22]

RESULTS AND DISCUSSION

Two different preliminary experiments were run to evaluate the effects of external mass transfer on the chemical reaction rate to be studied on the nonporous 50–60 mesh glass beads. In the first experiment, conversions from a plug flow reactor were compared at given space time (W/F) but at three different catalyst weights. Figure 2 shows the results obtained. Since the data for all three catalyst weights overlap, conversion is evidently not affected by flow velocity and it can be concluded that external film resistance is negligible.

In the second experiment, reaction rates from a differential recycle reactor were compared at a given feed rate (F) but at different total
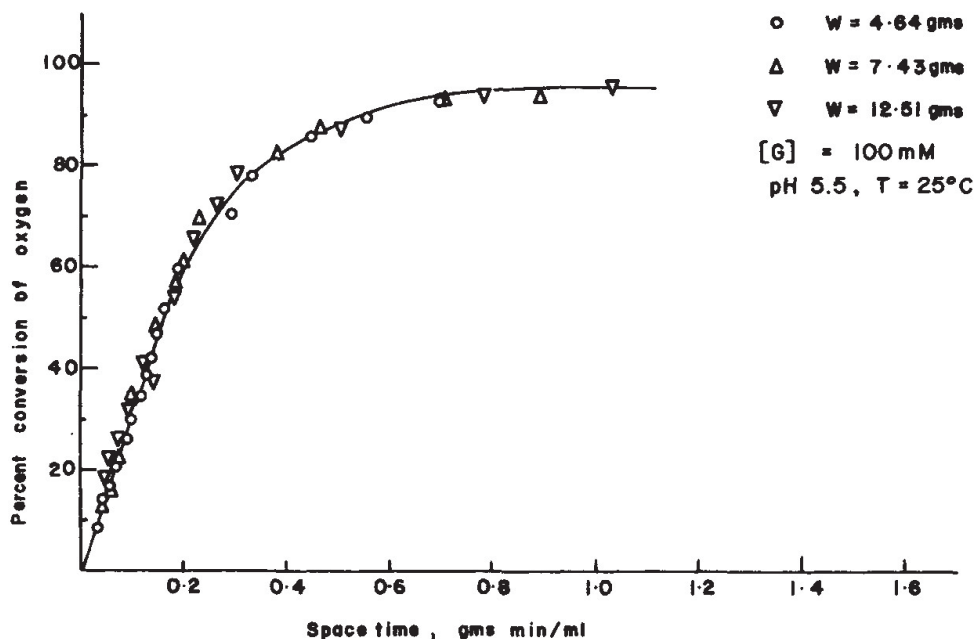
Fig. 2. Dependence of oxygen conversion on space time.

flow rates $(F + R)$.   Data obtained at four different feed rates from 8.4 to 41.1 cc/min and over a range of recycle rates from 360 to 1640 cc/min showed that the reaction rate is independent of total flow. It can therefore be concluded as before that the external mass transfer resistance is negligible above a total flow rate of 360 cc/min, corresponding to a linear velocity of 1 cm/sec.   In all subsequent experiments the reactor was operated well above this flow velocity.

Using the correlation of Wilson and Geankoplis[23] for mass transfer in packed beds, surface concentrations of oxygen and gluconic acid were calculated, corresponding to each of the experimental conditions.   In all the cases, surface concentration of oxygen was found to be only slightly different from that of the bulk concentration ($\ll 1\%$), confirming that external mass transfer effects are negligible for this system.   The surface concentration of gluconic acid was similarly found to be nearly the same as that of the bulk, indicating that the microenvironment near the glass was not different from that of the bulk.

Experiments were conducted with different amounts of immobilized enzyme to test whether axial dispersion effects and end effects are significant.   Reaction rates were measured at 25°C and 10mM glucose concentration at different oxygen concentrations and over a threefold range of bed weight.   The data were fitted to eq. (4) and the parameters $V_{\max}$ and K_M were estimated.   The kinetic constants
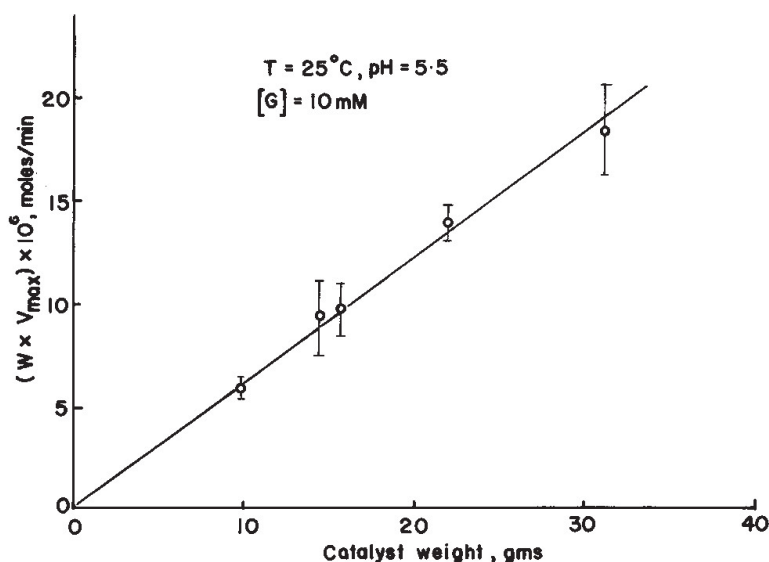
Fig. 3. Effect of bed weight on the reaction rate.

are independent of bed weight, indicating that dispersive effects are not significant. Figure 3 shows the same data, but plotted in a form that would produce a straight line passing through the origin, if axial dispersion and end effects were negligible. The results clearly support such a conclusion.

pH Profiles

The effect of pH upon the activities of the enzyme in solution and in the immobilized form was studied at a glucose concentration of 100mM. At this concentration level, the values of V_{max} estimated by applying eq. (4) are essentially the true maximum velocities when oxygen and glucose are in great excess. The results for both soluble and immobilized enzymes are compared in Figure 4 for pH between 3 and 8 on normalized scales.

It can be seen from Figure 4 that the optimum pH = 5.5 of the immobilized enzyme is the same as that of the enzyme in solution, however, the immobilized enzyme seems to be more susceptible to the changes in pH of the bulk solution. Below a pH of 3 the immobolized glucose oxidase completely and irreversibly lost activity. The results of this work are consistent with the findings of Broun et al.[16] for glucose oxidase crosslinked on cellophane and also with the findings of Weetall and Hersh[17] for glucose oxidase covalently linked on nickel oxide screens. These effects have been attributed to charges on the water insoluble carrier or unidentified chemical modification, but there exists no experimental verification for these speculations.
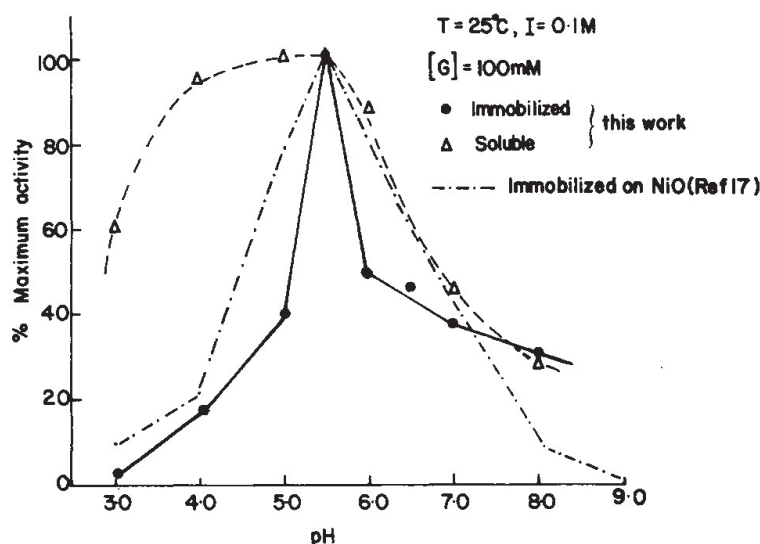
Fig. 4. pH-Activity profiles of soluble and immobilized glucose oxidase.

Effect of Substrate Concentrations

At 25°C and at pH = 5.5 a series of runs were made to evaluate the effects of glucose and oxygen concentrations on the kinetics of immobilized glucose oxidase. Reaction rates were measured at different oxygen concentrations for each of seven glucose levels between 5 and 100mM. The experimental results are shown in Figure 5 together with curves computed from eq. (3) by inserting best estimates of the various parameters. The estimated parameter values and their respective 95% confidence interval values are given in Table I along with the comparable literature results for the free enzyme and for glucose oxidase immobilized on porous glass.

Applying the standard statistical t-test for significant differences between means, a comparison of the kinetic constants for soluble and immobilized glucose oxidase shows no evidence that the former was affected by immobilization. It can be concluded that there is very

TABLE I

Comparison of Kinetic Parameters for Glucose Oxidase

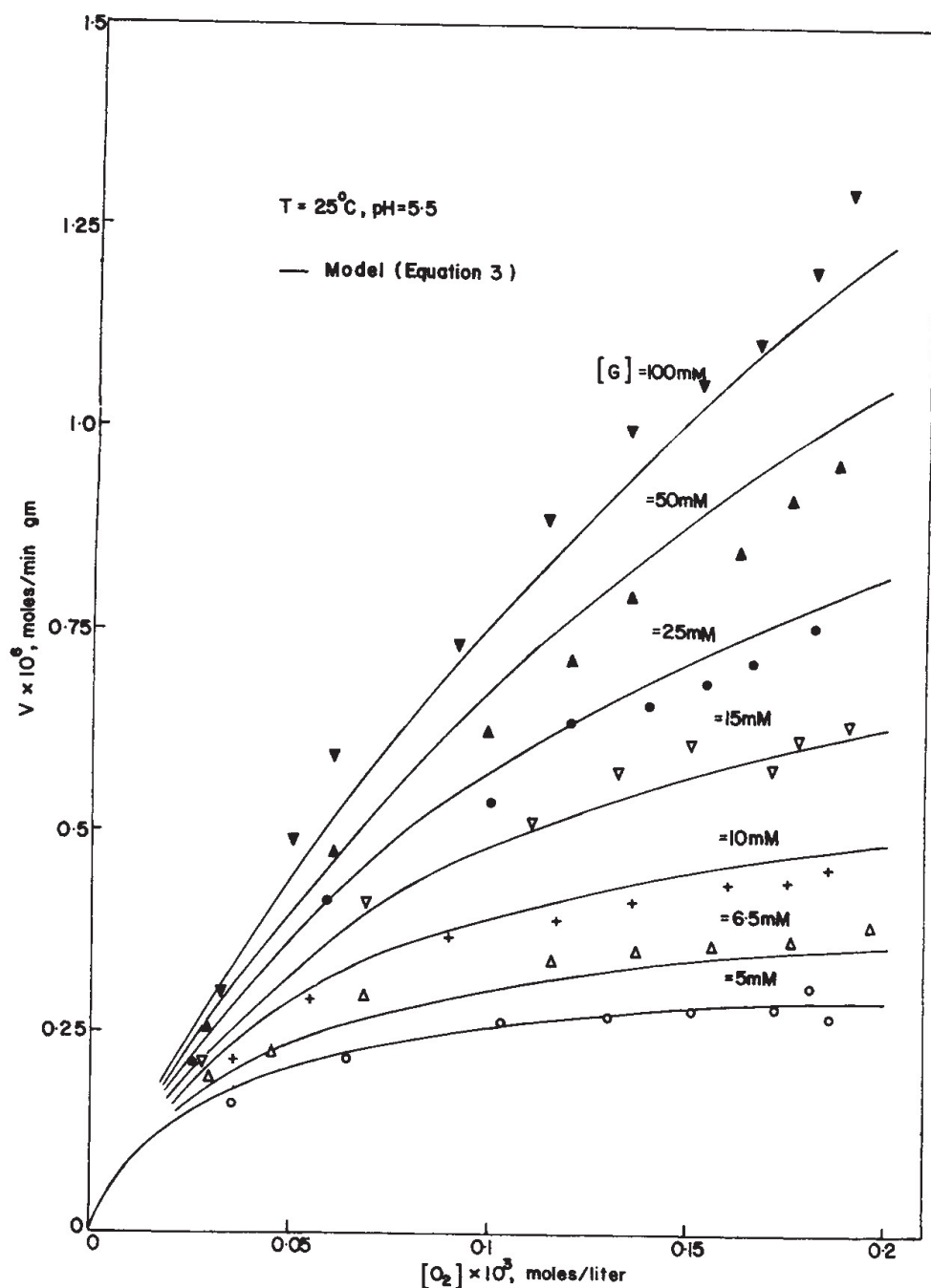| System	$k_{cat}/k_{ox} \times 10^3$ (mol/liter)	k_{cat}/k_{red} (mol/liter)	Ref.
Soluble enzyme	0.51	0.071	12
Bound to porous glass	0.50	0.005	13
Bound to nonporous glass	0.59 ± 0.177	0.08 ± 0.020	This work

Fig. 5. Effect of substrate concentration on the reaction rate at 25°C.

little or no interaction between the carrier and the active enzyme sites at this optimum pH = 5.5. However, it should be noted that the kinetic constant could be significantly different at other pHs, as suggested by the trends shown in Figure 4. The results obtained from this study are not in agreement with the results obtained by Weibel and Bright,[13] who observed a 14-fold increase in the value

of the apparent bimolecular constant, k_{red}. However, when the data of Weibel and Bright were corrected for external and internal diffusion effects,[24] the resulting constant was reduced by an order of magnitude. Moreover, when the data of the present work were analyzed by the inverse-plot method used by Weibel and Bright, it was found that the results could differ from the nonlinear parameter estimates by another factor of two.[22]

An order of magnitude estimate of the surface concentration of the enzyme can be calculated from the value of $k_{cat}E_T$ for the immobilized enzyme. The value of k_{cat} for the enzyme is readily available since it did not change upon immobilization, but an estimate is needed of surface area per gram of glass beads. Assuming the average diameter to be 0.2 mm for 40–60 mesh beads and the glass density to be 2.2 g/cc, the surface concentration was calculated to be 7.2×10^{-13} mol/cm^2. In actual preparation the surface concentration will be smaller than this, because the surface area of textured glass beads is higher than that of uniform spheres. To evaluate this result it is of interest to compare it to the surface concentration level that corresponds to monolayer coverage. Using a molecular diameter for the enzyme of 10^{-7} cm, monolayer coverage on 0.2 mm uniform spheres corresponds to a surface concentration of 13×10^{-13} mol/cm^2, a figure which is twice as high as the surface concentration estimated for the enzyme preparation used in this study. It may be concluded, therefore, that approximately 50% (or less) of the glass surface is involved in the enzyme catalysis.

Effect of Temperature

Reaction rates were measured at four different temperatures and the data for each temperature were fitted to eq. (3) to obtain the parameter estimates (with 95% confidence interval) shown in Table II. Since $k_{cat}E_T$ is the maximum velocity at excess concentration of glucose and oxygen, the rates are normalized for presentation as the temperature–activity profile in Figure 6. The corresponding profiles for soluble glucose oxidase and glucose oxidase immobilized on nickel oxide screen[17] are also shown. It can be seen from the figure that glucose oxidase immobilized on nonporous glass beads shows a sharper temperature–activity profile than those of glucose oxidase in solution or immobilized on a nickel oxide screen.

The decreased temperature sensitivity reported by Weetall and Hersh[17] may also be attributed to external diffusion effects. When such effects are significant, the enzyme molecules are not all efficiently used. As a result, denaturation due to increase in temperature
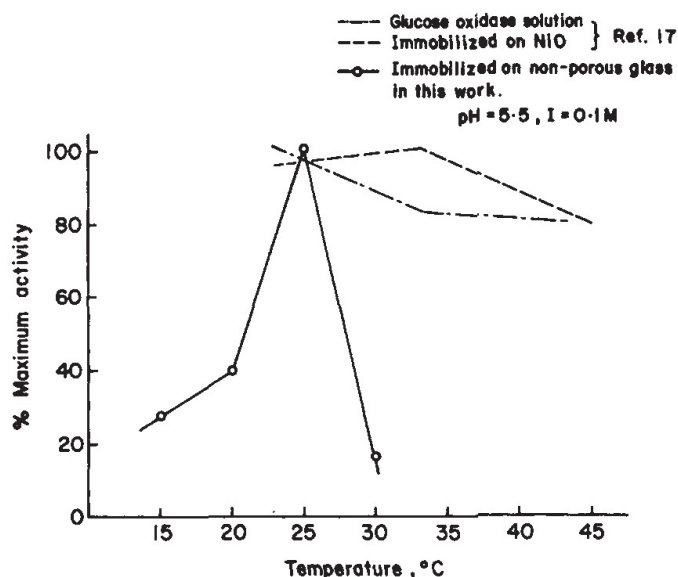
Fig. 6. Effect of temperature on the activity of immobilized glucose oxidase.

TABLE II

Kinetic Constants for Immobilized Glucose Oxidase at Various Temperatures

°C	$k_{cat}E_T \times 10^6$ (mol/g/min)	k_{cat}/k_{red} (mol/liter)	$(k_{cat}/k_{ox}) \times 10^3$ (mol/liter)
15	1.6 ± 0.43	0.051 ± 0.0026	0.41 ± 0.024
20	2.3 ± 0.36	0.036 ± 0.0017	0.27 ± 0.015
25	5.9 ± 2.47	0.080 ± 0.0199	0.59 ± 0.177
30	1.0 ± 0.12	0.006 ± 0.0002	0.11 ± 0.003

will not affect the overall reaction rate since the latter is in any case mass transfer limited. One may therefore, in agreement with Ollis,[25] anticipate a flatter temperature–activity profile and an increased optimum temperature when diffusional restrictions are present. A similar increase in the sensitivity of the temperature–activity profile upon immobilization was also observed for glucose oxidase crosslinked on cellophane membranes[16] and invertase covalently coupled to porous glass.[26]

Stability

Two runs which were made ten weeks apart with 10mM glucose solution at 25°C gave the following values for V_{max} (with 95% confidence interval):

Run no.	$V_{max} \times 10^6$ (mol/min g)
1	0.59 ± 0.036
42	0.63 ± 0.067

It can be concluded that the immobilized enzyme lost no activity upon storage for nearly ten weeks at 4°C.

The same batch of immobilized enzymes was used continuously for 60 hr with 10mM glucose at 25°C to obtain information on operational stability. At the end of each 15 hr, the oxygen level was varied inside the reactor to determine the kinetic constants K_M and V_{max} in eq. (4). The results, in Figure 7, show that K_M does not substantially change from run to run, indicating that the three-dimensional structure of the enzyme was not altered due to fluid motion near the glass surface. It can be seen from Figure 8, however, that the immobilized enzyme lost 40% of its original activity after 60 hr. Since K_M did not change upon continuous use for 60 hr, the change in V_{max} must be due to a change in E_T, the concentration of active enzyme. The loss in activity is due to denaturation rather than physical leaching, for if physical leaching had been responsible, the enzyme would have lost activity on storage. When the deactivation data were fitted to a first order model, the magnitude of the deactivation constant (with 95% confidence interval) was found to be $(7.6 \pm 3.09) \times 10^{-3} hr^{-1}$, corresponding to a half-life of 91.0 hr.

Effects of Different Preparations

Glucose oxidase was immobilized in two different batches to test whether both batches would give the same kinetic constants. Results obtained for 6.5mM glucose at 25°C and pH = 5.5 buffer are
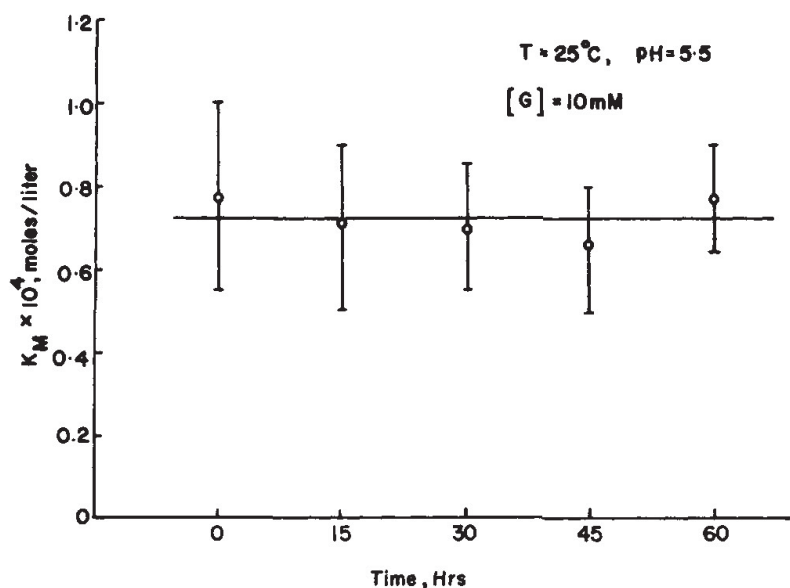

Fig. 7. Continuous operation of immobilized glucose oxidase, effect of K_M.
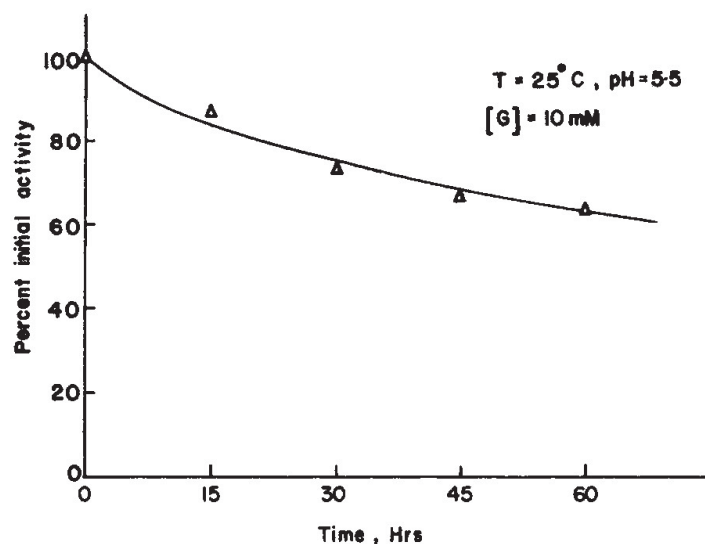
Fig. 8. Continuous operation of immobilized glucose oxidase, effect on V_{max}.

given in Table III. The K_M values for both batches are the same to within their 95% confidence intervals, however, the V_{max} for batch I is three times greater than that for batch II. That K_M did not change from batch to batch indicates that the binding mechanism for immobilization did not change. The difference in V_{max} is due to different amounts of enzyme immobilized on the glass surface.

TABLE III

Kinetic Constants for Different Immobilized Enzyme Preparations

Batch no.	$K_M \times 10^5$ (mol/liter)	$V_{max} \times 10^7$ (mol/min/g)
I	5.2 ± 2.87	5.3 ± 1.04
II	5.7 ± 2.41	1.8 ± 0.25

Nomenclature

E_T	total immobilized enzyme concentration, mol/g
F	feed rate, cc/min
$[G]$	average concentration of glucose, mol/liter
I	ionic strength of buffer, mol/liter
$k_1,\ k_{-1},\ k_2$	rate constants defined by eq (1)
k_4	rate constant defined by eq. (2)
k_{cat}	rate constant for the dissociation of enzyme glucose complex, min^{-1}
k_{ox}	rate constant for the reaction between oxygen and reduced form of enzyme, liter/mol/min

k_{red}	apparent bimolecular rate constant for the interaction of substrate and the oxidized form of enzyme, liter/mol/min
K_M	Michaelis-Menten constant, mol/liter
$[O_2]$	average oxygen concentration, mol/liter
$[O_2]_{in}$	oxygen concentration at the inlet of the reactor, mol/liter
$[O_2]_S$	saturated oxygen concentration, mol/liter
R	recycle rate, cc/min
$[S]_{in}$	substrate concentration at the reactor inlet, mol/liter
$[S]_0$	substrate concentration in the make-up feed, mol/liter
$[S]_{out}$	substrate concentration at the outlet stream, mol/liter
T	temperature, °C
v	reaction rate, mol/min/g
V_{max}	maximum velocity at a fixed concentration of glucose and excess concentration of oxygen, mol/min/g
W	catalyst weight, g
X	conversion

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