The pH Dependence of the Individual Steps in the Glucose Oxidase Reaction*

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SUMMARY

The kinetics of oxidation of D-mannose, 2-deoxy-D-glucose, and D-glucose by glucose oxidase from Penicillium notatum has been studied in the pH range from 3 to 8 at 25°, in the presence of 0.2 M KCl. The pH dependence of the individual steps in the catalytic mechanism was determined by stopped flow spectrophotometric measurements of each half-reaction, in conjunction with conventional steady state kinetic measurements of the over-all reaction. The following scheme, originally deduced by Gibson, Swoboda, and Massey (J. Biol. Chem., 239, 3927 (1964)) for the enzyme from Aspergillus niger at pH 5.6 and 25°, was found to accommodate the results at any value of pH in the range examined.

\[
E_0 + \text{S} \xrightleftharpoons[k_{-1}][k_1] E_0\text{S} \rightarrow E_r + \delta\text{lactone}
\]

where \(E_0, E_r, S, \) and \(P\) are, respectively, oxidized enzyme, reduced enzyme, substrate, and product.

The pH dependence and other characteristics of each step in turn are as follows.

1. The pH profile for \(k_1\) is sigmoid, and indicates that the combination of substrate with \(E_0\) is dependent upon a basic group (\(pK_1 = 5.00\) for mannose and glucose; \(pK_1 = 5.35\) for 2-deoxyglucose) in the enzyme. There is a small solvent deuterium isotope effect on \(k_1\). Halides have a specific effect on \(k_1\), causing it to increase markedly, but have no effect on \(k_{-1}\).

2. Flavin reduction, controlled by \(k_2\) and measured with 2-deoxyglucose, is relatively insensitive to pH in the range from 3 to 8. Halides also specifically decrease the rate of flavin reduction.

3. The pH profile for \(k_3\) is sigmoid and indicates that the combination of \(O_2\) with \(E_r\) is dependent upon an acidic group (\(pK_4 = 6.90\)) in the enzyme.

4. The pH profile for \(k_4\) is bell shaped and indicates that this terminal, first order process (which may consist of more than one step) is dependent upon an acidic (\(pK'_4 = 7.40\)) and a basic (\(pK_4 = 4.10\)) group in the enzyme.

Based on these findings, a kinetic scheme is presented which accounts for the pH dependence of the steady state velocity of oxidation of the three sugars in the pH range from 3 to 8. Analogue simulation of the partition of the enzyme between oxidized and reduced forms during turnover at different values of pH, with experimentally determined values of acid dissociation and rate constants, agreed with turnover patterns obtained in stopped flow spectrophotometric experiments at 450 mμ.

The flavoprotein glucose oxidase (EC 1.1.3.4) from Penicillium notatum and other fungi catalyzes the irreversible oxidation of a number of aldoses to the corresponding lactones according to Equation 1.

\[
\text{RCH(OH)} + \text{O}_2 \rightarrow \text{RCH} = \text{O} + \text{H}_2\text{O}_2
\]

Although some differences have been noted in physical and chemical properties of the enzyme from various fungi (2), no significant differences in kinetic properties appear to have been reported. Glucose oxidase from \(P. \text{notatum}\) has a molecular weight of 152,000, and contains 2 moles of firmly bound FAD (3). A recent review article on flavoproteins (4) includes a short discussion of glucose oxidase.

The kinetic mechanism of the reaction catalyzed by glucose oxidase from \(A. \text{niger}\) was convincingly deduced in 1964 by Gibson, Swoboda, and Massey (5) from the results of systematic stopped flow spectrophotometric experiments and manometric measurements carried out at pH 5.6 and over the temperature range from 0° to 38°. No evidence was obtained for kinetically significant flavin semiquinoid intermediates, and all their kinetic data could be quantitatively analyzed in terms of

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the partition of the enzyme between two spectrophotometric forms only, namely, fully oxidized (Eₐ) and fully reduced (Eᵢ). The results from all sugar substrates tested were accomplished by a general scheme, which consists of a reductive half-reaction (Equation 2) and an oxidative half-reaction (Equation 3).

\[
\begin{align*}
E₀ + S & \xrightleftharpoons{k₁} E₀S \xrightarrow{k₂} EᵢP₁ \xrightarrow{k₃} Eᵢ + \text{lactone} \\
Eᵢ + P₁ & \xrightarrow{k₄} EᵢP₂ \xrightarrow{k₅} Eᵢ + \text{H₂O₂}
\end{align*}
\]

The adequacy of this scheme was shown by quantitative regeneration of substrate competition and stopped flow turnover experiments. Nakamura and Ogura (6) have also studied this reaction by kinetic and other techniques, and their results, including the failure to identify semiquinoid intermediates, are in substantial agreement with those of Gibson, Swoboda, and Massey (5).

Using an experimental approach similar to that used at pH 5.6 by Gibson, Swoboda, and Massey (5), we have been able to determine the pH dependence of the individual steps in the catalytic mechanism at 25°C. We find that Equations 2 and 3 describe the kinetic behavior of glucose oxidase from P. notatum over the pH range from 3 to 8, and in the particular case of pH 5.6, our results are in agreement with those described previously (5). Our studies have been facilitated by the fact that the principal rate-determining steps, both in the reductive half-reaction and in the over-all reaction, vary remarkably depending upon the structure of the sugar substrate.

**EXPERIMENTAL PROCEDURE**

**Materials**

Glucose oxidase (P. notatum), A grade, obtained from Dohringer Mannheim, was commonly stored at 4°C, at concentrations ranging from 1 to 10 mg per ml, in 0.005 M potassium citrate, pH 5.5, containing 0.2 M KCl. Free flavin was effectively removed by repeated concentration of the enzyme solution in the Schleicher and Schuell collodion bag apparatus to a volume of about 0.5 ml. This method was also used, with good quantitative recovery, to reclaim enzyme from solutions used in stopped flow experiments. Solutions of the enzyme, and of all other reagents, were routinely treated with Millipore filters before use in any kinetic experiment.

2-Deoxy-d-glucose was obtained from Calbiochem (A grade and "glucose-free") and also from Sigma; d-mannose was obtained from Mann. Contamination of these chemicals by d-glucose was reduced to acceptable levels by methods described previously (5). Samples of 2-deoxy-d-glucose, from either purveyor, contained variable amounts of a yellow impurity which absorbed quite strongly in the ultraviolet region. This impurity had no effect on glucose oxidase kinetics, however, since 2-deoxyglucose from batch A having none or little of the impurity (either as received or after extensive charcoal treatment) behaved no differently than batches which were visibly contaminated.

D-Glucose, obtained from Pfannstiehl Laboratories, was used without further treatment.

Equilibrium mixtures of α and β forms of the sugars were used in all experiments, and the kinetic data are therefore expressed in terms of analytical sugar concentrations. The total concentration of sugar oxidized in the kinetic experiments was always much less than the equilibrium concentration of the β isomer, for which glucose oxidase is specific (7).

The following buffers were used: 0.02 M potassium citrate for pH 3.1, 4.75, and 5.4; 0.05 M potassium citrate for pH 3.5, 3.75, 4.5, and 5.1; 0.015 M potassium acetate for pH 4.0, 4.25, and 4.5; 0.02 M potassium phosphate for pH 6.0 and 6.4; 0.05 M imidazole hydrochloride for pH 6.65 and 7.0; and 0.02 M Tris hydrochloride for pH 7.8 and 8.5.

A variety of tests, involving the use of different buffers at different concentrations but at a constant ionic strength of 0.2, maintained with KCl, failed to reveal specific buffer effects. The enzyme is, however, subject to specific halide effects, particularly at pH values below 6.

**Methods**

**Enzyme Concentration**—Enzyme concentrations in stock solutions, expressed as molarity of E-FAD, were routinely estimated by assay of catalytic activity at 25°C, with the use of 0.1 M 2-deoxy-d-glucose and about 1 mM O₂ in 0.1 M potassium acetate, pH 5.4, containing 1 mM KCN. The details of such measurements are described below, under "Spectrophotometric Turnover Experiments." The advantage of this assay is that the reaction is fairly rapid and is zero order in O₂. The concentration of E-FAD in any stock enzyme solution which had been assayed in this manner was then determined by comparing its catalytic activity with that of a "standard" solution of glucose oxidase (freed of unbound flavin), the absorbance of which in a well aerated citrate-KCl solution (see "Materials") had been carefully measured at 450 nm. The extinction coefficient for E-FAD was taken to be ε₄₅₀ = 10.9 mM⁻¹ cm⁻¹ (2).

**Enzyme Stability**—The enzyme was stable for at least 8 hours, at 25°C and in the presence of 0.2 M KCl, over the pH range from 3 to 8. Therefore, within the pH range none of our results is attributed to irreversible, or slowly reversible, effects of solvent on the enzyme.

**Spectrophotometric Turnover Experiments** Those were conventional steady state kinetic experiments in which the rate of H₂O₂ formation was measured at 235 nm (ε₂₃₅ = 0.058 mM⁻¹ cm⁻¹) as a function of the concentrations of sugar and O₂ at selected pH values between 3 and 8. Measurements were made on a Cary 15 spectrophotometer with 0 to 0.1 absorbance slide-wire and with cell compartments and holders maintained by thermostats at 25°C. A slit width of 0.5 mm was used in all experiments. The effects of ionic strength, [H₂O₂], and slit width on ε₂₃₅ for H₂O₂ were found to be small. Furthermore, the species H₂O₂, which has a larger extinction coefficient than H₂O₂, was never present in significant amounts, since the pKₐ value for its formation is 11.7 (8).

Reactions were carried out in completely filled, and sealed, quartz cuvettes of 3.4 ml capacity containing a small glass bead to aid mixing. Purified oxygen was bubbled through the buffered sugar solution, containing 0.2 M KCl, for 10 min, before addition to the cuvette. Enzyme and KCN were then
added separately (usually in 0.02 ml volumes). KCN, at a
final concentration of 1 mM, was present in all turnover experi-
ments to inhibit traces of catalase in the glucose oxidase solu-
tions. The final concentration of glucose oxidase was in the
range from 7.0 \times 10^{-3} to 7.4 \times 10^{-7} M, depending on the nature
of the sugar (which determines the rapidity of turnover).

Each reaction was allowed to proceed until the O_2 had been
completely consumed. Since the initial concentration of sugar
always greatly exceeded that of oxygen, the sugar concentration
could be regarded as constant throughout the experiment. The
oxygen concentration at any time is equal to 0.068 (A_x - A_t)
M, with the initial value usually about 1 mM. No less than four
experiments, spanning at least a 5-fold range in sugar concen-
tration, were carried out at each pH value.

Because the reaction is for all practical purposes irreversible,
and because there is no significant product inhibition, any spec-
 trophotometric trace having curvature (owing to dependence
on the rate of the concentration of O_2) was accurately fitted to
the following equation.

\[
\frac{[E_{\text{m}}]}{v} = A + \frac{B}{[O_2]} \tag{4}
\]

The kinetic significance of A depends on the nature of the sugar,
whereas B is equal to 1/k_4 for all sugars. The values of 1/A
and B/A (the Michaelis constant for O_2) which best describe
the spectrophotometric traces were obtained by a digital computer
procedure.

Stopped Flow Experiments—These were carried out at 450
\text{nm} (corresponding to an absorption maximum of the oxidized
form of glucose oxidase) and 25^\circ with the Gibson-Durrum stop-
ped flow apparatus, with the use of adequate time constants.
The light path was 2.0 cm and the concentration of E-FAD
ranged from 4 to 8 \mu M after mixing. Ordinate millivolatages
were converted manually to \Delta A_{660} values.

Stopped flow measurements of the oxidative half-reaction are
based on the decrease in absorbance at 450 \text{nm} accompanying
the reduction of the enzyme by the sugar substrates. Buffered
enzyme and sugar solutions containing 0.2 M KCl were introduced
into the drive syringes from tonometers after having been deoxy-
genated by repeated evacuation and flushing with nitrogen. At
least four sugar concentrations were used at each pH value.

Stopped flow measurements of the oxidative half-reaction uti-
itize the increase in absorbance at 450 \text{nm} which occurs when
O_2 interacts with the reduced enzyme. An anaerobic solution of
buffered enzyme in 0.2 M KCl in a tonometer was reduced
over with mannose, the enzyme flavin remains completely oxi-
dized until oxygen is exhausted, whereupon the flavin becomes
completely reduced. This reduction is accompanied by a de-
crease in the extinction coefficient of enzyme-bound flavin at
235 \text{nm}.

At pH 5.6, the combination of mannose with E_0 is completely
stable, and at all mannose concentrations, each spectropho-
metric trace consisted of a linear increase in absorbance
which was terminated by a small and abrupt decrease in absorb-
ance just prior to the cessation of the reaction. This indicates
that the turnover rate is independent of the concentration of
oxygen within the sensitivity of the experiments. During turn-
over with mannose, the enzyme flavin remains completely oxi-
dized until oxygen is exhausted, whereupon the flavin becomes
completely reduced. This reduction is accompanied by a de-
crease in the extinction coefficient of enzyme-bound flavin at
235 \text{nm}.

When values of [E_{\text{m}}]/v were plotted against the reciprocal of the
concentrations of mannose, the experimental points fitted a
straight line passing through the origin at all pH values. These
results indicate that turnover was accurately bimolecular (first
order in enzyme and first order in mannose) at all pH values and
mannose concentrations used. The values of the apparent bi-
nolecular rate constants, k_{app}, were obtained from the slope
of the plots and are shown as a function of pH in Fig. 1.

Stopped Flow Reductive Half-reaction Experiments—The
kinetics of reduction of the enzyme by mannose at 450 \text{nm} was
measured spectrophotometrically at 25^\circ at several pH values
which were selected on the basis of the results from the turnover
experiments just described. Mannose was varied from 0.02
to 0.3 M, and the enzyme concentration was 6 \mu M. Double

\[
\frac{1}{k_{\text{obs}}} = \frac{[E_0]}{v} = \frac{1}{k_{\text{app}}[M]} \tag{6}
\]

Equation 4a is a special case of

\[
y = a + b \ln \frac{y_t}{y_0} = a + b \ln \sum_{n=0}^{N} C_n y^n \tag{4b}
\]

where a, b, and the subscript terms are constants. Equation
4b describes the time course of many steady state enzyme reac-
tions, and the constants a (-1/A) and b (- B/A) can be evaluated
with regression techniques.
FIG. 1. n-Mannose: pH dependence of $k_l$. Data are from anaerobic stopped flow measurements of the reductive half-reaction (©) and from spectrophotometric turnover measurements (●). The stopped flow result at pH 5.6 is from Gibson, Swoboda, and Massey (5). The lines are calculated with the use of $k_l = 15 \text{ M}^{-1} \text{sec}^{-1}$ and $pK_1 = 5.00$ (see Equations 16 and 17). The temperature was 25°C; ionic strength, approximately 0.25.

The reciprocal plots of the observed first order rate constants, $k_{obs}$, versus mannose concentration gave straight lines, extrapolating to the origin, at all pH values investigated. The values of the bimolecular rate constants, $k_{1,app}$, were obtained from the slopes of these lines. Thus, in agreement with our spectrophotometric turnover experiments and with earlier stopped flow data obtained at pH 5.6 (5), there is no significant first order process under the conditions used. Our stopped flow results, together with those of Gibson, Swoboda, and Massey (5), are included in Fig. 1.

$\text{2-Deoxy-d-glucose}$

Spectrophotometric Turnover Experiments—2-Deoxyglucose was varied from 0.015 to 0.40 M, and enzyme concentrations were in the range from 0.7 × 10^{-3} to 2.0 × 10^{-3} M. At all pH values less than about 7.5 the spectrophotometric traces were linear and resembled those obtained with mannose, indicating that the enzyme remains fully oxidized during turnover under these conditions.

At pH 5.6, the kinetic scheme required for 2-deoxyglucose turnover (5) involves only three of the five steps in the general scheme (Equations 2 and 3) and is the following

$$E_o + 2\text{DG} \xrightleftharpoons{\kappa_1}{\kappa_4} E_o - 2\text{DG} \xrightarrow{k_2} E_r + \text{lactone} \quad (7)$$

for which the rate equation is

$$\frac{1}{k_{obs}} = \frac{[E_o]}{v} = \frac{1}{k_2} + \frac{k_1 + k_3}{k_4[2\text{DG}]} + \frac{1}{k_4[O_2]} \quad (9)$$

However, the turnover experiments indicate that the $O_2$ term of Equation 9 is not significant at pH values less than 7.5. When values of $[E_o]/v$ were plotted against the reciprocals of the concentrations of 2-deoxyglucose, at all pH values less than 7.5, each set of experimental points fitted a straight line with a finite ordinate intercept, which, from the stopped flow measurements, is known to represent $1/k_2$. The pH dependence of $k_2$ is given in Fig. 2.

In the case of experiments performed at pH 7.85 the spectrophotometric traces were distinctly curved. This indicates that the rate of the oxidative half-reaction has been decreased relative to that of the reductive half-reaction, and that the concentration of oxygen now partially controls the turnover rate of the enzyme. These traces were analyzed by a digital computer procedure according to Equation 4.

The apparent Km values of 2-deoxyglucose, corresponding to infinite oxygen concentration, are plotted as a function of pH in Fig. 3.

Stopped Flow Reductive Half-reaction Experiments—2-Deoxyglucose was varied from 0.02 to 0.20 M, and the enzyme concentration was 6 μM. When the reciprocals of the values of $k_{obs}$ were plotted against the reciprocals of the 2-deoxyglucose concentrations at each pH, the points fitted a straight line which extrapolated to a finite value of $k_{obs}$. This value represents the first order rate constant, $k_2$, controlling the rate of absorbance decrease at 450 nm associated with flavin reduction. Our stopped flow values of $k_2$, together with the value of $k_2$ obtained previously by similar methods (5) at pH 5.6, are included in Fig. 2, and are in good agreement with the turnover results. Such agreement is important, since it indicates that the first order process controlling enzyme turnover with 2-Deoxyglucose at all pH values is indeed flavin reduction (or possibly some slower first order step preceding flavin reduction for which the absorb-
FIG. 3. 2-Deoxy-D-glucose: pH dependence of $K_m$. Data are from anaerobic stopped flow measurements of the reductive half-reaction (O) and from spectrophotometric turnover measurements (●). The stopped flow result at pH 5.6 is from Gibson, Swoboda, and Massey (5). The lines were calculated with the use of $K_m = 0.0286$ M and $pK_1 = 5.35$ (see Equations 13 and 15). The temperature was 25°C; ionic strength, approximately 0.25.

The curvature of traces from glucose turnover experiments can be increased (i.e. the combination of $O_2$ with $E_r$ becomes increasingly rate-limiting) either by increasing the concentration of glucose at a given pH value or by increasing the pH at a given concentration of glucose. The rationale for analysis of such spectrophotometric traces is based entirely on the kinetic scheme for glucose deduced from a variety of stopped flow spectrophotometric experiments at pH 5.6 (5). At 25°C, the kinetic scheme which is required again involves only three of the five steps in the general scheme (Equations 2 and 3), and is the following:

$$\begin{align*}
E_o + G & \xrightarrow{k_1} E_r + \text{lacitone} \\
E_r + O_2 & \xrightarrow{k_4} E_o - P - E_o + H_2O
\end{align*}$$

for which the rate equation is

$$\frac{[E_r]}{v} = \frac{1}{k_4} + \frac{1}{k_4(G)} + \frac{1}{k_4(O_2)}$$

The spectrophotometric traces from glucose turnover experi-

FIG. 4. D-Glucose: pH dependence of $k_t$. Data are from anaerobic stopped flow measurements of the reductive half-reaction (O) and from spectrophotometric turnover measurements (●). The stopped flow result at pH 5.6 and 27°C is from Gibson, Swoboda, and Massey (5). The lines were calculated with the use of $k_t = 12,000$ M$^{-1}$ sec$^{-1}$ and $pK_1 = 5.00$ (see Equation 18). The temperature was 25°C; ionic strength, approximately 0.25.

The curvature change at 450 mJ acts as a spectrophotometric indicator and not some other step which follows flavin reduction.

The plots of $k_{\text{obs}}$ versus the reciprocals of 2-deoxyglucose concentration also yielded apparent $K_m$ values. These are included in Fig. 3, together with the apparent $K_m$ at pH 5.6 determined previously (5). Once again, the agreement between the results of turnover and those of stopped flow experiments is quite good.

**d-Glucose**

Spectrophotometric Turnover Experiments—Glucose was varied from 0.03 to 0.8 M, and enzyme concentrations were in the range from $5.1 \times 10^{-9}$ to $2.0 \times 10^{-8}$ M. Curvature in the glucose traces becomes significant at pH values greater than 4.2. In the cases of 2-deoxy-D-glucose, for which the reductive half-reaction is much slower than that for D-glucose, analogous curvature in the spectrophotometric traces became evident only at much higher pH values (around 7.8). These facts by themselves, taken together with the pH dependence of the first and second steps in the reductive half-reaction, strongly suggest that $O_2$ must combine with a protonated form of $E_r$.

The curvature of traces from glucose turnover experiments can be increased (i.e. the combination of $O_2$ with $E_r$ becomes increasingly rate-limiting) either by increasing the concentration of glucose at a given pH value or by increasing the pH at a given concentration of glucose. The rationale for analysis of such spectrophotometric traces is based entirely on the kinetic scheme for glucose deduced from a variety of stopped flow spectrophotometric experiments at pH 5.6 (5). At 25°C, the kinetic scheme which is required again involves only three of the five steps in the general scheme (Equations 2 and 3), and is the following:

$$\begin{align*}
E_o + G & \xrightarrow{k_1} E_r + \text{lacitone} \\
E_r + O_2 & \xrightarrow{k_4} E_o - P - E_o + H_2O
\end{align*}$$

for which the rate equation is

$$\frac{[E_r]}{v} = \frac{1}{k_4} + \frac{1}{k_4(G)} + \frac{1}{k_4(O_2)}$$

The spectrophotometric traces from glucose turnover experi-

FIG. 5. The pH dependence of $k_t$. Data are from stopped flow measurements of the oxidative half-reaction (O), and from spectrophotometric turnover measurements with D-glucose (●) and 2-deoxy-D-glucose (X). The stopped flow result at pH 5.6 and 27°C is from Gibson, Swoboda, and Massey (5). The stopped flow result at pH 8.5 fits the log-log plot quite well, although this is not shown. The lines are calculated with $k_t = 2.5 \times 10^6$ M$^{-1}$ sec$^{-1}$ and $pK_1 = 6.90$ (see Equation 19). The temperature was 25°C; ionic strength, approximately 0.25.
the marked pH dependence of the individual steps in the reaction, which merits at all pH values greater than 4.2 were analyzed by the digital computer method, yielding the optimal values of the parameters $A$ (which from Equation 12 is $1/k_2 + [1/k_2([I])]$) and $B$ ($1/k_2$) for each of four concentrations of glucose. A plot of $A$ versus $1/[G]$ gave a straight line, with ordinate value $1/k_2$ and slope $1/k_2$. Figs. 4 to 6 show the pH dependence of $k_{1,app}$, $k_{4,app}$, and $k_{5,app}$, respectively, determined from the glucose spectrophotometric turnover experiments. The results obtained previously (5) at pH 5.6 are also included in these figures. Below pH 4.2, $k_{4,app}$ could not be evaluated with glucose because of insufficient dependence of the turnover rate on $O_2$ concentration. It was also found that the precise evaluation of $k_{4,app}$ at the highest pH values is quite difficult, owing to the extensive curvature of the spectrophotometric traces.

Stopped Flow Reductive Half-reaction Experiments—Glucose was varied from 0.002 to 2.02 mM, and the enzyme concentration was 6 nM. As with mannose, the kinetics of reduction of glucose oxidase by glucose, within the time resolution of stopped flow experiments, gives no evidence for a rate-determining first order process involving flavin reduction. The values of $k_{1,app}$ for glucose determined in stopped flow experiments are plotted as a function of pH in Fig. 4. The agreement with the results from spectrophotometric turnover experiments is satisfactory.

Stopped Flow Oxidative Half-reaction Experiments—Because of the marked pH dependence of the individual steps in the reaction, we have reinvestigated the possibility that proton transfer might be rate-limiting in one or more of these steps by spectrophotometric turnover experiments in $\mathrm{H}_2\mathrm{O}$ (0.9 mole fraction $\mathrm{H}^+$) at 25°. The value of $k_{1,app}$, measured with glucose at pH and pD 6.0, was found to be decreased about 30% in $\mathrm{H}_2\mathrm{O}$. At pH and pD 7.0, the value of $K_{m,app}$ [($k_{-1} + k_2$)/$k_1$] for 2-deoxyglucose was increased about 30% in $\mathrm{H}_2\mathrm{O}$, while there was a negligible effect on $k_1$ measured with this sugar.3 Since the pH and pD values used are close to the plateau region for $k_{1,app}$ (see Fig. 4), the solvent isotope effect observed here is probably due to a decrease in $k_1$ itself, and not to the increase in $pK_1$ which is anticipated in $\mathrm{H}_2\mathrm{O}$ (9). Neither $k_4$ nor $k_5$, measured with glucose, was significantly affected by $\mathrm{H}_2\mathrm{O}$, as reported previously (10).

DISCUSSION

Step 1: Combination of Sugar with Enzyme—2-Deoxyglucose is the only sugar of the three examined for which there is kinetic evidence for the existence of a conventional Michaelis complex of the form $E_{0,S}$. However, it should be noted that this is a reflection only of the relative rates of formation and decomposition of the complex, and not of any intrinsic difference in the chemical pathways of oxidation of the three sugars. Thus glucose-1-2H (Equation 8 of Reference 10) behaves kinetically like 2-deoxyglucose (Equation 9 of this paper) as a result of a 10- to 15-fold kinetic isotopic effect on the rate of decomposition ($k_2$) of $E_{0,S}$.

From Equation 9, the limiting value of $K_m$ at high pH values (Fig. 3) is ($k_1 + k_2$)/$k_1$. Since $k_2$ is essentially insensitive to pH (Fig. 2), the pH dependence of $K_m,app$ can be accounted for by a scheme involving the obligatory combination of the sugar with a basic form of the enzyme.

$$
\begin{align*}
\text{H}^+ & \overset{k_1}{\longrightarrow} E_0 \\
E_0 & \overset{k_{1,2DG}}{\longrightarrow} k_{1,app} \quad \text{[2DG]} \\
E_0 & \overset{k_2}{\longrightarrow} E_1 + \text{lactone}
\end{align*}
$$

(13)

The value of $pK_1$ from Fig. 3, is 5.35. The pH dependence of the kinetics of oxidation of 2-deoxyglucose, both in the over-all reaction (except for pH values above 7.5) and in the reductive half-reaction, is therefore expressed by Equation 14

$$
\frac{1}{k_{obs}} = \frac{[E_1]}{v} = \frac{1}{k_2 + k_{1,2DG}} \left[ 1 + \frac{[H^+]}{K_1} \right]
$$

(14)

from which the pH dependence of $K_{m,app}$ (Fig. 3) is seen to be

$$
K_{m,app} = \frac{k_2 + k_{1,2DG}}{k_1} \left(1 + \frac{[H^+]}{K_1} \right)
$$

(15)

The interpretation of the pH dependence of $K_m$ for 2-deoxyglucose as the consequence of the combination of the sugar with a basic form of the enzyme (Equation 13) is entirely substantiated by all the kinetic results obtained with both mannose and glucose. For each of these sugars it has been established (5) that $k_4 > [k(S)]_{k_{-1}}$. In the case of mannose, the rather bizarre, but not unique, finding (5) that the combination of sugar with enzyme is entirely rate-limiting in turnover at pH 5.6 (with the result that $V_{max}$ cannot be experimentally evaluated) has been found to be true for all pH values. The pH dependence of $k_{4,app}$ for mannose (Fig. 1) reveals a $pK_4$ value of 5.00, and is simply accommodated, in analogy with the treatment of the 2-deoxyglucose results, by the following scheme, which describes both turnover and the reductive half-reaction (although, of course, in the latter case $O_2$ is not present)

$$
\begin{align*}
\text{II}^+ & \overset{K_1}{\longrightarrow} E_0 \quad \text{[O}_2\text{]} \overset{k_{1,2DG}}{\longrightarrow} E_0 + \text{lactone} + \text{II}_2\text{O}_2
\end{align*}
$$

(16)

for which the rate equation is

$$
\frac{1}{k_{obs}} = \frac{[E_1]}{v} = \frac{[H^+]}{K_1} + 1
$$

(17)
The spectrophotometric turnover data for glucose, when interpreted in terms of the special case of the general scheme represented by Equations 10 and 11, yield values of $k_{4,app}$ for this sugar which show a pH dependence (Fig. 4) which is identical with that observed for $k_{1,app}$ in the mannose reaction. Thus pK1 for glucose is 5.00, even though the value of $k$ for glucose is 10$^9$ times larger than $k_1$ for mannose. The pH dependence of the combination of glucose with the enzyme is therefore entirely analogous to that required for the combination of mannose with the enzyme.

$$\text{H}^+ + E_0 \xrightarrow{k_1} E_0 \xrightarrow{k_4[O_2]} E^- \cdot P_1$$  \hspace{1cm} (18)

Step 2: Flavin Reduction—The rate of reduction of flavin, relative to the rates of other steps in the over-all reaction, is sufficiently small to be a rate-limiting step only in the cases of 2-deoxyglucose and glucose-1$^2$H (5, 10). Within the accessible pH range, namely from 3.2 to 7.85, $k_2$ showed very little pH dependence, as shown in Fig. 2. It should be noted, from the results of the glucose experiments, that $k_{4,app}$ probably controls the turnover kinetics of 2-deoxyglucose to a small extent at the extreme values of pH. The precision of the data shown in Fig. 2 is not sufficient to show the decrease in the turnover number (at the highest and lowest values of pH) which would be anticipated if $k_{4,app}$ were to become important.

Step 3: Terminal First Order Process in Reductive Half-reaction—The terminal first order process governed by $k_4$ in the reductive half-reaction, which has a relatively large heat of activation (5), is kinetically important only in glucose turnover at temperatures below 15°C. Although a terminal first order process in the reductive half-reaction involving no spectrophotometric change was necessary for the interpretation of stopped flow turnover experiments, it was not positively identified as a product release step, nor is it required to be such (5). Since all of the experiments described here were carried out at 25°C, the pH dependence of $k_4$ has not been studied.

Step 4: Combination of O$_2$ with Reduced Enzyme—In contrast to the combination of sugar with $E_0$, the combination of O$_2$ with $E_-$ is an acid-catalyzed process. The evidence for this conclusion is the pH profile of $k_{4,app}$ shown in Fig. 5. The evaluation of $k_4$ from spectrophotometric turnover experiments with glucose is again based on the kinetic turnover scheme for this sugar represented by Equations 10, 11, and 12. It is interesting to note in Fig. 5 that the evaluation of $k_{4,app}$ from 2-deoxyglucose turnover experiments, which is possible only at pH 7.8 and above, is consistent with the idea that $k_4$ (and probably $k_5$) are identical for all sugar substrates, although these two steps are in general too rapid to exert any kinetic control in the turnover of sugars other than glucose.

The interaction of O$_2$ with $E_-$ is most simply explained by the obligatory combination of O$_2$ with an acidic form of $E_0$, as follows.

$$E_- \xrightarrow{k_5} E_0 + \text{H}^+ \xrightarrow{k_{10}[O_2]} E_0 \cdot P_1$$  \hspace{1cm} (19)

The value of pK$_4$ from the profile given in Fig. 5 is 6.90.

Step 5: Terminal Step in Oxidative Half-reaction—Fig. 6 shows that $k_{4,app}$, computed from glucose turnover experiments on the basis of Equation 8, has a bell shaped pH profile. Whether this is a consequence of two consecutive intramolecular conversions of the oxidized enzyme (one being base-catalyzed and the other acid-catalyzed) or concerted acid-base catalysis in a single elementary step cannot be decided by the types of kinetic measure-

Although there are thermodynamic constraints governing the values of equilibrium constants in a loop, we have no evidence that the two acid dissociation constants labeled $K_a$, for example, are numerically different. The values of pK$_4$ and pK$_5$ necessary to generate the profile of Fig. 6 are 4.10 and 7.40, respectively.

Complete pH Dependence of Over-all Reaction—Taken together, the kinetic results from all sugars tested by Gibson, Swoboda, and Massey (5) required the general scheme represented by Equations 2 and 3. Using the evidence that we have presented for each step in turn, we may now express the kinetic behavior of the general scheme in the pH range from 3 to 8 as follows.

$$[E_0^+] + [H^+] + [O_2] + [S] \xrightarrow{k_{10}} \frac{k_{10}k_{5} + k_{4}k_{5} + k_{5}k_{10}}{k_{5}k_{10} + k_{4}k_{5} + k_{5}k_{10}} \frac{[H^+] + [S]}{k_{5}[O_2]}$$  \hspace{1cm} (20)

The values of the acid dissociation constants and rate con-
**Table I**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_1$</th>
<th>$\frac{k_1 + k_2}{k_3}$</th>
<th>$k_2$</th>
<th>$k_4$</th>
<th>$k_5$</th>
<th>$pK_1$</th>
<th>$pK_4$</th>
<th>$pK_5$</th>
<th>$pK_6$</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-Glucose</td>
<td>12,600</td>
<td>3.400</td>
<td>40</td>
<td>2.5 x 10^6</td>
<td>1,200</td>
<td>5.00</td>
<td>5.35</td>
<td>4.00</td>
<td>7.40</td>
</tr>
<tr>
<td>2-Deoxy-d-glucose</td>
<td>1,400</td>
<td>0.029</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Mannose</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

* a Rough estimate based on temperature dependence of $k_2$ for 2-deoxyglucose (5) and on estimate of $k_2$ for d-glucose at 3° (10).

b Determined in the absence of KCl.

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![Graph](http://www.jbc.org/)

**Fig. 7.** Analogue computer simulation (lines) of stopped flow turnover experiments (points) with 4.65 μM glucose oxidase, 10^{-2} M d-glucose, and 2.5 x 10^{-4} M O2 (all concentrations after mixing). The temperature was 25°; ionic strength, approximately 0.25. The scheme which was simulated involved the steps $k_1$, $k_4$, and $k_5$ of Equation 21. The values used for $k_1$, $k_4$, $k_5$, $pK_1$, $pK_4$, $pK_5$, and $pK_6$ are given in Table 1.

---

We have tested the adequacy of the over-all scheme (Equations 21 and 22) by analogue computer regeneration of stopped flow turnover experiments with glucose at 25° and selected pH values. In these experiments, which are shown in Fig. 7, the oxidized fraction of the enzyme ($E_o + E_o P$) is measured as a function of time at 450 μm. The turnover patterns reflect primarily the dominance of $k_{on}^{app}$[G] at low pH values and the dominance of $k_{on}^{app}$[O2] at high pH values. The step controlled by $k_{on}^{app}$ is of some importance only at high pH values with the particular substrate concentrations used. The rapid reduction of the fully oxidized enzyme in the presence state portion of the reaction seen at pH 5.0 and 7.8 corresponds to $k_{on}^{app}$[G]. Except for the results at pH 7.8, the correspondence between the computed and experimental traces is excellent. The discrepancy at pH 7.8 may be due to a trace of uninhibited catalase.

In comparing our results with the bell shaped initial velocity-pH profile obtained by Keilin and Hartree (3) at 39° with 0.056 M glucose and approximately 1.8 x 10^{-4} M O2, we noted that we could qualitatively regenerate the basic limb but that the computed acidic limb was displaced approximately 1 pH unit in the basic direction. This discrepancy is too great to be caused by the heats of ionization of the two residues (corresponding to $pK_1$ and $pK_4$) which have to be in the conjugate base form for catalysis to occur. We therefore re-evaluated $K_1$ and $k_1$ under experimental conditions similar to those used by Keilin and Hartree, namely, 0.2 M acetate and phosphate-citrate buffers with no KCl. Under these conditions the value of $pK_1$ in the case of mannanse was 4.0 (compared to 5.0 in the presence of 0.2 M KCl), whereas the value of $k_1$ was unchanged. The presence of 0.2 M KCl therefore shifts the acidie limb of the initial velocity-pH profile approximately 1 pH unit upwards. KCl, NaCl, and KBr, at a concentration of 0.2 M, were found to inhibit mannanse turnover to the same extent at pH 4.15. These results indicate that it is the anion, rather than the cation, which interacts with the enzyme and stabilizes the conjugate acid species, $+\text{HE}_2$. Chloride ion effects have also been noted with L-amino acid oxidase (11).

The effects of added salt on the first two steps of the reductive half-reaction were investigated more fully. With 2-deoxyglucose at pH 3.65 (0.02 M citrate), the values of $k_3$ and $K_{m,app}$ were unaffected by the addition of 0.167 M K$_2$SO$_4$. This indicates, barring fortuitous compensatory effects on rate and equilibrium constants, that $K_1$, $k_3$, $k_{a,app}$, and $k_6$ are not sensitive to ionic strength. The insensitivity of $k_1$ to ionic strength (and to Cl-) was confirmed with mannanse at pH 7.3 with the use of 0.1 M imidazole hydrochloride. However, Cl$^-$ was shown to markedly decrease $k_2$ and $K_m$ to approximately the same extent in the case of 2-deoxyglucose at pH 7.3. This result suggests that $K_m$ = $k_2$/$k_5$, in which case we can estimate that the value of $k_5$ for 2-deoxyglucose is 1400 M$^{-1}$ sec$^{-1}$. This is approximately one-tenth of the rate at which glucose combines with $E_o$. In summary, therefore, halides have a specific acid-weakening effect on the enzyme, causing pH$^*_T$ to increase markedly, and also specifically inhibit the rate of flavin reduction, controlled by $k_6$, whereas $K_1$, $k_3$, $k_{a,app}$, and $k_6$ are almost completely insensitive to ionic strength.

Our results clearly show that the shape and position of the velocity-pH profile will be determined by the relative contributions of three pH-dependent processes. Furthermore, since two of these processes ($k_3$ and $k_5$) are bimolecular steps, the shape and position of the profile will in general be very dependent on the substrate concentrations as well as the values of pK$_1$, pK$_4$, pK$_5$, and pK$_6$ and the relative values of $k_3$, $k_5$, $k_{a,app}$, and $k_6$. This is illustrated for each sugar in Fig. 8 for the range of pH used here. The pH corresponding to the maximum value of v/LET (if this exists) may be readily computed by differentiation of Equation 22. Thus, for d-glucose, at 25°, we have the following.
The identification of the four ionizable groups involved in the catalytic mechanism on the basis of their $pK_a$ values is equivocal because of the possibility of perturbation by the protein environment. The following assignments are therefore tentative. The $pK_1$ of around 4, which is increased to 5 in the presence of chloride, most probably represents the ionization of a carboxyl group. The value of $pK_2$, namely 6.90, is in the range most frequently spanned by the ionization of the imidazolium group of histidine in proteins, although an unusually acidic sulfhydryl or ammonium group might be responsible. However, as we shall discuss later, $pK_4$ may represent the ionization of reduced flavin. The value of 4.10 for $pK_3$ is again most likely to be due to the carboxylate group, whereas $pK_{4}^*$, with a value of 7.40, may be due to imidazolium, with sulfhydryl and ammonium remaining as possibilities. In all cases, these ionizable groups may either function directly as general acid-base catalysts in the mechanism, or they may be involved in some structural transition in the protein which is essential for catalytic activity.

The combination of the sugar with the enzyme, which is characterized by $pK_1$ and does not involve an oxidation, may involve an ionization of the hydroxyl group of carbon atom 1 of the substrate which is assisted by a carboxylate group at the active site. The solvent deuterium effect on $k_1$ would be consistent with this idea. Examples of base-catalyzed proton abstraction preceding oxidation are known (12), and it is of interest that the nonenzymatic reduction of flavins by dihydrolipoic acid is both specific and general base catalyzed (13).

The evidence bearing on the mechanism of flavin reduction is that $k_2$ is independent of pH in the range from 3 to 8 and the process involves the breaking of the bond between hydrogen and carbon 1 (10). Hydrogen atom transfer from carbon atom 1 seems unlikely because of the consistent failure to obtain any evidence for semiquinoid intermediates and because the stabilization of a glucose radical would be difficult. Arguments were advanced to support the idea that hydrogen atom 1 is removed as a proton following a glycoside linkage with the flavin and concurrent with electron transfer into the flavin (10). These arguments were in the form of analogies, drawn from a mechanism for bromine oxidation of glucose (14) and from a scheme proposed for $N$-amino acid oxidase which was based on the isolation of an enzyme-substrate derivative after borohydride treatment and hydrolysis (15). Subsequently, this derivative was shown to be that expected if a Schiff base were formed between the $\alpha$-keto (or $\alpha$-imino) acid product and the $\varepsilon$-amino group of a lysine residue in the enzyme (16). The mechanistic significance of such a structure is not at all clear, especially since borohydride treatment was later shown to have no effect upon the catalytic activity of the enzyme (17). In any case, the fact that flavin reduction per se is not general base-catalyzed (at least within the limits of our measurements) makes the idea of proton abstraction from carbon 1 of the substrate in the glucose oxidase reaction less attractive. The alternative mechanism, namely, hydride transfer to flavin from carbon 1 of the sugar anion formed in the first step of the reaction, has the advantageous feature of expulsion of a negative species from a negative, rather than a neutral, substrate. Furthermore, calculations quoted in Reference 18 favor hydride transfer in the oxidation of methoxide and other species. The pH insensitivity of $k_2$ may indicate that the developing negative charge on the flavin, resulting from hydride transfer from substrate, is neutralized by proton transfer from H$_2$O.

The function of the acid group with an apparent $pK_a$ of 6.85 which is essential for the reduction of oxygen in the step con-
trolled by $k_4$ is particularly interesting since this represents one of the few cases where the crucial biological function of $O_2$, namely its reduction (albeit at the 2-electron level), can be studied in a stepwise fashion. Two experimental facts are worth emphasizing. First, this step, although far from being diffusion-controlled, is very insensitive to temperature (5). This suggests that electron transfer, rather than the breaking of a covalent bond, may be involved; in addition, since the major kinetic barrier in the interaction of $E$, with $O_2$ must be entropic, a significant structural rearrangement is implicated. The flattening of the flavin molecule which occurs upon oxidation (together with protein conformation changes which might be induced by this process) may account for these results. Second, comparison of the rate of this bimolecular process with the corresponding rate of the nonenzymatic oxidation of FMNH$_2$ (19) suggests that no enzymatic catalysis may be involved. Furthermore, since the observed $pK_a$ of 6.85 corresponds rather closely with the $pK_a$ measured for free FMNH$^+$ (20), it would be of great interest to know whether the $pH$ dependence of the oxidation of free FADH$_2$ resembles the $pH$ dependence we have obtained with reduced glucose oxidase. Until this question is resolved, it is not possible to conclude whether the enzyme provides a general acid catalyzed pathway for the combination of bound FADH$_2$ with $O_2$, or whether such a pathway is rather an intrinsic property of FADH$_2$ oxidation.

The interpretation of the bell shaped $pH$ dependence of $k_{app}$ is likewise difficult because the nature of this process is unknown, except for the fact that it involves the interconversion of two oxidized forms of the enzyme (5). Thus the process might consist of the final step (or steps) in the synthesis of free H$_2$O (including its release from the enzyme) or a structural isomerization of the enzyme. An answer to this question has to await the measurement of the rate of formation of free H$_2$O in the oxidative half-reaction. It is of interest to note that the nonenzymatic oxidation of reduced flavin by $O_2$ involves in part a saturation phenomenon (19, 21) indicative of complex formation between $O_2$ and reduced flavin, with a subsequent first order decay of this complex which involves a solvent deuterium effect (21).

It was pointed out previously (10) that a possible reason for the fact that $E_1$-$O_2$ (the analogue of FMNH$_2$-$O_2$ in the nonenzymatic reaction) is not a kinetically significant species in the glucose oxidase oxidative reaction is that the rate of conversion of $E_1$-$O_2$ to $E_0$-$P_2$ may be much greater than the largest value of $k_d([O_2])$ which is attainable experimentally. If this is the case, the catalytic efficiency of the enzymatic pathway can be judged from the fact that, whereas the rate of the nonenzymatic conversion of FMNH$_2$-$O_2$ to FMN and H$_2$O$_2$ is 24 sec$^{-1}$ (19), the rate-limiting first order process in the enzymatic oxidative half-reaction (controlled by $k_4$, and involving the interconversion of two oxidized forms of the enzyme) proceeds at a rate of about 1150 sec$^{-1}$ under similar conditions. Thus, if the enzymatic pathway involves the step $E_1$-$O_2$ $\rightarrow$ $E_0$-$P_2$, the rate of this process must be at least 100 times greater than the nonenzymatic decay of FMNH$_2$-$O_2$.

It is clear that further studies of the $pH$ and temperature dependence of the nonenzymatic oxidation of reduced flavin by $O_2$, as well as the determination of whether H$_2$O$_2$ is released enzymatically in the step controlled by $k_4$, or in that controlled by $k_d$, would help to clarify the extent and mechanism by which the oxidation of E-FADH$_2$ by $O_2$ is aided by the protein.

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REFERENCES