The Glucose Oxidase Mechanism. Enzyme Activation by Substrate

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Abstract: Glucose oxidase, in its catalysis of the reaction between glucose and oxygen, is shown to be consistent with the reactions (1) $E + G \rightarrow RL$; (2) $RL \rightarrow R + L$; (5) $RL + G \rightarrow R + L + G$; (3) $R + O_2 \rightarrow E \cdot H_2O_2$; and (4) $E \cdot H_2O_2 \rightarrow E + H_2O_2$, where E is oxidized enzyme, G is β-D-glucose, R is reduced enzyme, and L is gluconolactone; RL and $E \cdot H_2O_2$ are complexes involving enzyme and products. The rate constants k_1 and k_5 are determined at 0 and 15°. At 30°, k_2 and k_5 become too large to determine and the three-parameter mechanism involving only k_1, k_3, and k_4 reproduces the data. Oxygen-uptake data and steady-state stopped-flow experiments lead to identical results, within experimental error.

Glucose oxidase (EC 1.1.3.4) catalyzes the reaction between β-D-glucose and oxygen to yield hydrogen peroxide and δ-gluconolactone. The mechanism has been studied by Nakamura and Ogura[2a] and more recently in a more definitive fashion by Gibson, Swoboda, and Massey.[2b] The latter concluded that the mechanism involved steps 1–4

$$E + G \xrightarrow{k_1} RL \quad (1)$$

$$RL \xrightarrow{k_2} R + L \quad (2)$$

$$R + O_2 \xrightarrow{k_3} E \cdot H_2O_2 \quad (3)$$

$$E \cdot H_2O_2 \xrightarrow{k_4} E + H_2O_2 \quad (4)$$

where E is oxidized enzyme, R is reduced enzyme, G is β-D-glucose, and RL and $E \cdot H_2O_2$ are complexes between enzyme and products, gluconolactone[1] and hydrogen peroxide. Gibson and Bright,[3] using deuterated glucose, have been able to demonstrate conclusively that a reaction, $E + G \rightleftarrows EG$, is involved and shows up clearly when the hydrogen atom transfer between enzyme and glucose is slowed by the presence of the isotope. This step is not included in our studies, because, as shown earlier,[2b] hydrogen atom transfer is so rapid with ordinary β-D-glucose that EG cannot be detected kinetically.

Gibson, *et al.*,[2b] employed a Warburg apparatus to study the rate of oxygen uptake in the reaction, and they used steady-state stopped-flow methods,[4] monitoring the 450-mμ peak of the flavin in the enzyme. The invention of the rapidly responding oxygen electrode as is used in the Y.S.I. biological oxygen monitor provides a method for obtaining homogeneous oxygen uptake rate data very much more rapidly and, usually, more precisely than can be done with the Warburg device. The use of the oxygen electrode in this work, then, allowed for a more detailed analysis of a large number of data points than was available to Gibson, *et al.*;[2b] this advantage was sufficient to show clearly that an additional reaction is necessary to complete the mechanism

$$RL + G \xrightarrow{k_5} R + L + G \quad (5)$$

The constants are the rate constants for each step.

Experimental Section

The glucose oxidase from *Aspergillus niger* was purchased from Mann Research Laboratories. The glucose was obtained from Pfanstiehl Laboratories, Inc. Other chemicals used in preparing buffers and the like were of ACS reagent quality.

Oxygen monitor experiments were done on a YSI biological oxygen monitor by placing the anomer-equilibrated glucose dissolved to appropriate concentration in buffer consisting of 0.1 *M* sodium acetate adjusted to pH 5.6 with acetic acid; the solution was saturated with oxygen, the electrode was put in place in the cuvette, and the enzyme solution was then added by means of a Hamilton syringe calibrated in microliters. The oxygen concentration was recorded as a function of time on a Heath Model EUW-20A strip chart recorder equipped with a variable speed drive. The enzyme was standardized by spectrophotometric titration with glucose, the absorbency index difference between oxidized and reduced flavin being found to be $1.3 \times 10^4\ M^{-1}\ cm^{-1}$ by limited oxygen oxidation of partially reduced enzyme. Enzyme concentration, expressed as moles of active flavin per liter, was accurately known and of the order of 10^{-8} *M*. In the work at 0°, the recommended low-temperature YSI membrane was used to ensure fast response. In all cases, 10^{-3} *M* NaCN was present in the solution to suppress catalase action and to sequester any heavy metal ions which might inhibit the enzyme. The concentration of glucose studied ranged from 0.001 to 0.6 *M*, where these are concentrations of the equilibrated anomeric glucose.

The steady-state stopped-flow experiments were done using the Gibson–Durrum apparatus. In these experiments, the enzyme concentration, accurately known from run to run, was about 5×10^{-6} *M*. Acetate buffer and cyanide were identical with those used in the oxygen monitor experiments. Actual concentrations of glucose, and enzyme and starting oxygen concentrations used, were carefully recorded, the oxygen being determined on the biological oxygen monitor. Plots of oxidized enzyme concentration *vs.* time were prepared from photographs of the per cent transmission *vs.* time traces on the oscilloscope. Temperatures were controlled to within ±0.1°.

Results

Gibson, Swoboda, and Massey found the mechanism of glucose oxidase action to follow reactions 1–4, where G is glucose, E is oxidized enzyme, R is reduced enzyme, L is gluconolactone, and RL is reduced enzyme–gluconolactone complex; $E \cdot H_2O_2$ is a complex involving oxidized enzyme and hydrogen peroxide. These reactions lead to the rate equation (steady state)

$$\frac{E_T}{V} = \frac{1}{k_1[G]} + \frac{1}{k_2} + \frac{1}{k_3[O_2]} + \frac{1}{k_4}$$

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(2) (a) T. Nakamura and Y. Ogura, *J. Biochem.* (Tokyo), **52**, 214 (1962); (b) Q. H. Gibson, B. E. P. Swoboda, and V. Massey, *J. Biol. Chem.*, **239**, 3927 (1964).

(3) H. J. Bright and Q. H. Gibson, *ibid.*, **242**, 994 (1967).

(4) B. Chance, *ibid.*, **151**, 553 (1943).

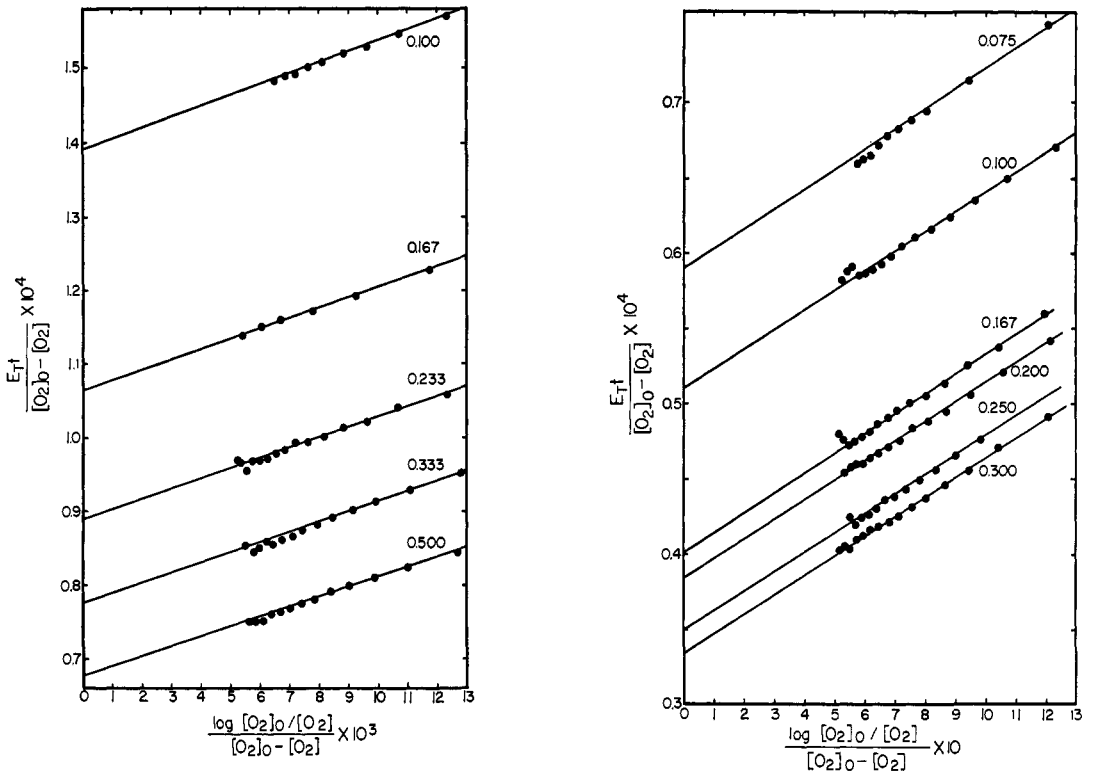

Figure 1. Plot to evaluate k_3 and $1/k_1[G] + 1/(k_2 + k_5[G]) + 1/k_4$ (intercept) from the equation

$$\frac{E_1 t}{[O_2]_0 - [O_2]} = \frac{1}{k_1[G]} + \frac{1}{k_2 + k_5[G]} + \frac{1}{k_3} + \frac{\ln [O_2]_0/[O_2]}{[O_2]_0 - [O_2]} + \frac{1}{k_4}$$

The slope is $1/k_3$. Data from $[O_2]$ *vs.* t curves were obtained from a biological oxygen monitor. Time is expressed in minutes; (a, left) 0°; (b, right) 15°.

We have found that an additional reaction is necessary to complete the mechanism (eq 5). This reaction

$$RL + G \xrightarrow{k_5} R + L + G$$

is a catalytic dissociation of the complex RL, the catalyst being the substrate, glucose.

The qualitative necessity for each of the equations as well as the quantitative evaluation of each of the rate constants involved were determined using data from the biological oxygen monitor. The data consist of oxygen concentration as a function of time at various glucose and enzyme concentrations.

Steady-state treatment leads to eq 6, where E_T is total

$$\frac{E_T}{V} = \frac{1}{k_1[G]} + \frac{1}{k_2 + k_5[G]} + \frac{1}{k_3[O_2]} + \frac{1}{k_4} \quad (6)$$

enzyme concentration. The oxygen monitor data are integral data. Therefore $-dt/d[O_2]$ was substituted for $1/V$ and the equation integrated, leading to eq 7

$$\frac{E_T t}{[O_2]_0 - [O_2]} = \frac{1}{k_1[G]} + \frac{1}{k_2 + k_5[G]} + \frac{1}{k_4} + \frac{1}{k_3}\left(\frac{\ln ([O_2]_0/[O_2])}{[O_2]_0 - [O_2]}\right) \quad (7)$$

where [G] is in large excess of $[O_2]_0$, the starting oxygen concentration; $[O_2]$ is the concentration of oxygen at any time, t. Plots were prepared of $E_T t/([O_2]_0 - [O_2])$ *vs.* $\ln([O_2]_0/[O_2])/([O_2]_0 - [O_2])$ at various [G] (Figure 1). When [G] is in large excess over $[O_2]$, these lines may be seen to have a slope of $1/k_3$ and an intercept of $1/k_1 + 1/(k_2 + k_5[G]) + 1/k_4$. Thus k_3 is determined quantitatively.

The intercepts, I, were then plotted *vs.* $1/[G]$. This results in the plots shown in Figure 2. The slope of this line approaches $1/k_1 + 1/k_5$ as $1/[G]$ approaches zero, but it is a curve, as would be expected if k_2 were involved. From the limiting slope at $1/[G] = 0$, the sum $1/k_1 + 1/k_5$ was estimated. The intercept at $1/[G] = 0$ is $1/k_4$.

The precise evaluation of k_1 can be done by taking advantage of the difference in magnitude between k_1 and k_3; that is, if the starting glucose concentration in a run is made small (of the order of magnitude of the starting oxygen concentration), then $1/k_1[G]$ in eq 6 becomes much larger than $1/k_3[O_2]$, or, indeed, than the terms $1/(k_2 + k_5[G])$ and $1/k_4$, both of which are or become independent of either substrate concentration at low glucose concentrations. Stated differently, the rate depends almost entirely on the reaction $E + G \rightarrow RL$ at low [G]. However, the control of the rate by this chemical reaction becomes complete only when $[G] \rightarrow 0$. Yet another statement of this effect is that as $[G] \rightarrow 0$, $[E] \rightarrow E_T$. Since, from steady-state treatment, $V = k_1[E][G]$, then $-d[G]/dt = k_1[E][G]$ and $-d[G]/dt \sim k_1 E_T[G]$ at low [G]; then plots of ln [G] *vs.* time yield lines whose initial slopes are very easily measured. The extrapolation to $[G] = 0$ is done as follows. If the initial slope of ln [G] *vs.* t plots is S, then $S = k_1[E]$; inverting and multiplying the result by E_T yields $E_T/S = E_T/k_1[E]$. From steady-state treatment $E_T/V = 1/k_1[G] + 1/(k_2 + k_5[G]) + 1/k_3[O_2] + 1/k_4$ and $E/V = 1/k_1[G]$. Thus, $E_T/[E]$ may be written

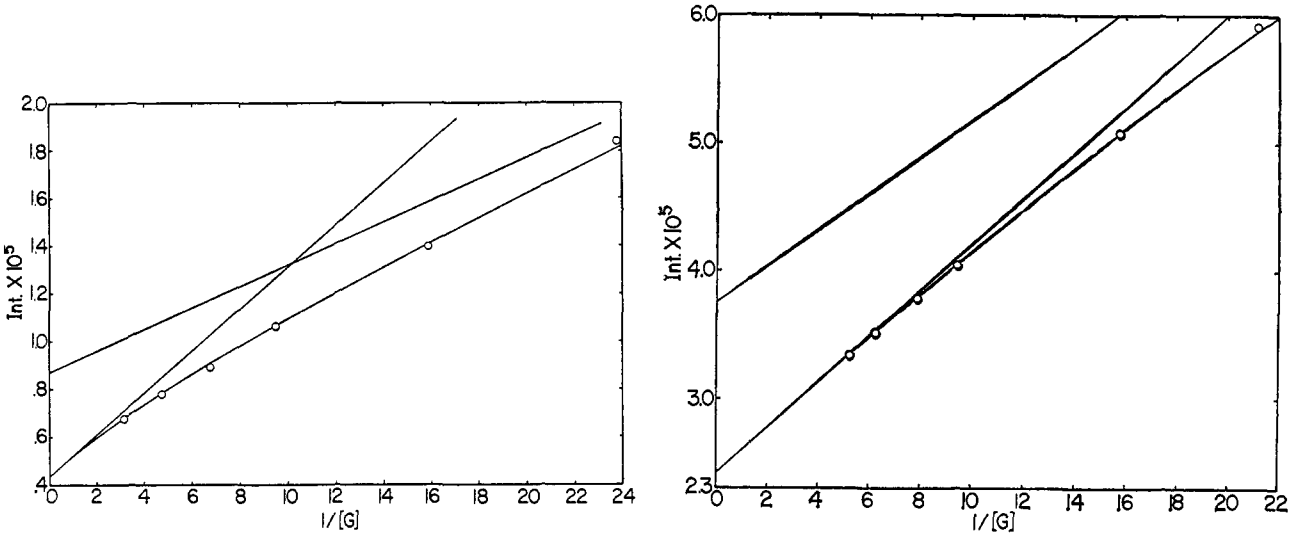

Figure 2. Plots to determine k_4 and to estimate k_2 and k_5. "Int." are the intercepts of plots of $E_T t/([O_2]_0 - [O_2])$ *vs.* log $([O_2]_0/[O_2])/([O_2]_0 - [O_2])$, where Int $= 1/(k_1[G]) + 1/(k_2 + k_5[G]) + 1/k_4$. The upper straight line would be expected if $k_5 = 0$ (Gibson, *et al.*,[2] mechanism) and has a slope of $1/k_1$ and an intercept of $1/k_4 + 1/k_2$. The lower straight line would be expected if $k_2 = 0$ and has a slope of $1/k_1 + 1/k_5$ and an intercept of $1/k_4$. The curved line is calculated from the final values taken from the constants and the points represent experimental data. Time is expressed in minutes; (a, left) 0°; (b, right) 15°.

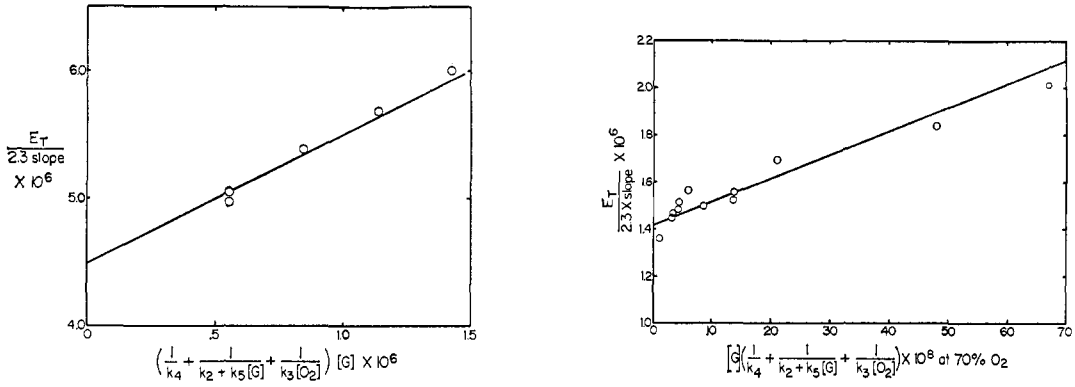

Figure 3. Plot to evaluate k_1, where $E_T/[S] = 1/k_1 + [G]\{1/(k_2 + k_5[G]) + 1/k_3[O_2] + 1/k_4\}$. The slope is drawn as unity, the intercept is $1/k_1$, and the points are calculated from experimental data. Time is expressed in minutes; (a, left) 0°; (b, right) 15°.

in terms of the constants and substrate concentrations and this result substituted in the E_T/S equation to yield $E_T/S = 1/k_1 + [G]\{1/(k_2 + k_5[G]) + 1/k_3[O_2] + 1/k_4\}$. Here k_3 is known, k_4 has been estimated from the intercepts of the experimental lines in Figure 2, k_2 and k_5 have been estimated from the slope and curvature of the experimental lines in Figure 2, and the quantity $[G]\{1/(k_2 + k_5[G]) + 1/k_3[O_2] + 1/k_4\}$ can be estimated; when this quantity is plotted *vs.* E_T/S, the resulting straight line *must* have a slope of unity and an intercept at $[G] = 0$ of $1/k_1$. Such plots are shown in Figure 3.

Finally, with a good value for k_1, eq 8 follows directly

$$\left[\frac{E_T t}{[O_2]_0 - [O_2]} - \frac{1}{k_1[G]} - \frac{1}{k_3}\left(\frac{\ln ([O_2]_0/[O_2])}{[O_2]_0 - [O_2]}\right) - \frac{1}{k_4}\right]^{-1} = k_2 + k_5[G] \quad (8)$$

from eq 7, and the left-hand side of the equation may be calculated. Then a plot of

$$\left[\frac{E_T t}{[O_2]_0 - [O_2]} - \frac{1}{k_1[G]} - \frac{1}{k_3}\left(\frac{\ln ([O_2]_0/[O_2])}{[O_2]_0 - [O_2]}\right) - \frac{1}{k_4}\right]^{-1}$$

Table I. Rate Constants[a]

	Oxygen monitor	Stopped flow	GSM
	0°		
k_1, M^{-1} min^{-1}	$2.22 \pm 0.02 \times 10^5$	2.2×10^5	2.0×10^5
k_2, l. min^{-1}	$2.3 \pm 0.15 \times 10^4$		3.9×10^4
k_3, M^{-1} min^{-1}	$7.8 \pm 0.34 \times 10^7$	7.5×10^7	7.8×10^7
k_4, l. min^{-1}	$2.3 \pm 0.02 \times 10^4$	2.0×10^4	2.2×10^4
k_5, M^{-1} min^{-1}	$2.6 \pm 0.26 \times 10^5$		
	15°		
k_1, M^{-1} min^{-1}	$7.0 \pm 0.08 \times 10^5$	5.5×10^5	5.6×10^5
k_2, min^{-1}	$7.0 \pm 0.79 \times 10^4$		2×10^5
k_3, M^{-1} min^{-1}	$1.26 \pm 0.01 \times 10^8$	1.2×10^8	1.1×10^8
k_4, min^{-1}	$4.1 \pm 0.03 \times 10^4$	4.3×10^4	4.6×10^4
k_5, M^{-1} min^{-1}	$2.8 \pm 0.95 \times 10^6$	(1.6×10^6)	
	30°		
k_1, M^{-1} min^{-1}	$1.43 \pm 0.04 \times 10^6$		1.1×10^6
k_3, M^{-1} min^{-1}	$1.60 \pm 0.08 \times 10^8$		1.3×10^8
k_4, min^{-1}	$8.6 \pm 0.28 \times 10^4$		7.5×10^4

[a] Rate constants calculated for β-D-glucose concentration in moles and minutes. The column headed GSM contains constants calculated from ref 2b, extrapolated in some cases from the nearest temperature. The limits of precision are standard deviations. The accuracy of the constants is estimated to be about ±20%, based on agreement between different runs by different people in this laboratory and by comparison with the values of Gibson, *et al.*[2b]

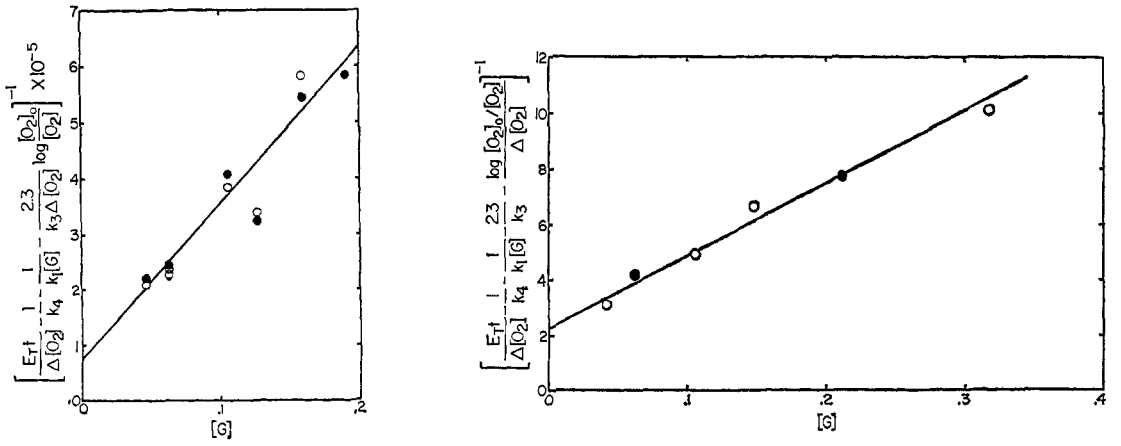

Figure 4. Plot to evaluate k_2 and k_5. Since $E_T t/([O_2]_0 - [O_2]) = 1/k_1[G] + 1/(k_2 + k_5[G]) + (1/k_3)\{\ln([O_2]_0/[O_2])/([O_2]_0 - [O_2])\} + 1/k_4$, then $(E_T t/([O_2]_0 - [O_2]) - 1/k_1[G] - 1/k_3)\{\ln([O_2]_0/[O_2])/([O_2]_0 - [O_2])\} - 1/k_4)^{-1} = k_2 + k_5[G]$, and the plot has a slope of k_5 and intercept of k_2. Black dots are at infinite oxygen, open circles are at 10^{-4} M oxygen. Time is expressed in minutes; (a, left) 0°; (b, right) 15°.

vs. [G] yields a line with a slope of k_5 and an ordinate intercept of k_2 (see Figure 4).

As the numerical values of the constants were improved, successive approximations were used until the interdependent constants underwent no further change. The constants so determined are displayed in Table I. Table II compares randomly selected oxygen concentrations and times, where the experimental times are compared with calculated times. It may be seen that the agreement is excellent.

The work was then repeated, now using steady-state stopped-flow procedures. Here, plots of E_{ox} (=[E] + [E·H_2O_2]) *vs.* t are obtained. Steady-state treatment leads to

$$\frac{E_{ox}}{V} = \frac{1}{k_1[G]} + \frac{1}{k_4}$$

The area A_0 under the E_{ox} *vs.* t curve is

$$\int_0^{t([O_2]\approx 0)} E_{ox}dt$$

But $1/V = -dt/d[O_2]$, so that $E_{ox}\, dt/d[O_2] = -(1/k_1[G] + 1/k_4)$, and

$$\int_0^{t([O_2]\approx 0)} E_{ox}dt = A_0 = -\int_{[O_2]_0}^{0}\left(\frac{1}{k_1[G]} + \frac{1}{k_4}\right)d[O_2] = \left(\frac{1}{k_1[G]} + \frac{1}{k_4}\right)[O_2]_0$$

It may be seen that $A_0/[O_2]_0 = E_{ox}/V = 1/k_1[G] + 1/k_4$ provided $[G]_0$ is in large excess over $[O_2]_0$ and remains constant during the run. Furthermore, it may be seen that, in general

$$\int_0^t E_{ox}dt = A_t = -\int_{[O_2]_0}^{[O_2]_t}\left(\frac{1}{k_1[G]} + \frac{1}{k_4}\right)d[O_2]$$

or

$$A_t = (1/k_1[G] + 1/k_4)([O_2]_0 - [O_2])$$

where A_t is the area swept out at time t at which time the oxygen concentration is $[O_2]$.

Dividing the equation for A_t by that for A_0, one obtains $A_t/A_0 = ([O_2]_0 - [O_2])/[O_2]_0 = 1 - ([O_2]/[O_2]_0)$; then $1 - A_t/A_0 = (A_0 - A_t)/A_0 = [O_2]/[O_2]_0$; and $[O_2] = [(A_0 - A_t)/A_0]\,[O_2]_0$. Plots of E_T/E_{ox} *vs.* $1/(A_0 - A_t)$ yield lines with slope $1/k_3$ because $(E_T/E_{ox})\cdot(A_0/[O_2]_0) = (E_T/E_{ox})(E_{ox}/V) = E_T/V$, and $A_0/[(A_0 - A_t)[O_2]_0/A_0] = 1/[O_2] = (A_0/[O_2]_0)[1/(A_0 - A_t)]$. In taking the slope E_T/E_{ox} *vs.* $1/(A_0 - A_t)$, the factor $A_0/[O_2]_0$ cancels and the plot has the slope of E_T/V *vs.* $1/[O_2]$. Such plots have been shown to have the slope $1/k_3$. The intercepts, E_T/E_{ox}, multiplied by $A_0/[O_2]_0$ *vs.* $1/[G]$ are the same as E_T/V *vs.* $1/[G]$ plots obtained from the oxygen monitor data. At infinite $[O_2]$, the intercept is $1/k_4$ and the slope is $1/k_1 + 1/k_5$. These plots, along with E_{ox}/V (equivalent to $A_0/[O_2]_0$) *vs.* $1/[G]$ plots, are shown in Figure 5. The slope of the E_{ox}/V *vs.* $1/[G]$ is $1/k_1$, while the intercept is $1/k_4$. Note that, within experimental error, the intercepts are the same while the slopes are quite different for the two curves, confirming the necessity for the reaction involving k_5.

The constants k_1, k_3, and k_4 have now been determined. No attempt was made to separate the constants k_2 and k_5 in the 0° case and k_2 was not determined at 15°. The oxygen monitor data were considered to be more reliable for the determination of k_2 and k_5.

Discussion

The difference between the mechanism presented here and that of Gibson, *et al.*, is the addition of the parameter k_5 and the reaction RL + G → R + L + G. The necessity for the additional parameter is shown in two ways: first, the slopes of the lines in Figure 2 as glucose concentration approaches infinity, when compared with the slope of the E_{ox}/V *vs.* $1/[G]$ plot in Figure 5; Figure 5 has the slopes $1/k_1$ at the pertinent temperature, while the slopes in Figure 2 are greater than this as $1/[G] \to 0$. The latter slopes in the present mechanism are $1/k_1 + 1/k_5$. The other manner of showing this is in the slope of E_T/V *vs.* $1/[G]$ plots from the stopped-flow data when compared with the E_{ox}/V *vs.* $1/[G]$ plots (upper curve slopes compared with the lower curve slopes in Figure 5) from the same data. The fact that the parameter, k_2, is needed arises from the curvature of the plots (points) in Figure 2.

It is worthwhile at this point to consider what can be rather definitely concluded from the data. First, as previously shown,[2b] there are two oxidized forms of the enzyme, only one of which reacts with glucose and neither of which reacts with oxygen. This is proven by Figure 5. Here, we see that at infinite glucose and

Table II. Calculated and Observed Times[a]

% O_2 sat.	t_{obsd}	t_{calcd}	% O_2 sat.	t_{obsd}	t_{calcd}
$G_T = 0.50\ M$; $E_T = 1.61 \times 10^{-8}\ M$			$G_T = 0.010\ M$; $E_T = 6.42 \times 10^{-8}\ M$		
81.9	0	...	89.8	0	...
75.0	0.70	0.69	82.6	2.00	1.99
70.0	1.19	1.18	68.5	6.00	6.04
60.0	2.19	2.20	55.3	10.00	10.03
50.0	3.24	3.23	42.8	14.00	13.97
40.0	4.32	4.31	31.1	18.00	17.78
30.0	5.46	5.43	20.2	22.00	21.62
20.0	6.67	6.64	$G_T = 0.30\ M$; $E_T = 0.879 \times 10^{-8}$		
10.0	8.08	8.08	88.2	0	...
$G_T = 0.333\ M$; $E_T = 2.14 \times 10^{-8}\ M$			80.0	0.53	0.53
80.5	0	...	70.0	1.18	1.18
70.0	0.89	0.90	60.0	1.87	1.86
60.0	1.76	1.77	50.0	2.56	2.56
50.0	2.63	2.64	40.0	3.30	3.29
40.0	3.54	3.55	30.0	4.11	4.08
30.0	4.51	4.50	20.0	5.00	4.98
20.0	5.52	5.52	10.0	6.17	6.16
10.0	6.71	6.71	$G_T = 0.075\ M$; $E_T = 1.47 \times 10^{-8}$		
6.0	7.31	7.31	87.9	0	...
$G_T = 0.10\ M$; $E_T = 4.29 \times 10^{-8}\ M$			75.0	0.81	0.82
85.4	0	...	65.0	1.46	1.46
80.0	0.40	0.40	55.0	2.12	2.12
70.0	1.14	1.13	45.0	2.82	2.80
60.0	1.87	1.88	35.0	3.53	3.51
50.0	2.63	2.63	20.0	4.71	4.66
40.0	3.40	3.41	10.0	5.69	5.62
30.0	4.18	4.20	$G_T = 0.0174\ M$; $E_T = 0.586 \times 10^{-7}\ M$		
20.0	5.02	5.02	84.2	0	...
10.0	5.94	5.95	70.0	0.58	0.59
$G_T = 0.020\ M$; $E_T = 6.42 \times 10^{-8}\ M$			60.0	1.01	1.01
85.3	0	...	50.0	1.44	1.45
78.7	1.00	1.01	40.0	1.86	1.89
65.9	3.00	2.97	30.0	2.32	2.34
53.4	5.00	4.93	10.0	3.36	3.31
41.2	7.00	6.90	$G_T = 0.0020\ M$; $E_T = 1.03 \times 10^{-7}$		
29.3	9.00	8.88	88.4	0	...
18.0	11.00	10.80	80.0	1.60	1.61
7.6	13.00	12.71	70.0	3.84	3.79
			55.0	8.02	7.85
			45.0	11.60	11.37
			35.0	16.35	16.05

[a] Calculation of t *vs.* $[O_2]$ from rate constants. For G_T concentration of 0.1 M and higher, the working equation is

$$t = \frac{1}{E_T}\left\{\left(\frac{1}{k_4} + \frac{1}{k_1[G]} + \frac{1}{k_2 + k_3[G]}\right)([O_2]_0 - [O_2]) + \frac{2.303}{k_3}\log\frac{[O_2]_0}{[O_2]}\right\}$$

For G_T concentrations less than 0.1 M, the working equation is

$$t = \frac{2.303}{E_T}\left\{\frac{[O_2]_0 - [O_2]}{2.303k_4} + \frac{1}{k_1}\log\frac{[G]_0}{[G]} + \frac{1}{k_5}\log\frac{k_2 + k_3[G]_0}{k_2 + k_3[G]} + \frac{1}{k_3}\log\frac{[O_2]_0}{[O_2]}\right\}$$

O_2 refers to per cent of saturated oxygen at time t. Time is in minutes.

finite oxygen concentration an appreciable fraction of the enzyme remains in the oxidized form (ordinate intercept on Figure 5). The straight line in these figures shows that the reaction of the active oxidizing form with glucose is first order in glucose and that a single rate constant describes the totality of the glucose oxidation. Rate constants k_1 and k_4 adequately accommodate these data.

Second, the straight lines in Figure 1 show that a single rate constant suffices to describe the reoxidation of the reduced enzyme at varying oxygen and glucose concentrations; this graph shows that the reoxidation is first order in oxygen and involves only one reduced species which is capable of reacting with oxygen. Figure 2 is essentially a plot of E_T/V at infinite oxygen *vs.* 1/[G]. Under these conditions, the reduced form of the enzyme which is capable of reacting with oxygen is eliminated ($1/k_3[O_2] \rightarrow 0$). It has already been shown that one of the oxidized forms of the enzyme is unreactive with glucose; therefore, the curvature found in Figure 2 must be due to a reaction of glucose with a reduced form of the enzyme; that this reaction must involve a reduced form which does not react with oxygen is shown by the fact that the reaction occurs at infinite oxygen concentration, where the form which does react with oxygen is no longer present.

There remains, then, the question of the nature of the various oxidized and reduced forms and the chemistry involved in the reaction of the nonoxidizable (by oxygen) reduced form with glucose. Gibson, *et al.*,[2b] put forth two hypotheses concerning the nature of the various forms of enzyme. The first and preferred postulate is that the nonoxidizable reduced form is RL, a complex between the reduced enzyme and the lactone, which must dissociate before oxidation can take place. Correspondingly, the nonreducible oxidized form is $E \cdot H_2O_2$,

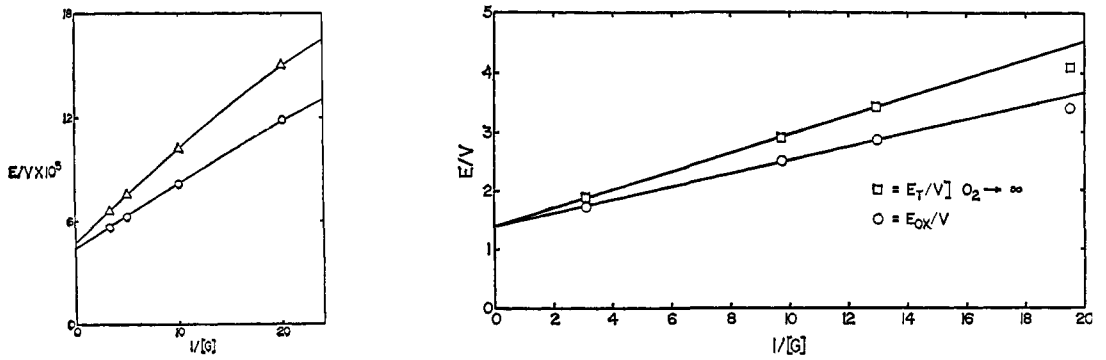

Figure 5. Lower lines are plots of E_{ox}/V *vs.* 1/[G] and have slopes of $1/k_1$ and intercepts of $1/k_4$ within experimental error; upper lines are E_T/V *vs.* 1/[G] and have limiting slopes (1/[G] → 0) of $1/k_1 + 1/k_5$ within experimental error. Lines are not calculated but drawn to best fit experimental points. Time is expressed in minutes; (a, left) 0°; (b, right) 15°.

a complex involving oxidized enzyme and hydrogen peroxide, which must also dissociate before its reduction can occur. The second postulate is that the nonoxidizable reduced form differs from the oxidizable form in conformation, that is, the two forms are conformational isomers. Correspondingly, the two oxidized forms would be conformational isomers. Considerable further work needs to be done to distinguish these postulates.

In regard to the chemistry involved in the reaction of the nonoxidizable reduced form with glucose, several comments should be made. First, this reaction with glucose results in the same product as is obtained in a parallel reaction which does not involve either substrate. That is, the reaction R → R′ and the reaction R + G → R′ + G yield the same products. Therefore, the product R′ cannot contain glucose. Further proof of this is provided by Gibson, *et al.*,[2b] who showed that enzyme reduced by a *stoichiometric* quantity of glucose reoxidized with the same rate constant as enzyme having an excess of glucose present. Further, they showed that the reoxidation rate using 2-deoxyglucose was, within experimental error, the same as that found with glucose. Thus, the glucose must react with the first formed reduced enzyme to catalyze the change from a form not oxidized by oxygen to that which reacts with oxygen. If the nonoxidizable form is RL, then the catalysis could be accomplished by a displacement reaction: RL + G → RG + L, followed by a very rapid dissociation of RG such that no kinetically detectable quantity of RG accumulates. If the nonoxidizable form is a conformational isomer of the oxygen-reactive reduced form, one need only postulate that glucose associates with the oxygen-unreactive reduced enzyme and that this complex rapidly isomerizes with dissociation of the glucose. If the first formed reduced enzyme has the conformation of the oxidized enzyme (this seems sensible because of the rapidity of the reduction process), glucose might well be attracted to this conformation as it is to the oxidized enzyme.[3] Then, because of the rapidity of the reoxidation of R′, the stable reduced configuration, one would obtain E′, having the conformation of the reduced form. This then would have to isomerize to the stable oxidized form in order to oxidize glucose. Further work is necessary to distinguish these possibilities.