The Mechanism of Action of Xanthine Oxidase*

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SUMMARY

The changes in optical and EPR spectra observed during the anaerobic titration of xanthine oxidase with dithionite can be accounted for by simple oxidation-reduction equilibria involving Mo(VI) ↔ Mo(V) ↔ Mo(IV); FAD ↔ FADH → FADH₂, and the 1-electron reduction of each of the two iron-sulfur centers. The equilibrium constants so obtained permit the calculation of the relative probabilities of the 36 possible states that occur on addition of a given quantity of reductant. pH jump experiments with this enzyme (EDMONDSON, D., BALLOU, D., VAN HEUVELEN, A., PALMER, G., and MASSEY, V. (1973) J. Biol. Chem. 248, 6129–6135) show that internal electron transfer is much more rapid than turnover and we therefore assume that the distributions described by these equilibrium constants are maintained during catalysis.

By adopting a model in which 3 moles of xanthine each donate a pair of electrons in a consecutive irreversible process, and using the equilibrium distributions described above, it is possible to calculate accurately the time course of reduction of both the flavin and iron-sulfur centers and to account for the increase in the rate of bleaching of deflavoenzyme observed experimentally. By invoking the existence of enzyme-xanthine complexes we can account for (a) the participation and time dependence of “very rapid” and “rapid” molybdenum and (b) the deuterium isotope effects observed during steady state and transient state kinetics (EDMONDSON et al.).

The same equilibrium model accounts for the optical and EPR data obtained during the reaction of reduced enzyme with oxygen; the biphasic kinetics are readily explained by assuming that FADH₂ is the species reacting rapidly with O₂ in the fast phase.

From these results we conclude that the iron-sulfur centers act as an electron reservoir functioning to maintain molybdenum as Mo(VI) (for efficient reduction) and flavin as FADH₂ (for efficient oxidation).

Perhaps the most striking feature of the reactions of milk xanthine oxidase with either xanthine or oxygen is the apparent kinetic simplicity of the observed absorbance changes. In the case of anaerobic reduction, the time course of the decrease in absorbance at 450 nm exhibits almost exponential behavior after a slight initial lag. The reaction of reduced enzyme with oxygen is only slightly more complex exhibiting two distinct kinetic phases. The more rapid oxidation phase appears to reflect the removal of 5 electrons per enzyme-bound FAD and the slow phase, the removal of the remaining electron. The dependence of substrate concentration on both the rate observed in the fast phase of oxidation and that observed in reduction is readily interpreted in terms of a very rapid formation of an enzyme-substrate complex which subsequently decays more slowly. This simple interpretation of the individual half-reactions is entirely compatible with the observed steady state kinetic pattern.

In no case is there any indication that intramolecular electron transfer between the various oxidation-reduction groups of the enzyme is rate-limiting (1, 2). Rather, the limiting step in both oxidation and reduction appears to be the decay of an enzyme-substrate complex which is accompanied by a much faster redistribution of electrons among molybdenum, the iron-sulfur centers, and FAD. Thus the key to describing the absorbance changes and the appearance or disappearance of EPR signals during the reaction of xanthine oxidase with substrates is the determination of the equilibrium distribution of electrons among the various oxidation-reduction groups. The rate of change of this distribution appears to depend on only a limited number of kinetic parameters which describe the formation and disappearance of enzyme-substrate complexes.

At present, the best evidence that the distribution of electrons is determined solely by the relative reduction potentials of the various acceptor groups comes from an examination of the appearance and disappearance of the EPR signals which have been assigned to the two types of iron-sulfur centers. For example, Orme Johnson and Beinert (3) and later Lowe et al. (4) have shown in static experiments that Fe/S² II exhibits a higher reduction potential than Fe/S I. In agreement with these observations, reduced Fe/S II appears more rapidly than reduced Fe/S I during kinetic anaerobic reduction experiments (1). When the kinetics of oxidation was examined, the signal representing Fe/S I disappeared much more rapidly than that of Fe/S II, this order being that predicted by the relative reduction potential of the two groups (2).

The original purpose of the present study was to investigate the validity of the idea that the various reactions of xanthine oxidase can be explained solely in terms of the relative reduction potential is supported by the satisfactory agreement observed experimentally. Thus, by using the data presented here, we should be able to answer questions concerning the nature of the electron acceptors in this enzyme.

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‡ The abbreviation used is: Fe/S, non-heme iron-sulfur center.
potentials of the various electron acceptor groups and a limited number of kinetic constants. As the work progressed, it became clear that this rather simple mathematical formulation was capable of explaining not only the gross features of the oxidation and reduction reactions but also a variety of other experimental observations which had previously been considered as anomalous behavior. However, it should be emphasized that the importance of the mathematical models are not so much that they are capable of describing a variety of observations, but rather that they make certain predictions which can be tested experimentally. If nothing else, at least a framework is provided within which a given problem can be defined more precisely in terms of what parameters are known and which need to be measured.

MATERIALS AND METHODS

Most experimental details are given in the preceding paper (2). Unless indicated otherwise, all experiments were conducted in 0.1 M pyrophosphate, pH 8.5, at 25°C.

Combined optical and EPR dithionite titrations were performed in a special all glass anaerobic vessel which contained an attached cuvette for absorbance readings, a calibrated exit port which allowed the anaerobic transfer of a known volume of sample solution to an EPR tube, and an entry port to which a Hamilton syringe containing stock dithionite solution could be attached. The exact design of the apparatus and the preparation of anaerobic solutions will be presented elsewhere. It is important to note that after each addition of reducing equivalents the titration apparatus was placed in a recording spectrophotometer and the final reading was taken only after no further slow absorbance changes occurred.

Simulation of kinetic data was performed on a PDP 8E digital computer using standard mathematical expressions for consecutive, irreversible reactions. Programs were written in 8K Focal language and computer time was kindly provided by Dr. C. H. Williams.

RESULTS AND DISCUSSION

Xanthine oxidase appears to contain two active sites, each of which contains 1 molybdenum atom, two distinct iron-sulfur centers, and 1 molecule of FAD (5). At present there is no evidence which suggests that electrons are distributed among a minimum of 12 electron-accepting groups. On the contrary, the most plausible interpretation is that each 380,000 molecular weight protein molecule contains two noninteracting active centers, each composed of molybdenum, FAD, and iron in a ratio of 1:1:4 (1, 5). Throughout this paper, the term enzyme molecule will refer to a single active center which is capable of accepting only 6 electrons during catalysis.

In our previous study of the oxidation of reduced xanthine oxidase, the amount of reaction occurring in the fast phase was found to be markedly dependent on both the extent of reduction prior to mixing and the wavelength of observation. These results were interpreted in terms of the inability of 1-electron-reduced enzyme molecules to form FADH2 (2). Although not discussed, there was also a noticeable nonproportionality between the total absorbance changes at 450 nm and those at 550 nm when enzyme reduced with various amounts of dithionite was reoxidized with oxygen (Fig. 1A, A), and a similar effect was seen in static dithionite titration experiments. At low levels of reduction, the percentage of change observed at 500 nm is consistently greater than that at 450 nm; however, after the addition of about 1 mole of dithionite per total FAD, the percentage of change at 550 nm is smaller than that at 450 nm. Identical results were found when earlier titration studies were examined in terms of the relative absorbance changes at different wavelengths (Fig. 1A, A, and Ref. 6).

This relationship between changes at 450 and 550 nm is not limited to static titrations with dithionite. Similar nonlinearity was observed when dithionite was added as achieved by light irradiation in the presence of glycine (Fig. 1B). Another way of demonstrating this relationship is to plot the ratio of the absorbance changes at 450 and 550 nm against the amount of reducing equivalents added (Fig. 1B). The value of this ratio approaches 2.0 when small amounts of dithionite are added and is identical with that measured for the complete reduction of deflavoxanthine oxidase (2), suggesting that only the iron centers contain reducing equivalents in 1-electron-reduced enzyme molecules. As the titration proceeds, the reduction of flavin predominates and is expressed by a sharp rise in the ratio of the absorbance changes, which reaches a maximum value of about 5.0. Then, as the remaining electrons are added the ratio drops to about 4.0, the final value for the complete reduction of xanthine oxidase. This final decrease in the ratio suggests that once FAD is fully reduced, the remaining electrons are taken up by the iron and molybdenum centers. This pattern of electron uptake is identical with that predicted from the kinetic features of the oxidation reaction (2).

Anaerobic Titrations with Dithionite—The results given in Fig. 1 and those described in the preceding paper (2) indicate a fixed relationship between the reduction potentials of the iron-sulfur centers and FAD. In order to quantify this relationship and to determine the relative reduction potentials of the various states of molybdenum, a new set of titration experiments were performed in which both the absorbance and electron paramagnetic resonance properties of the enzyme were measured after each addition of dithionite. In agreement with the results presented in Fig. 1, a nonlinear relationship between the absorbance changes at 450 nm and those at 550 nm was observed (Fig. 2A). The pattern for the appearance of the flavin radical, iron, and molybdenum signals (Fig. 2B) is also consistent with previous dithionite titration experiments in which only EPR spectra were recorded (11).

It is generally believed that the "rapid" molybdenum signal represents Mo(V) (7) and that the fully oxidized enzyme contains molybdenum in the VI oxidation state. However, the oxidation...
for this reason, it will be assumed that only 6 electrons can be taken up by a given active site and that if further reduction does take place, the groups responsible exhibit reduction potentials which are much more negative and, therefore, can be neglected.

The amount of flavin semiquinone present at any level of reduction is extremely small, about 7% of the total FAD present (Fig. 2B), and thus the contribution of this species to the observed absorbance changes is insignificant. Similarly, the contribution of absorbance changes due to the formation of Mo(V) and Mo(IV) are also negligible since even when changes attributable to molybdenum have been observed, their magnitude is quite small in comparison to the extinction difference between oxidized and reduced enzyme (9). Therefore, the contribution of flavin at 450 nm can be derived from a comparison of the spectral properties of deflavo- and native xanthine oxidases. The extinction difference between oxidized and fully reduced native enzyme was found to be 29,600 M⁻¹ cm⁻¹ at 450 nm whereas the corresponding difference for deflavoenzyme was found to be 14,400 M⁻¹ cm⁻¹. The difference of 12,200 M⁻¹ cm⁻¹ was taken to represent the extinction change between enzyme-bound FAD and FADH₂; this value is typical of flavoproteins.

The relative extinction changes of Fe/S I and II were determined in two ways. A comparison of the time course of the absorbance changes at 450 and 550 nm, measured in the stopped flow apparatus, with that of the two iron centers, measured by rapid freeze EPR techniques during oxidation of reduced enzyme, allows a calculation of the proportion of the absorbance changes due to Fe/S I and Fe/S II (2). Alternatively, if xanthine oxidase is reacted with a very small amount of reductant so that only 1-electron-reduced enzyme molecules are generated, the resultant absorbance changes can be partitioned between the two iron centers by a ratio equal to that observed in the very early stages of the combined EPR-optical titrations shown in Fig. 2. The best values of the extinction changes at 450 and 550 nm for Fe/S II were found to be 2,800 and 1,300 M⁻¹ cm⁻¹, respectively, while those for Fe/S I were 11,600 and 5,600 M⁻¹ cm⁻¹ at 450 and 550 nm, respectively. As a check on the validity of these extinction coefficients, the proportion of reduced Fe/S I (%F₁) was calculated by using the following formula:

\[
\%F₁ = \frac{6,900}{5,600} \times \frac{(550 - 1,300)}{6,900} \times (s \text{ signal at } g = 2.12)
\]

where 6,900 represents the total extinction change at 550 nm and 5,600, 1,300, the extinction changes due to Fe/S I and II, respectively; \( \% \Delta A_{560} \) the per cent absorbance change at 550 nm; and \( s \text{ signal at } g = 2.12 \), the amount of reduced Fe/S II. There is a good agreement between the values of \( F₁ \) calculated in this manner and those determined directly from the EPR signal at \( g = 1.95 \) (Fig. 3A).

Using a similar equation, the amounts of FADH₂ were calculated from the absorbance changes at 450 nm. Since the proportion of reduced Fe/S I can be calculated from either the absorbance changes at 550 nm or from the EPR signals at \( g = 1.05 \), two sets of points for FADH₂ were generated and found to agree reasonably well (Fig. 3B).

Assuming each enzyme unit is capable of accepting 6 electrons, the only reduced species which cannot be observed experimentally is Mo(IV). Thus by combining the absorbance and EPR data we can calculate the fractional amount of the species FADH₂, FADH₂, Fe/S I, Fe/S II, and Mo(IV) as a function of the degree of reduction. These are the data points shown in Fig. 3. By comparing the number of electrons accepted with the number added we see that substantial amounts of Mo(IV) do not accumulate until after the addition of 4 electrons.
Calculation of Relative Reduction Potentials—The simplest interpretation of the results given in Fig. 3 is that the distribution of electrons among the various acceptor groups is determined by a single set of six relative reduction potentials. This assumption requires that the electron affinities of the various groups are independent of the number of reducing equivalents accepted by an enzyme molecule, that is, the reduction potential of a given acceptor group is not affected by the presence of electrons in any of the other groups; then, the fractions of reduced components after each addition of dithionite are determined by a single set of six relative reduction potentials. This was achieved by starting with a set of arbitrary values for Y ranging from 10 to 0 and five trial values for the equilibrium constants. Then the various fractions of the components at equilibrium during the titration of xanthine oxidase with dithionite were calculated in terms of the constants defined in Table I and Equation 2. The dotted line described in the text. The solid lines in both panels represent theoretical curves calculated using the relative electron affinity constants and equations used in deriving the amount of reduced components in terms of the binding of an electron, and the association equilibrium constants were determined for the conditions given in Fig. 2, 0.1 m pyrophosphate, pH 8.5, at 25°. The binding of an electron to Fe/S II was assigned an affinity constant equal to unity. The remaining constants are relative to this value.

| Table I | Electron affinity constants for six acceptor groups within xanthine oxidase |
|---|---|---|
| Reaction | Electron affinity | Relative reduction potential |
| Fe/SIIox | Fe/SIIred | K | μV |
| Fe/SIox | Fe/SIred | K | μV |
| FADH + H+ | FADH+ | K | μV |
| FADH+ + H+ | FADH2 | K | μV |
| Mo(VI) | Mo(V) | K | μV |
| Mo(V) | Mo(IV) | K | μV |

Each reductive step is defined in terms of the binding of an electron, and the association equilibrium constants were determined for the conditions given in Fig. 2, 0.1 m pyrophosphate, pH 8.5, at 25°. The binding of an electron to Fe/S II was assigned an affinity constant equal to unity. The remaining constants are relative to this value.

where (FeII) and (FeI) represent Fe/S II and Fe/S I and the subscripts, the reduced and oxidized forms of these 1-electron acceptor groups. The total number of electrons per FAD is then given by:

\[ e^-/FAD = \frac{1}{1 + Y + \frac{K_1}{K_2 + K_3 + 2K_4K_5}} + \frac{K_4}{D_2} + \frac{2K_4K_5}{D_2} \]

and Y is defined as (1 minus fraction of reduced Fe/S II)/(fraction of reduced Fe/S II). From left to right, the six terms in Equation 3 represent the fractions of electrons present as reduced Fe/S II, reduced Fe/S I, FADH+, FADH2, Mo(V), and Mo(IV), respectively. Values of the five equilibrium constants were derived by fitting the observed results in Fig. 3 to Equation 3. This was achieved by starting with a set of arbitrary values for Y ranging from 10 to 0 and five trial values for the equilibrium constants. Then the various fractions of the components and the total number of electrons per FAD unit were computed, and the resultant curves were compared to those observed experimentally. This process was repeated until a satisfactory agreement between the observed and calculated results was achieved. The best values for the equilibrium constants are given in Table I. A comparison of the data and calculated curves is shown in Fig. 3, and the agreement is quite good, particularly when the scatter in the experimental data is considered.

The small amount of flavin radical observed in the titration
experiments is simply a consequence of the low affinity of oxidized flavin for a single electron, whereas the semiquinone form exhibits a 100-fold greater affinity. Similarly, the small amount of "rapid" molybdenum signal reflects the greater affinity of Mo(V) for an electron than that of Mo(VI). As expected from earlier work (1, 2, 4), the equilibrium constant for the reduction of Fe/S I was found to be about 40% of that for the reduction of Fe/S II.

In the preceding paper (2), inactivation of xanthine oxidase by cyanide treatment was shown to have no effect on the reaction of reduced enzyme with oxygen. This was true for both the rates of oxidation and the detailed spectral properties of the reaction, and suggests strongly that the equilibrium distribution of electrons within inactive and active enzyme molecules is the same. Thus, as shown in Fig. 4, the equilibrium appearance of the reduced iron centers FADH, and FADH2 within cyanide-inactivated xanthine oxidase follows a pattern analogous to that observed for native enzyme. The major difference is the lack of any detectable amount of "rapid" molybdenum. Again, significant amounts of "slow" molybdenum do not occur until after 6 or 7 electrons have been added, suggesting that it represents an oxidation state lower than IV. As with native enzyme, no further absorbance change was observed after the addition of 6 electron eq. Since the reduced iron centers and fully reduced flavin account for only 4 electrons, the remaining 2 electrons are assumed to be present as Mo(IV). The lack of any detectable "rapid" molybdenum signal can then only be interpreted as meaning that 1-electron reduction of Mo(VI) is extremely unfavorable relative to 2-electron reduction; in terms of Equation 2, \( K_s \) must be at least 100 times greater than \( K_r \). This implies that one major effect of cyanolysis of the active center persulfide groups is a dramatic decrease in the thermodynamic stability of Mo(V). This apparently lower value for \( K_s \) also suggests that the persulfide group is located in close physical proximity to the molybdenum atom in agreement with earlier absorbance measurements (10).

![Graph showing observed fractions of reduced components at equilibrium during the titration of cyanide-inactivated xanthine oxidase with dithionite.](http://www.jbc.org/)

Calculation of Electron Distributions within Discrete Enzyme Molecules—In the preceding discussion, the various fractions of reduced components were assumed to represent the distribution

<table>
<thead>
<tr>
<th>No. of Electrons (j)</th>
<th>Fraction of Total Acceptor Group</th>
<th>Corrected Macroscopic Electron Affinity Constant ((L_j))</th>
<th>Total Extinction Change ((\text{mM}^{-1}\text{cm}^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\text{Mo}^V)</td>
<td>(\text{Mo}^IV)</td>
<td>(\text{Fe}/\text{S} \ II)</td>
</tr>
<tr>
<td>1</td>
<td>0.062</td>
<td>0.060</td>
<td>0.625</td>
</tr>
<tr>
<td>2</td>
<td>0.087</td>
<td>0.017</td>
<td>0.349</td>
</tr>
<tr>
<td>3</td>
<td>0.094</td>
<td>0.027</td>
<td>0.683</td>
</tr>
<tr>
<td>4</td>
<td>0.244</td>
<td>0.078</td>
<td>0.879</td>
</tr>
<tr>
<td>5</td>
<td>0.481</td>
<td>0.519</td>
<td>0.856</td>
</tr>
<tr>
<td>6</td>
<td>0.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
</tbody>
</table>
of electrons at equilibrium. The mathematical formulation
used to describe these distributions also assumed that there is no
cooperative interaction between the four types of acceptor groups.
It was not necessary to consider discrete enzyme molecules con-
taining 1 to 6 electrons; only the five equilibria defined in Equa-
tion 2 were required for a description of the titration data.
However, for an analysis of kinetic measurements, the concen-
trations of 1- to 6-electron-reduced enzyme molecules must be
considered, particularly since the distribution of electrons within
these discrete enzyme molecules determines the observed ab-
sorbance and EPR signal changes. Fortunately, the values of
the 6 relative electron affinity constants given in Table II de-
fine not only the equilibrium distribution of electrons but also
the equilibrium populations of 1- to 6-electron-reduced enzyme
molecules and the fractional distribution of electrons within these
molecules.

The 36 possible states of xanthine oxidase are shown in Fig. 5.
The probability of formation of an intermediate state, i, is:

$$P(i) = \frac{e^{(-\Delta G_i/RT)}}{Z}$$

where $\Delta G_i$ is the free energy of formation for the ith state and
$Z$ is the sum of probabilities for all intermediates containing the
same number of electrons as the ith intermediate. As $\Delta G_i =
-RT \ln K_i$, $P_i = K_i/Z$; for those intermediates containing more
than 1 electron $K_i$ is the product of all the individual affinity
constants used in the formation of the intermediate. For ex-
ample the fractional amounts of intermediates 6 through 13
(Fig. 5) for a 1-electron-reduced enzyme, i.e. $P(6)$ to $P(13)$ are
given as $K_1K_2K_3K_4/2$, $K_2K_3K_4K_5/2$, $K_3K_4K_5K_6/2$,
$K_4K_5K_6K_7/2$, $K_5K_6K_7K_8/2$, and $K_6K_7K_8K_9$, respectively, where the partition function, $Z$, is
equal to $K_6K_5K_4 + K_5K_4K_3 + K_4K_3K_2 + K_3K_2K_1 +
K_2K_1K_0$. The distribution of electrons among the various acceptor
groups is given by the sum of the fractional amounts of the ap-
propriate intermediate states. Again using 2-electron-reduced
enzyme as an example, the fractional amount, $f$, of each group
present, is given by the following relationships

$$f_{MoIV} = P(6)$$
$$f_{MoV} = (P(7) + P(8) + P(9))$$
$$f_{FeII} = (P(7) + P(10) + P(11))$$
$$f_{FeI} = (P(8) + P(10) + P(12))$$
$$f_{FADII} = (P(9) + P(11) + P(12))$$
$$f_{FADH_2} = P(13)$$

A compilation of the fractional distribution of electrons within
enzyme molecules containing 1 to 6 electrons was calculated in
this manner (Table II) and the results are given as the fraction
of each reduced component. In most cases, the number of elec-
srons in each acceptor group is equal to the fraction of reduced
species present; however, since Mo(IV) and FADH_2 represent
the uptake of 2 electrons, the fractional amounts of these species
must be doubled when summing up the number of electrons
within a given reduced enzyme unit.

The fractional amounts of the various intermediate states,
$P(i)$, were also used to calculate macroscopic values of the re-
duction potentials of xanthine oxidase. The reduction of an
enzyme unit can be described in terms of the stepwise binding of
6 electrons. For each step, the binding of an electron is de-
defined by the following equilibrium:

$$E(j-1) + e^- \rightarrow E(j) \quad j = 1 \text{ to } 6$$

where $E(j)$ represents an enzyme unit containing $j$ electrons and
$L_j$, a statistical electron-binding constant. The relative value
of $L_j$ is determined by the fractional amounts of the various
intermediate states in $E(j-1)$ and the values of the individual
affinity constants of the specific acceptor groups listed in Table
I. For example, in the case of the formation of 2-electron-re-
duced enzyme, intermediates 6, 7, 8, and 9 can be considered to
be generated by the addition of an electron to intermediate 2,
intermediate 10 by the addition of an electron to intermediate 3,
and intermediates 11, 12, and 13 by the addition of an electron to
intermediate 5. Therefore, the numerical value of $L_j$ is equal to
(1.0 + $K_1 + K_2 + K_3$) $P(2) + K_1P(3) + (1.0 + K_1 +
K_2)P(5)$ and is, of course, defined as relative to the electron af-
finity of Fe/S II. The relative values of $L_j$ calculated in this
manner are defined by Equation 4, and therefore include a sta-
tistical factor which is equal to the ratio of the total number of
possible intermediates in $E(j)$ to the total number in $E(j-1)$.
This statistical factor reflects the configurational entropy change
between the two over-all states of reduction. In order to com-
pare the intrinsic constants on a per electron basis, the values of
$L_j$ for $j$ equal to 1 to 6 were divided by 4, 8/4, 10/8, 8/10, 4/8
and 1/4, respectively, and the results are given in Table II.

In addition to allowing the calculation of expected EPR sig-
als, the results presented in Table II also determine the ab-
sorbance changes which occur when 1- to 6-electron-reduced en-
zyme molecules are produced. The extinction differences be-
tween oxidized and reduced enzyme molecules at 450 and 550
nm were calculated from the following equations:

$$\Delta A_{450}(j) = 2.8000F_{FeI}(j) + 11.6000F_{FeII}(j) + 12.2000F_{FADH_2}(j)$$
$$\Delta A_{550}(j) = 1.2000F_{FeI}(j) + 6.6000F_{FeII}(j)$$

where $j$ represents the number of electrons within the reduced
enzyme unit, $f$ the fraction of each reduced species, and the co-
efficients are the values of the molar absorbance difference be-
tween the oxidized and reduced forms of each species derived
earlier. As shown in Table II, the ratio of the extinction changes
at 450 nm to those at 550 nm follows a pattern which is observed
experimentally (Fig. 1).

Slow Phases during Partial Reduction by Substrates—Several
workers have reported that even when substoichiometric amounts
of substrates are used to reduce xanthine oxidase, absorbance
and EPR signal changes occur which are considerably slower than
those which can be accounted for in terms of the formation and
decay of an enzyme-substrate complex (7, 11). An example of
these types of changes is shown in Fig. 6A. When enzyme is
reduced with an amount of xanthine equivalent to about 0.3
electron per active center and the oxidized minus reduced differ-
ence spectrum recorded as a function of time, one observes a very
rapid decrease in absorbance which is expressed by a broad dif-
FIG. 5. Possible reduction states of xanthine oxidase. From left to right the seven vertical columns represent enzyme molecules containing 0 to 6 electrons, respectively. The various reduced intermediates within each vertical column represent the possible distinct configurational states for an enzyme molecule containing an integral number of electrons. With the exception of fully oxidized enzyme, only the reduced forms of the various oxidation-reduction groups are shown. Fe I and Fe II represent reduced iron centers I and II, respectively. As indicated by the subscripts, 36 possible intermediates can be generated during the reduction of xanthine oxidase.

Both the fact that slow changes are observed and the nature of these slow spectral changes are predicted from the results presented in Table II. Xanthine and similar substrates donate electrons 2 at a time; however, the small amount added ensures that, to a good approximation, only 1-electron-reduced and fully oxidized enzyme molecules will be present at equilibrium; this can be demonstrated quantitatively using the macroscopic electron-binding constants listed in Table II. Therefore, initially, a population of 2.5% 2-electron-reduced enzyme molecules is generated which subsequently equilibrates with oxidized enzyme to form a population of 5% 1-electron reduced molecules. Since the equilibration process must proceed by an intermolecular electron transfer process, it might be expected to be much slower than the rate of reduction by substrate. Furthermore, a decrease at 450 nm and an increase at 550 nm in the absorbance differences are also predicted from the calculated extinction coefficients in Table II. The calculated ratio of extinction changes at 450 nm to those at 550 nm for 1-electron reduced enzyme is 5.6 whereas that for 1-electron-reduced enzyme is 2.1. These values are in reasonable agreement with the ratios of 5.0 and 2.2 observed for the initial and final difference spectra shown in Fig. 6A.

When the xanthine to FAD ratio was increased to about 1, the magnitude of the slow absorbance changes was observed to be proportionately much smaller than that observed with less substrate (Fig. 6B). This observation is also consistent with the results in Table II. At this level of reduction the equilibrium
FIG. 6. Slow spectral changes accompanying partial reduction with xanthine. All reactions were carried out anaerobically. Base-lines were run with oxidized enzyme in both the reference and sample compartments. Difference spectra were recorded with the partially reduced enzyme samples in the reference compartment of the spectrophotometer. All reactions were carried out with the same enzyme preparation which exhibited an AFR value of 147. A, approximately 5% reduction by xanthine. Enzyme (3.9 X 10⁻⁵ M total FAD) was mixed anaerobically with 5.7 X 10⁻⁴ M xanthine and difference spectra were recorded 2, 39, 55, 85, 163, and 342 min after mixing. The absorbance difference between oxidized versus reduced enzyme monotonically decreased at 450 nm and increased at 550 nm. The inset shows the absolute absorption spectrum of oxidized enzyme (top curve), 5% reduced enzyme (middle curve), and enzyme reduced with excess dithionite (bottom curve). B, approximately 33% reduction by xanthine. Enzyme (3.54 X 10⁻⁶ M total FAD units) was mixed with 3.3 X 10⁻⁵ M xanthine and difference spectra were recorded immediately, and 18, 35, and 190 min after mixing. Again, the absorbance difference monotonically decreased at 450 nm and increased at 550 nm.

The existence of slow changes accompanying incomplete reduction is not limited to xanthine-like substrates but appears to be a general phenomenon shown by any substrate which donates 2 electrons at a time. Slow changes were also observed when cyanide-inactivated xanthine oxidase was reacted with a small amount of NADH (Fig. 7). In this case, the rate of initial reduction by substrate is slow and was not complete by the time the first spectrum was recorded. Therefore, the absorbance difference at 450 nm is observed to rise to a maximum and then decrease at a rate consistent with that observed for slow changes after reduction with xanthine, whereas at 550 nm an increase in absorbance is seen. However, the final difference spectrum is virtually identical with that observed with xanthine.

A summary of results from a series of partial reduction experiments using a variety of reducing agents is shown in the inset of Fig. 7. The extinction differences were calculated assuming that the concentration of reducing equivalents was equal to the concentration of 1-electron-reduced enzyme molecules. Therefore, each curve represents an independent determination of the spectrum of this enzyme species. The difference spectrum...
but more complex changes in the EPR spectrum of xanthine oxidase represents the reduction of inactive enzyme by active enzyme. Widespread distinct kinetic phases and suggested that the slow phase of xanthine oxidase by excess substrate occurs in two electron steps. Morel (12) showed by absorbance measurements that the reduction of xanthine oxidase by excess substrate occurs in two electron steps. The results in Figs. 1, 6, and 7 suggest strongly that the final equilibrium distribution of electrons is independent of the source of reducing equivalents. The slow redistribution of electrons which is observed with xanthine and NADH simply reflects a difference between the kinetically determined population of reduced enzyme molecules produced by the addition of electron pairs and the final equilibrium population. In the case of both dithionite and glycine-light reduction, the magnitude of the absorbance difference between the two species increases continuously during the entire reaction. The inset shows expected oxidized minus reduced difference spectra for 1-electron-reduced enzyme molecules which were derived from a variety of independent experiments. In the case of partial reduction experiments (less than 50% total reduction), the concentration of electron-reducing equivalents was taken as equal to the concentration of 1-electron-reduced enzyme molecules. The lower solid curve was calculated from the partial reduction by xanthine experiment shown in Fig. 6A. The dotted line was calculated from the results for the partial reduction of inactive enzyme by NADH. The dashed line was calculated from an experiment in which native enzyme was partially reduced by irradiation with visible light in the presence of 0.03 m glycine. For this latter curve, the per cent reduction at 450 nm and the total enzyme concentration were used to calculate the number of 1-electron-reduced enzyme molecules. The expected absorbance changes derived from the results given in Table II which were obtained from an analysis of dithionite titration experiments. The difference spectra of the slow phase observed in the stopped flow apparatus for the reaction of fully reduced enzyme with oxygen. This slow phase has previously been assigned to the reaction of 1-electron-reduced enzyme with oxygen (2).

**Fig. 7.** Spectral changes accompanying partial reduction with NADH. The reaction was carried out anaerobically using cyanide-inactivated xanthine oxidase (AFR = 0.05). Difference spectra were recorded as described in Fig. 6. Enzyme (3.1 × 10^{-4} mol total FAD) was mixed with 3 × 10^{-4} mol NADH and spectra were recorded 7, 17, 38, 92, and 220 min after mixing. The lower curve represents the spectrum recorded 7 min after mixing and reflects the fact that the initial reaction of oxidized enzyme with NADH is considerably slower (about 10^4 times) than that observed with xanthine under similar conditions. The absorbance difference at 450 nm reaches a maximum at 17 min after mixing and then slowly decreases to the equilibrium value. In contrast, the absorbance difference at 550 nm increases continuously during the entire reaction. The inset shows expected oxidized minus reduced difference spectra for 1-electron-reduced enzyme molecules which were derived from a variety of independent experiments. In the case of partial reduction experiments (less than 50% total reduction), the concentration of electron-reducing equivalents was taken as equal to the concentration of 1-electron-reduced enzyme molecules. The lower solid curve was calculated from the partial reduction by xanthine experiment shown in Fig. 6A. The dotted line was calculated from the results for the partial reduction of inactive enzyme by NADH. The dashed line was calculated from an experiment in which native enzyme was partially reduced by irradiation with visible light in the presence of 0.03 m glycine. For this latter curve, the per cent reduction at 450 nm and the total enzyme concentration were used to calculate the number of 1-electron-reduced enzyme molecules. The expected absorbance changes derived from the results given in Table II which were obtained from an analysis of dithionite titration experiments. O, the difference spectra of the slow phase observed in the stopped flow apparatus for the reaction of fully reduced enzyme with oxygen. This slow phase has previously been assigned to the reaction of 1-electron-reduced enzyme with oxygen (2).

**Slow Phases during Reduction by Excess Substrate**—In 1952, Morell (12) showed by absorbance measurements that the reduction of xanthine oxidase by excess substrate occurs in two widely distinct kinetic phases and suggested that the slow phase represents the reduction of inactive enzyme by active enzyme.

In addition to absorbance changes, there are also equally slow but more complex changes in the EPR spectrum of xanthine oxidase reduced with excess substrate. These EPR signal changes have recently been described in great detail by Swann and Bray (13), who also interpreted the observed changes as due to a slow reduction of inactive xanthine oxidase by reduced, active, enzyme molecules.

The results presented in Table II agree with both their observations and interpretations. Assuming that the transfer of electrons between active and inactive molecules occurs by 1-electron steps, the following simplified set of equations may be used to interpret the slow changes accompanying reduction by excess substrate:

$$\begin{align*}
E(6) + E^*(i) & \underset{\text{slow}}{\rightarrow} E(5) + E^*(i+1) \\
E(6) + E^*(i) & \underset{\text{slow}}{\rightarrow} E(4) + E^*(i+1) \\
E(4) + \text{xanthine} & \underset{\text{fast}}{\rightarrow} E(6) + \text{uric acid}
\end{align*}$$

where \(E(i)\) and \(E^*(i)\) represent active and inactive enzyme molecules containing \(i\) electrons. The first step after the complete reduction of active enzyme units is production of 5-electron-reduced active molecules and 1-electron-reduced inactive molecules. As shown in Table II, this would be accompanied by a marked increase in the amount of Mo(V) and, therefore, "rapid" molybdenum signal. However, the net change in the amount of reduced iron centers and FADH₂ would be much...
smaller, and little or no absorbance change at 450 nm would be expected. If only Mo(VI) is capable of accepting 2 electrons from xanthine, a condition which is necessary if each enzyme unit can accept only 6 electrons and reduction proceeds by a hydride transfer (1, 9), then 5-electron-reduced active enzyme molecules cannot react with xanthine since only reduced states of molybdenum are present. To get further net reduction, an additional bimolecular reaction involving the production of 4-electron-reduced active enzyme molecules is necessary. This would account for the fact that the rate of the decrease in absorbance at 450 nm and the disappearance of "rapid" molybdenum is measurably slower than the increase in "rapid" molybdenum in what Swann and Bray (13) have called phase III of reduction.

The reduction of xanthine oxidase by NADH also occurs in two kinetically distinct phases (5). The amplitude of the slower phase contributes about 30% of the total absorbance change and the rate of this phase is very similar to that observed for the slow phase with xanthine. In contrast to reduction with xanthine, however, the amplitude of the slow phase for reduction with NADH is independent of the activity to flavin ratio of the enzyme preparation. Edmondson et al. (6) have reported, and we have confirmed, that the reduction of cyanide-inactivated enzyme by NADH also occurs in two phases, about 30% of the absorbance change occurring very slowly. Since reduction of NADH is thought to be mediated by the flavin moiety, this observation of two phases appears to be inconsistent with present ideas concerning active and inactive xanthine oxidase. However, the electron distributions in Table II provide a simple interpretation for this apparently anomalous situation.

It is reasonable to assume that NADH is only capable of reacting with fully oxidized FAD since 2 electrons must be donated to this chromophore. Both oxidized and 2-electron-reduced enzyme molecules can react with NADH fairly rapidly, but the fractional amount of FAD present in 4-electron reduced enzyme units is only about 2% of the total flavin present. Therefore, the last step in reduction by NADH would be expected to be at least 50 times slower than the first step. Even the first step of reduction is quite slow (about 0.2 min⁻¹ for 4 × 10⁻⁴ M NADH) compared to that of xanthine reduction so that the rate of NADH reduction of 4-electron-reduced enzyme would be expected to be of the same order of magnitude as the rates of intermolecular electron transfer between enzyme molecules. Since the electron distributions within cyanide-treated enzyme appear to be similar to those of native enzyme (Fig. 3), the amount of slow phase during NADH reduction of inactive xanthine oxidase would also be expected to contribute about 1/5 of the total absorbance change. The ability of the calculated results in Table II to explain the reaction of enzyme with NADH is particularly satisfying and seems to indicate that the mathematical formulation used to derive the various electron distributions is valid.

Rapid Phases during Reduction with Xanthine—Although the reduction of xanthine oxidase by xanthine has been examined extensively by both rapid freeze EPR techniques and stopped flow absorbance spectrometry (1, 9, 15, 16), there has been no serious attempt to quantitate the observed signal and absorbance changes in terms of a specific reaction mechanism. This is due, in part, to the fact that 3 substrate molecules must react to achieve complete reduction. The requirement of a minimum of three consecutive reactions greatly complicates any formulation of a kinetic mechanism. Recently, Edmondson et al. (1) have suggested that the rate-limiting step in reduction is the release of product, which occurs by the breakdown of a persulphide-xanthine bond, and that the rate of this step is the same for each of the three consecutive steps in the overall reduction process.

When this idea of three consecutive reactions exhibiting identical rates is examined in more detail, it becomes apparent that, while still only an approximation, a model of this sort is capable of explaining a number of apparently anomalous experimental observations. First, although the disparity is small, the value of \( V_{\text{max}} \) calculated from an analysis of the rates of anaerobic reduction and the oxidation of fully reduced enzyme is consistently smaller than the extrapolated value obtained by steady state experiments in which product formation is measured directly (2). Second, since the individual steps in either reduction or oxidation are thought to occur by the formation and decay of an enzyme-substrate complex, the rates of either partial reduction or partial oxidation, under conditions in which the enzyme is in excess, should be consistent with the over-all rates measured in the presence of excess substrate. Therefore, data determined in the presence of excess enzyme should fit on the same line as that obtained by plotting the reciprocal rate against the reciprocal of the concentration of excess substrate. However, this relationship is not observed for either reduction or oxidation.

A series of reduction experiments in which the ratio of xanthine to active FAD was varied from 0.6 to 10.0 is shown in Fig. 8A. Neglecting for the moment the initial lag, the remaining portion of the time course for a xanthine to active enzyme ratio of 0.5 can be described by a single exponential whose rate of decay is only about 50% smaller than that observed for complete reduction in the presence of excess xanthine. When the concentration of xanthine was doubled, more absorbance change was observed, but the shape of its time course was nearly identical with that observed at the lower substrate concentration. This result
Fig. 9. Normalized time courses for the reduction of native and deflavoxanthine oxidase by excess xanthine. Reactions were monitored at 450 nm. Conditions after mixing: 10^{-4} M xanthine, 10^{-6} M active sites for native enzyme (O), and 1.6 \times 10^{-6} M active sites for deflavoxanthine (●). Solid lines are theoretical and were calculated using values for $k_{-1}/k_1$ and $k_2$, defined in Equation 6, equal to $1.3 \times 10^{-4}$ and 25 s^{-1}, respectively, and the required extinction coefficients given in Table II. These calculations, particularly those for deflavoxanthine, are discussed more thoroughly in the text. The dotted line was drawn through the points for deflavoxanthine to indicate the discrepancy between the observed and predicted results.

indicates that the effective concentration of reactive enzyme unit is not greatly altered by the first 2-electron reduction step and is, of course, what would be expected for an enzyme molecule that is capable of accepting 6 electron eq. Only when the ratio of xanthine to active enzyme approaches 3 are obviously second order conditions observed. Finally, as the ratio of xanthine to active FAD was raised to 10.0, pseudo-first order conditions were again observed, and, in this case, the concentration of xanthine determined the observed rate. In another experiment in which the concentration of enzyme was kept much smaller than that of substrate, the dependence of the over-all rate on xanthine concentration was examined (Fig. 8B). The results are reasonably consistent with a mechanism that postulates the rapid formation and slow decay of an enzyme-substrate complex. However, it is clear that the rate of partial reduction with limiting substrate is significantly greater (by a factor of about 2) than that predicted from the results with excess xanthine. This discrepancy, coupled with the lower value of $V_{max}$ calculated from the rates observed in the individual half-reaction, suggests that the rate of at least the first step in reduction is greater than that estimated from the rate of the over-all reaction. A result of this type is particularly puzzling since a small lag is still observed when the over-all time course of reduction is examined in the presence of excess xanthine.

A third apparently anomalous experimental finding is the greater rate of reduction of deflavoxanthine by xanthine than that of native enzyme (1, 14). A comparison of the time courses for the reduction of native and deflavoxanthine shows that deflavoxanthine is reduced about 1.5 times more rapidly by excess xanthine than is native enzyme (Fig. 9). This difference was initially rationalized in terms of a conformational change produced by the removal of FAD. However, as will be shown, this result is entirely consistent with the idea that the rates of the three steps required for complete reduction of native enzyme are nearly equal and not significantly different from the rates of the two steps required for the complete reduction of deflavoxanthine.

The simplest possible formulation of anaerobic reduction is to assume that each 2-electron step occurs by the rapid formation of an enzyme-substrate complex which subsequently decays by a first order process in which product is released and electrons are donated to the enzyme molecule. Assuming that the rates of both formation and decay of this complex are independent of the reduction state of the enzyme, each step may be written as:

$$E(1) + X \xrightarrow{k_1} EX(1) \xrightarrow{k_2} E(1 + 2) + U \quad i = 0, 2, 4$$

where $E(i)$ and $EX(i)$ represent enzyme molecules containing $i$ electrons, and $X$ and $U$, xanthine and uric acid, respectively.

The values of $k_1$ and $k_{-1}$ are assumed to be much greater than $k_2$ so that the observed rate of reduction, $k$, equals $k_2[X]/(K_d + [X])$, where $K_d$ equals $k_{-1}/k_1$. Therefore, at high substrate concentrations the over-all reduction reaction can be considered in terms of three consecutive irreversible reactions:

$$E(0) \xrightarrow{k_{-1}} E(1) \xrightarrow{k_2} E(1 + 2) \xrightarrow{k_{-1}} E(0)$$

Analytical expressions for the time dependence of the concentrations of each enzyme reduction state were obtained by solving the appropriate differential equations. The simplest case is to assume that each addition of an electron pair contributes an equivalent absorbance change; then the normalized time course for the over-all reduction of xanthine oxidase by excess substrate is given by

$$\frac{\Delta A}{\Delta A_0} = (1 + 2/3 \times k_2 t + 1/6 \times k_2^2 t^2) e^{-k_1 t}$$

where $\Delta A_0$ is the total absorbance change ($A_0 - A_\infty$), $\Delta A = A_1 - A_\infty$, and $k$ is defined above. The most striking result of this analysis is the fact that the time course of reduction is not predicted to be a simple exponential process but, rather, accelerates with a rate which increases from $\frac{1}{3} k$, initially, to $k$ at extremely long reaction times. Even more important is the fact that the half-time of the over-all three-step reaction (determined numerically) is about 2.3 times longer than $\ln (2/k)$ which would represent the half-time if only a single step were observed.

The predicted accelerating time course and underestimation of the individual rate constants result from assigning an equal probability to each step in Equation 7. This assignment is a consequence of the idea that the rate-limiting step in reduction is the release of product subsequent to the distribution of 2 electrons among the four types of acceptor groups. In terms of the present simple model, this requires that the probability of xanthine reacting with $E(0)$ is equal to its probability of reacting with $E(4)$, not 3 times greater as would be expected if the electron acceptor sites were independent. Therefore, initially, the rate of the overall change in absorbance is $\frac{1}{3}$ the rate of change of $E(0)$.

The elementary model described by Equation 8, in which $k_1 = k_2 = k_3$ and each electron pair yields an equivalent absorbance change, is not adequate for a description of the detailed features of the reduction reaction. However, it does serve to demonstrate certain basic principles. Most important is the fact that it predicts that rate constants measured from a simple analysis of anaerobic reduction experiments in the presence of excess substrate will be an underestimate of the true values for the individual steps. This result provides an explanation for the fact that the value of $V_{max}$ calculated from the rates of the individual half-reactions is smaller than the value observed by steady state measurements and that the rates observed for
partial reduction experiments appear to be inconsistent with those observed for complete reduction (Fig. 8B) (2). In addition, this model provides a qualitative explanation for the faster rate of reduction of deflavoenzyme. After removal of flavin, each active enzyme unit is capable of accepting only two electron pairs. Assuming the two steps in the reduction of deflavoenzyme occur at rates equal to those of native enzyme, the half time for the reduction of flavin-free enzyme would be 1.6 x In (2/6); that is, the observed rate will be about 1.4 times greater than that of native enzyme, and such a relationship is observed experimentally (Fig. 9). This result is simply a reflection of the fact that only two steps are required for the reduction of deflavoenzyme and, therefore, need not be explained in terms of a conformational change produced by the removal of FAD. Finally, although Equation 8 predicts an amount of acceleration greater than that observed, it is important to note the time course of reduction by xanthine does consistently exhibit an initial lag, even at 450 nm (1, 5).

Keeping the simple formulation of Equation 5, the following more realistic scheme was adopted. First, the values for $k_1$ and $k_{-1}$ were assumed to be much greater than $k_2$ and their ratio, $K_d$, was assumed to be independent of the state of reduction of the enzyme. Second, the extinction coefficients given in Table II were used. These values do not predict an equal absorbance increment per electron pair added. Rather, most of the absorbance change has taken place by the time 4-electron-reduced enzyme is generated. Third, the value of $k_2$ was made to depend on the fraction of Mo(VI) present in $EX(i)$ and can be expressed as $k_2 \times f_{Mo(VI)}(i)$, where $f_{Mo(VI)}(i)$ is the fractional amount of oxidized molybdenum in $i$-electron reduced enzyme and $k_2$ is an intrinsic constant for the decay of the enzyme-substrate complex. The reason for modifying the values of $k_2$ in this manner arises from the idea that for each step in reduction, xanthine must first donate a pair of electrons to oxidized molybdenum. For this slightly more complicated model, the values of $k_1$, $k_2$, and $k_3$ in Equation 7 are not equal but are in a ratio of 1:0.896:0.678 (Table II, Columns 1 and 2).

Comparison between the observed and calculated absorbance time courses are shown in Figs. 9 and 10A. The only parameter varied was $k_2$, which when set equal to 25 s⁻¹ gave a reasonable fit to the observed data. Clearly the model presents a good description of the absorbance changes at both 450 and 550 nm (Fig. 10A). The increased amount of lag at 550 nm is a consequence of the proportionally smaller extinction coefficient assigned to the formation of 2-electron-reduced enzyme molecules at this wavelength. Since the extinction change for the formation of FADH₂ cannot be derived independently, no attempt was made to fit the results at 600 nm.

The simulated time course for the reduction of deflavoenzyme given by the solid line in Fig. 9 was calculated by deriving electron distributions for 2- and 4-electron-reduced enzymes molecules which contain no flavin. The values of the relative electron affinity constants for the remaining iron centers and molybdenum species were kept the same as those used for native enzyme (Table I). New extinction coefficients at 450 nm were calculated on the basis of these electron distributions for deflavoenzyme; 0.25, 0.50
enzyme. The value of \( k_3 \) was also set equal to \( 25 \, \text{s}^{-1} \) for the first step and modified by the fraction of \( \text{Mo(VI)} \) present in 2-electron-reduced deflavoenzyme for the second and final step in reduction. There is a very reasonable agreement between the observed reduction time course for deflavoenzyme and that predicted from parameters which were originally derived for native enzyme (Fig. 9). Again, the major reason that the reduction of flavin-free enzyme appears to be faster is the fact that only two steps are involved.

Although the rates measured from over-all time courses of reduction underestimate the true value of \( k_3 \) for the first step in reduction, the value of \( K_d \) determined from reciprocal plots will be identical with the value derived by a more complicated analysis since the substrate concentration dependence of each step in Equation 7 is the same. Using the value of \( K_d \) determined from the observed data and the value of \( k_2 \) (Equation 5) derived from fitting the model to the observed time courses, a theoretical concentration dependence of the rate of reduction for the first step \( (s) \) was derived and is given by the dotted line in Fig. 8B. As shown, this theoretical result agrees well with the observed rates of reduction measured in the partial reduction experiments and provides an explanation for the apparently anomalous greater rate measured when enzyme is in excess. Similarly, the greater value of \( k_3 \) derived from the model compared to that obtained from the over-all time course of reduction offers an explanation for the discrepancy between the high value of \( V_{\text{max}} \) observed in steady state experiments and that calculated from transient state kinetics. Therefore, to a reasonable approximation, the absorbance changes which occur for each step in reduction may be explained in terms of the very rapid formation and slow decay of an enzyme-substrate complex which is accompanied by a rapid distribution of 2 electrons among the various acceptor groups. This electron distribution is determined solely by the relative reduction potentials of these groups which can be measured in static experiments.

In spite of the good agreement between the theory and experimental results presented in Figs. 8, 9, and 10A, a closer examination of detailed features of the reduction reaction indicates that this simple model (Equation 5) is still inadequate. This is particularly evident from the rapid freeze EPR results (Fig. 10C) and the partial reduction experiments (Fig. 11). The model does not account for the almost instantaneous formation of the "very rapid" molybdenum signal which decays more rapidly than either FADH or "rapid" molybdenum. Further, the fact that the molybdenum signals observed are a function of the type of substrate used is not taken into account. Bray and co-workers (7, 17) have shown that the "very rapid" molybdenum is only observed with xanthine homologues and also that the "rapid" molybdenum signal changes shape with differing substrates. Therefore, xanthine, or its product, must be bound to reduced states of molybdenum and perturb the immediate environment of these atoms.

However, the simple model does accurately describe the formation of the iron signals (Fig. 10B); as expected from the results in Table II, Fe/S II appears at a slightly faster rate than iron center I. Edmondson et al. (1) reported that at pH 8.5 the appearance of the two reduced iron centers was identical. How-

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**Fig. 11.** Partial reduction of xanthine oxidase by limiting xanthine. The symbols represent experimental data and the solid lines, predicted theoretical time courses calculated as described in Fig. 10 and in the text. The dotted lines were drawn through data points when the deviation between the observed and predicted time courses was substantial or when no theoretical curve was calculated. The symbols in each panel correspond to those defined in Fig. 10. A, absorbance changes observed when enzyme \((1.0 \times 10^{-4} \, \text{M active FAD})\) was reacted with \(5 \times 10^{-6} \, \text{M xanthine}\) in the stopped flow apparatus. B, appearance of iron-sulfur center signals when enzyme \((9.3 \times 10^{-4} \, \text{M active FAD})\) was reacted with \(5 \times 10^{-5} \, \text{M xanthine}\) in the rapid freeze apparatus. C, appearance of "very rapid" molybdenum, "rapid" molybdenum, and flavin radical signals during the reduction reaction described for B.
ever, they used the $g = 1.90$ signal to estimate Fe/S, and this signal contains a large contribution from Fe/S II. When their data were replotted using the $g = 1.95$ signal to represent iron center I, the resultant time courses were similar to those reported here. In the case of the FADH- and “rapid” molybdenum signals, both the observed integrated intensities and rates of appearance deviate significantly from those predicted (Fig. 10C). The maximum intensity for “rapid” molybdenum is close to that predicted but it appears earlier in time, whereas more flavin radical is observed and appears more slowly than predicted. It is important to note that all of the species represented in Fig. 10C account for no more than about 10% of the total electrons accepted by each enzyme unit. Thus, the simple model does, in fact, account for the uptake of most of the electrons during reduction and, with the exception of the detailed features of the time courses for molybdenum and flavin radical, is quite adequate for a description of the over-all reduction reaction. Furthermore, the observation of small intensities for flavin radical and molybdenum signals is consistent with the results in Table II since even in static experiments only relatively small amounts of FADH$^{-}$ and MO(V) were observed (Figs. 3 and 4).

The discrepancy between observed and calculated results can be seen more clearly in partial reduction experiments. Equation 6 predicts that the absorbance time course of reduction with limiting xanthine should be described by a single exponential at all wavelengths. However, the observed absorbance curves (Fig. 11A) exhibit a distinct nonlinearity in logarithmic plots, with an initial lag phase. This is particularly striking at 600 nm. Similarly, the time course for the appearance of all EPR signals should exhibit exponential behavior. This is observed for the iron centers, FADH$^{-}$, and “rapid” molybdenum signals. Even the extents of reduction are reasonably close to those predicted considering the small signals that were measured experimentally. However, the most striking observation is the rapid appearance and subsequent disappearance of the “very rapid” molybdenum signal when only one step of the reduction reaction is examined. This indicates clearly that the “very rapid” signal is not a unique property of 2-electron-reduced enzyme molecules. Rather, this signal must represent the properties of a transient species which is formed during each step in reduction. This idea is also consistent with the absorbance results in Fig. 10A. The “bowed” shape of the observed time courses is characteristic of a two-step reaction mechanism in which the formation of the first intermediate is accompanied by little or no absorbance change.

All of these results indicate that an additional species must be written into the formulation described by Equation 5. This requires the following more complex mechanism:

$$E'(X(i+2)) \rightarrow E'(X(i)) \rightarrow E'(X(i))$$

$$E'(X(i)) \rightarrow E'(X(i+2))$$

where $E'(X(i+2))$ is the new species; $K_d$, the dissociation constant of the initial enzyme substrate complex; $k_1$, the formation of $E'(X(i))$, which depends on the fraction of Mo(V1) present and therefore the reduction state of enzyme; and $k_2$, the simple first order decay of $E'(X(i))$. The effective rate of formation of $E'(X(i+2))$ is equal to $k_2 \cdot [E'(X(i+2))]/([K_d + [X]])$. The principal problem of this formulation is the assignment of the initial absorbance within and, therefore, the properties of $E'(X(i+2))$. This distribution must allow for a significant amount of Mo(V) in order to explain the “very rapid” molybdenum signal, but the amounts of reduced iron centers and FADH$_2$ must be small since no initial rapid decrease in absorbance is observed in either complete or partial reduction experiments.

This problem was resolved by adopting the chemical interpretation shown in Fig. 12 for the intermediates defined in Equation 9, and is based in part on the earlier mechanisms proposed by Edmondson et al. (1) and Stiefel (18). The first step involves the simple binding of xanthine to the active site. Then, attack of the persulfide group causes the splitting of the carbon-hydrogen bond which is accompanied by the donation of 2 electrons to Mo(VI). The rate of this process is presumed to be fast and dependent on the fraction of the Mo(VI) present. The hydrogen atom originally at position 8 of the xanthine molecule is then transferred to the Fe/S center (18), which is also liganded to the molybdenum atom. In addition, the oxidized xanthine molecule is assumed to bind tightly to the reduced state of the molybdenum atom, thereby greatly stabilizing these species and preventing the transfer of electrons to the iron centers and flavin. The final step involves the attack of a water molecule which hydrolyzes the persulfide-xanthine linkage causing the release of product. The major rate-determining step in this series of reactions involves the breaking of the persulfide bond and the release of product. Since the observed values of $V_{\text{max}}$ do not vary greatly with pH, this step probably does not involve the direct attack of OH$^{-}$ (2, 6).

The two important features of the mechanism are the rapid
breakage of the C—H bond during the formation of $E'X$ and the binding of oxidized xanthine to the reduced states of molybdenum within this intermediate. The major effect of substituting a deuterium atom for the hydrogen atom at position 8 of xanthine appears to be an approximately 2-fold decrease in the observed value of $K_m$ for xanthine with little or no effect on $V_{\text{max}}$ (1). In addition, the observed rates of appearance and signal intensities of "very rapid" and "rapid" molybdenum are altered. These deuterium effects are readily explained in terms of Equation 9 and Fig. 12. The resultant calculated distributions for the two states are shown in Table III along with the expected extinction changes.

The chemical interpretation of these transient intermediates is given in Fig. 12 and discussed in the text. The distributions were calculated using the following relative electron affinity constants for the acceptor groups defined in Table I: $K_1 = 0.4, K_2 = 0.1, K_3 = 10.0, K_4 = 100.0$, and $K_5 = 9.0$. Calculations of the rates and extinction changes for the formation of reduced components and the oxidized versus reduced extinction changes were carried out as described for the results in Table II.

**Table III**

<table>
<thead>
<tr>
<th>No. of electrons</th>
<th>Fraction of total acceptor group in E'X(j)</th>
<th>Extinction change at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>450 nm</td>
</tr>
<tr>
<td></td>
<td>Mo (V)</td>
<td>Mo (IV)</td>
</tr>
<tr>
<td>2</td>
<td>0.143</td>
<td>0.856</td>
</tr>
<tr>
<td>4</td>
<td>0.094</td>
<td>0.906</td>
</tr>
</tbody>
</table>

Again assuming $k_1, k_2, k_3, k_4$, and $k_5$ are much greater than any of the other rate constants, the slope of a plot of the reciprocal rates versus $1/[X]$ is equal to $k_1/(k_2 k_3 k_4 k_5)$. If $k_1$ is much greater than either $k_2$ or $k_3$, the value of $V_{\text{max}}$ is simply $k_1 k_2/(k_3 + k_5)$. Since $k_2$ represents the rate of the breakage of the C—H bond, the observed value for $K_m$ for xanthine would be expected to decrease markedly with deuterated substrates, but little effect is expected on the observed value of $V_{\text{max}}$. Similarly, since reduced states of molybdenum are also produced during this step, an effect on the amount and rate of appearance of the corresponding EPR signals is expected. The close proximity of the protonated basic group to the molybdenum atom is in accord with the effects of deuterated substrates on the fine structure of the spectrum of "rapid" molybdenum (1, 9).

The amount of "very rapid" molybdenum signal is strongly dependent on pH with much more signal being observed at high pH. In addition, the spectrum of this species exhibits no deuterium effect. These results are also compatible with the scheme in Fig. 12, if the pK of the basic group is about 9 and the protonation-deprotonation equilibria are very rapid (1). "Very rapid" molybdenum then represents the deprotonated form of $E'X$ and "rapid" molybdenum, the protonated form. Within this framework, however, the pK of the basic group must be dependent both on the presence of bound substrate and the number of electrons present within the enzyme unit as a whole. In order to explain the rapid decay of the "very rapid" signal, the pK must increase markedly both after the release of product and after the addition of more than 2 electrons so that the deprotonated form is observed only during the first of these over-all steps in reduction (1, 18).

In order to calculate the expected time course of reduction, it was first necessary to determine the electron distributions with $E'X(2)$ and $E'X(4)$. This was achieved by setting the electron affinity constants of Mo(VI) and Mo(V) equal to 100 and 9, respectively, when oxidized xanthine is attached to the persulfide group. The remaining electron affinity constants for the iron centers and flavin were kept the same. The values for $K_1$ and $K_4$ were given these larger values in order to express the idea that covalently attached xanthine binds to the reduced states of molybdenum and prevents, in large part, the redistribution of electrons among the other acceptor groups. The resultant calculated distributions for the two $E'X$ intermediates are shown in Table III along with the expected extinction changes.

Predicted time courses were calculated by fixing $k_1$ at 25 s$^{-1}$, varying the rate for the formation of $E'X$ and the amount of unprotonated $E'X(2)$. There are only two slow steps in the over-all reduction reaction since, after the formation of $E'X(6)$, no further signal or absorbance changes are possible (Equation 9). The rate of formation of $E'X(4)$ and $E'X(6)$ were attenuated by the fractions of Mo(VI) present in 2- and 4-electron-reduced enzyme. The predicted results for reduction by excess xanthine (Fig. 13A) agree reasonably well with all of the observed results, although for the purpose of clarity only the fit to the experimental absorbance changes is shown. Again, the predicted intensity of the FADH- signal is somewhat lower than that observed (Fig. 10), but the order of appearance of all the signals is identical with that which is observed experimentally. For these calculations the best value of $k[X]/(K_d + [X])$ was found to be about 200 to 250 s$^{-1}$ and the amount of unprotonated $E'X(2)$ was set equal to 65% of the total present. Using these parameters, "very rapid" molybdenum reaches a maximum value about 10 ms after mixing and decays much more rapidly than any of the other signals. This is followed by the appearance of "rapid" molybdenum and then FADH-. The half-time for the formation of the iron signals is slower and agrees well with the data in Fig. 10. This order of Mo $\rightarrow$ FADH- $\rightarrow$ Fe is in agreement with all previously reported reduction experiments (1, 7, 18).

Even more satisfying is the agreement of the calculated partial reduction time courses with those observed experimentally. The predicted changes at 450 nm do exhibit the observed lag phase, and the overall time course is in excellent agreement with that measured experimentally (Fig. 13B). Again, the
of the observed over-all rate of anaerobic reduction should not depend linearly on $1/[X]$ but rather the observed rate should decrease more rapidly with decreasing substrate concentrations than that predicted by a linear relationship. Indeed such curvature is seen in the observed results shown in Fig. 8B but was originally ignored. In effect then, the rate-limiting step in reduction does depend on the concentration of substrate. In the partial reduction experiments (Figs. 8A and 11A) the rates of formation and decay of $E'X$ are roughly equal because of the low concentration of enzyme used. However at either high substrate or enzyme concentrations, product release, $k_3$, is rate limiting as was originally suggested by Edmondson et al. (1).

Therefore, under the latter conditions, which are those most commonly examined, the more simple model defined by Equation 5 does produce a good approximation for the time courses of the absorbance changes and the appearance of reduced iron-sulfur centers. The complex model (Equation 9) is required only when the time courses of the molybdenum signals or those for partial reduction at low enzyme concentration are examined in more detail.

**Reaction of Reduced Enzyme with Oxygen**—During the oxidation of reduced xanthine oxidase by molecular oxygen two phases are observed in both stopped flow absorbance measurements and rapid freeze EPR experiments (2). In the case of 6-electron reduced enzyme, the fast phase accounts for the removal of 5 electrons and the slow phase, the removal of the remaining electron. The spectral properties of the slow component are nearly identical with those predicted in Table II for 1-electron-reduced enzyme molecules (Fig. 7, inset). This is true not only of the characteristic features of the observed difference spectrum, which, as predicted, resembles deflavoenzyme, but more importantly, the magnitudes of the observed extinction changes are nearly identical with those predicted for 1-electron-reduced enzyme (Table II). Therefore, in the case of completely reduced xanthine oxidase, all of the enzyme molecules are converted rapidly to the 1-electron-reduced state which then slowly reoxidizes, presumably by the reaction of oxygen with the small amount of flavin radical present (2).

The observed rates of the fast phase followed a kinetic pattern consistent with the formation and decay of a reduced flavin-oxygen complex. This can be expressed by the formulation used for the oxygen reaction in Equation 10, with the observed fast rate being equal to $k_3[O_2]/(k_{-1}/k_4 + [O_2])$, provided $k_{-1}$ and $k_4$ are much greater than $k_3$. This same hyperbolic relationship should hold if enzyme is in excess and the concentration of enzyme is substituted for that of oxygen. However, as in the case of reduction, the rates observed in partial reoxidation experiments appear to be greater than those predicted under conditions in which oxygen is in excess.

The results in Fig. 14A represent a series of experiments in which xanthine oxidase was mixed with a series of oxygen concentrations. With $O_2/total$ FAD $\leq 1$, a purely first order reaction was observed, the rate of which is not dependent on the absolute concentration of oxygen. When the ratio is increased to about 2.3, a decelerating time course is observed; this reflects second order conditions due to the decrease of both oxygen and free enzyme during the course of the reaction. Finally, at ratios greater than 3.0, the two distinct phases characteristic of the over-all oxidation reaction were observed (2). Reciprocal plots of the rates of the fast phase at high oxygen concentrations (Fig. 14B) agree well with those previously reported with a limiting rate of about 7000 min$^{-1}$ and a $K_d$ for oxygen binding equal to $7 \times 10^{-4}$ M. However, the rates observed when en-

![Image of predicted time courses for the reduction of xanthine oxidase by excess and limiting xanthine concentrations. The solid lines represent theoretical time courses calculated using Equation 9 as described in the text.](http://www.jbc.org/.../FADH')
an individual step. Since there is little absorbance change, and the observed rate should be slower than that predicted for complete reduction, several steps are represented by the rapid behavior. These observations are also consistent with the present interpretation. In the limiting case, at low levels of reduction, the time course for this phase no longer exhibits completely first order behavior. Rather, with complete reduction, logarithmic plots of the fast phase curve downward.

With less reduction a greater rate is observed, and the time course of the fast phase exhibits more closely exponential behavior. These observations are also consistent with the present interpretation. In the limiting case, at low levels of reduction, only one rapid step is involved in the production of 1-electron-reduced enzyme molecules and, therefore, the observed rate represents a true value of the rate for an individual step. After complete reduction, several steps are represented by the rapid phase in oxidation so that exponential behavior is not predicted and the observed rate should be slower than that predicted for an individual step. Since there is little absorbance change, particularly at 450 nm, in going from 6- to 4-electron-reduced enzyme (Table II), the accelerating behavior observed for the fast phase in oxidation should be more pronounced than that observed for reduction. This does appear to be the case and is a reflection of the fact that the iron centers and flavin exhibit greater reduction potentials than molybdenum (Table III).

Fig. 14. Oxidation of fully reduced xanthine oxidase by various amounts of molecular oxygen. A, dithionite-reduced enzyme (1.1 \times 10^{-4} M total FAD) was reacted with several concentrations of oxygen and the time courses were monitored at 450 nm. The molar ratio of O_2 to total FAD is given beside each curve. B, dependence of the observed rate of the fast phase of oxidation on oxygen or enzyme concentration. \( k_0 \) rates measured in the presence of excess oxygen. The same set of experiments described for A but are not shown. C, rates measured with limiting oxygen (the lower two curves in A). The solid line was drawn through the data points determined with excess O_2. The dotted line represents a theoretical curve for the presence of oxygen or enzyme concentration. The oxygen or enzyme concentration dependence of the observed rate for the first step in the oxidation of fully reduced enzyme using values for \( k_0 \) (Equation 10) equal to 7 \times 10^{-4} M and 205 s^{-1}, respectively.

enzyme was in excess appear to be about 2 times greater than that expected when oxygen is in excess (Fig. 14B).

The apparent discrepancy between results from partial and complete oxidation experiments are readily interpreted in terms of the principles developed in the analysis of reduction experiments. The fast phase in oxidation represents a multiple step reaction but, again, the probability of each of the steps is approximately the same, since in this case only FADH_2 is capable of reacting with oxygen. This is again expressed by a time course which exhibits acceleration and a half-time greater than that predicted from the rate constant which describes an individual oxidation step (mix. Equation 8). Thus, the observed over-all rate of the fast phase is predicted to be about 2 times slower than that expected if only a single step were examined.

This type of argument also provides an explanation for the observed dependence of both the shape and rate of the fast oxidation phase on the amount of reduced enzyme present initially. As described in the preceding paper (2), the rate of the fast phase decreases with increasing reduction of the enzyme, and after complete reduction (6 electrons per FAD) the time course for this phase no longer exhibits completely first order behavior. Rather, with complete reduction, logarithmic plots of the fast phase curve downward.

With less reduction a greater rate is observed, and the time course of the fast phase exhibits more closely exponential behavior. These observations are also consistent with the present interpretation. In the limiting case, at low levels of reduction, only one rapid step is involved in the production of 1-electron-reduced enzyme molecules and, therefore, the observed rate represents a true value of the rate for an individual step. After complete reduction, several steps are represented by the rapid phase in oxidation so that exponential behavior is not predicted and the observed rate should be slower than that predicted for an individual step. Since there is little absorbance change, which includes both 2- and 1-electron oxidation steps. The rate for each step (excluding the \( E(1) \rightarrow E(0) \) reaction which is determined by the slow second order rate) is given by \( k[O_2]/(K_d(i) + [O_2]) \) where \( k \) is the rate of decay of the FADH_2-O_2 complex and \( K_d(i) \) is the dissociation constant of this complex which is dependent on the fraction of FADH_2 present in i-electron-reduced enzyme molecules. The upper and lower horizontal pathways, \( E(6) \rightarrow E(4) \rightarrow E(2) \rightarrow E(0) \) and \( E(5) \rightarrow E(3) \rightarrow E(1) \), represent 2-electron oxidation in which the product is hydrogen peroxide; the diagonal arrows represent 1-electron oxidations in which superoxide is generated.

The simplest model for oxidation is to assume that the FADH_2-O_2 complex breaks down to give a mixture of \( O_2^- \) and \( \text{H}_2\text{O}_2 \) and that the proportion of each is determined kinetically by the different rates of 1- and 2-electron transfers from fully reduced flavin to oxygen. In the case of 1-electron-reduced enzyme molecules, FADH_2 cannot be formed and, therefore, a much slower rate is observed. The amount of this slow phase is diagnostic of the relative amounts of 1- and 2-electron transfers to oxygen during the rapid phase of oxidation. In an attempt to determine independently the relative rates of 1- and 2-electron transfers, a sample of enzyme was partially reduced with a limiting amount of xanthine, immediately mixed with oxygen, and the absorbance changes at 450 nm recorded. As shown in Fig. 15, there is a dramatic difference in the amplitude of the slow phase when the time course for the reoxidation of enzyme partially reduced with xanthine is compared to that for enzyme partially reduced with dithionite. In the latter case, reduction with dithionite was allowed to come to completion (as monitored by no further slow absorbance changes) before the sample was mixed with oxygen. The distribution of electrons within the dithionite-reduced sample is determined primarily by the distribution within 1-electron-reduced enzyme molecules which is the major species generated at equilibrium under these conditions. As expected then, the reaction with oxygen is predominantly slow. Furthermore, the small amount of fast phase that is observed (about 15 to 20% of the total) is consistent with the predicted equilibrium amounts of 2- and 3-electron-reduced enzyme molecules.

In the case of partial reduction with xanthine, only 2-electron-reduced enzyme molecules are generated initially although eventually other states will be generated by intermolecular electron transfer so that the final equilibrium distribution of reducing equivalents will be similar to that in the case of dithionite reduction. However, this equilibration process is slow, (half-time of the order of 30 to 50 min) (Fig. 6) and, since the
The remaining electron is present at a reduced iron center which can be generated after the first l-electron transfer to oxygen since this process occurs until 2-electron-reduced enzyme molecules are present. In the case of enzyme containing more than 2 electrons, fully reduced flavin is regenerated by intramolecular electron transfer from the reduced iron centers. The extinction changes of the slow component observed in the oxidation of fully reduced enzyme are in excellent agreement with those expected for the oxidation of 1-electron-reduced enzyme (inset to Fig. 7). This requires that 6-electron-reduced enzyme is converted stoichiometrically to 1-electron-reduced enzyme in the fast phase of the reaction; fully oxidized enzyme is only produced by the slow reaction of the latter species with oxygen. Complete production of 1-electron-reduced enzyme is also observed even when initially only 2-electron-reduced enzyme molecules are present (Fig. 15) and is, therefore, presumably generated no matter what the initial reduction state of the enzyme.

The production of 1 electron reduced enzyme molecules in the fast phase suggests that oxidation may proceed solely by 1-electron transfer steps and would require that large amounts of O$_2^-$ be produced. For example, enzyme reduced with 2.4 moles of xanthine per total FAD would yield about 4 O$_2^-$ per FAD in the fast phase of the reaction. In contrast, the amount observed under these conditions was only about 0.10 O$_2^-$ per FAD (2). Even when the rate of disproportionation at pH 8.5 is considered (about 2 x 10$^4$ M$^{-1}$ s$^{-1}$ (20)), this value is still about 20 times smaller than that expected if oxidation is occurring totally by 1-electron steps. This small amount of O$_2^-$ production at pH 8.5 is also evident from turnover experiments. Similar small levels of superoxide were reported by Knowles et al. (21) and Orme-Johnson and Bainert (22) in single turnover experiments at this pH.

Fridovich (23) has shown that the fractional amount of 1-electron oxidation can be calculated by comparing the rate of cytochrome c reduction and the rate of uric acid production in catalytic assays. The specific activities for cytochrome c and xanthine reduction reported by Komai et al. (14) suggest that only about 25% of electron flux from xanthine to oxygen occurs by 1-electron oxidation steps during turnover in air-equilibrated buffers at pH 8.5. Thus, it is clear that during the fast phase of oxidation, most of the kinetically distinct steps must involve 2-electron transfer to oxygen.

In order to account for both the lack of O$_2^-$ production and the production of 1-electron-reduced enzyme molecules during the fast phase of oxidation, the following interpretation has been adopted. First, oxygen binds rapidly to fully reduced flavin to form a FADH$_2$-O$_2$ complex. Then 1 electron is transferred to the oxygen molecule. In the case of enzyme containing more than 2 electrons, fully reduced flavin is regenerated by intramolecular electron transfer from the reduced iron centers and molybdenum. If the rate of this process is greater than the rate of diffusion of O$_2^-$ out of the active center a second electron can be transferred from FADH$_2$ to O$_2^-$ to form H$_2$O$_2$. This process occurs until 2-electron-reduced enzyme molecules are generated. At this stage, however, FADH$_2$ can no longer be generated after the first 1-electron transfer to oxygen since the remaining electron is present at a reduced iron center which is incapable of reacting directly with either O$_2^-$ or oxygen. Therefore, only in the case of 2-electron-reduced enzyme is O$_2^-$ produced rapidly. It is, of course, clear that O$_2^-$ must be produced in the reaction of 1-electron-reduced enzyme molecules with oxygen. However, the rate of this process is quite slow in comparison to the rate of disproportionation at pH 8.5 so that no net O$_2^-$ production would be expected during this phase of the reaction.

This model may be summarized by the following set of reactions:

$$ FADH_2 + O_2 \rightarrow FADH_2^+ + O_2^- $$  (13)

$$ FADH_2^+ + O_2 \rightarrow FADH_2 + O_2 $$  (14)

$$ H^+ + e^- + FADH_2 + O_2 \rightarrow FADH_2^+ + O_2^- $$  (15)

$$ FADH_2 + O_2 \rightarrow FADH_2^+ + O_2^- $$  (16)

$$ H^+ + FADH_2 \rightarrow FADH_2 + H_2O $$  (17)

$$ H^+ + FADH_2 + O_2^- \rightarrow FADH_2 + O_2 + H_2O $$  (18)

$$ H^+ + FADH_2 + O_2^- \rightarrow FADH_2 + O_2 + H_2O $$  (19)

$$ FADH_2 + O_2^- \rightarrow FADH_2 + O_2 + H_2O $$  (20)

The dotted lines represent oxygen species still bound to the enzyme. The rate-limiting step is envisioned to be the original decay of the fully reduced flavin-oxygen complex (Equation 14). Previous work with flavin model systems have shown that O$_2^-$ does react rapidly with fully reduced flavin (20, 24). For example, there is a 4-fold decrease of the over-all rate of oxidation of reduced tetraacetylriboflavin in the presence of superoxide dismutase (24). This can only be interpreted by assuming...
that a process similar to that described by Equation 16 is very rapid. In the case of xanthine oxidase, however, no effect of superoxide dismutase was observed on the time course of oxidation measured either in the stopped flow apparatus or by rapid freeze techniques (2). Therefore, if Equation 10 represents an important step in the over-all process, the \( \text{O}_2^- \) must be bound to the enzyme. Previous work has also shown that the blue or neutral semiquinone is rather unreactive toward oxygen when compared with either fully reduced flavin or the anionic radical (24, 25). Therefore, the reactions described by Equations 18 to 20 would be expected to be slower than any of the others. Finally, for all situations the intramolecular electron transfer steps are assumed to be very rapid (Equation 15).

Application of these ideas to the general scheme outlined in Equation 12 results in the following formulation:

\[
\begin{align*}
E(0) & \rightarrow E(2) \rightarrow E(4) \rightarrow E(6) \\
E(1) & \rightarrow E(3) \\
E(5) & \rightarrow E(7)
\end{align*}
\]

(21)

in which only the oxidation of 2- and 1-electron-reduced enzyme molecules involves the formation of \( \text{O}_2^- \). In effect, each step is postulated to occur by a 1-electron transfer; however, the observable intermediates reflect 2-electron steps because of the rapid regeneration of \( \text{FADH}_2 \) and its subsequent rapid reaction with enzyme-bound \( \text{O}_2^- \) (Equations 15 and 16). Using the scheme outlined by Equation 21, it was possible to calculate time courses which agree very well with those observed. \( K_a \) and \( k \) as defined in Equation 12 were assigned values of \( 7 \times 10^{-20} \text{ M} \) and 205 \text{ s}^{-1}, respectively. The \( K_a \) values for the intermediates in Equation 21 were divided by the fractions of \( \text{FADH}_2 \) present in each intermediate in order to calculate the effective rate of each step. A second order rate constant of \( 9 \times 10^8 \text{ M}^{-1} \text{ s}^{-1} \) was assigned to the reaction \( E(1) \rightarrow E(0) \). Extinction coefficients were assigned to each intermediate according to the pattern given in Table II.

A comparison of observed and predicted time courses are shown in Figs. 15 and 16, and the agreement is excellent. The model predicts both the observed behavior at different wavelengths and at different states of reduction prior to mixing with oxygen (Fig. 15 and inset of Fig. 16). This model also explains the apparently greater rate of oxidation when enzyme is in excess (Fig. 14B). Although not shown, there is also reasonable agreement between the observed and expected time courses for the iron, \( \text{FADH}_2 \), and "rapid" molybdenum signals. A direct comparison of these time courses was not attempted since the high concentration of enzyme necessary to detect these signals requires that the reaction be carried out under second order conditions. However, the amounts of each signal in the rapid and slow phases were reasonably close to those observed. In addition an initial increase followed by a rapid decrease of both the molybdenum and \( \text{FADH}_2 \) signals is predicted, in agreement with the observed results (2). As with the reduction results, one noticeable problem involved the amplitude of the flavin radical. Again, less signal is predicted than was observed, but, as before, the absolute number of electrons represented by this species is extremely small. The major discrepancy, if there is one, involves the amount of \( \text{O}_2^- \) observed. The amount observed is still less than that predicted; however, the disparity (about a factor of 2) is much smaller than that if each step in reduction involved the production of \( \text{O}_2^- \). For the latter case, the observed amount of \( \text{O}_2^- \) is some 20 times less than that predicted. The reason for the small amount of observed \( \text{O}_2^- \) remains unclear. It may be that xanthine oxidase itself exhibits some dismutase activity, particularly at the high enzyme and substrate concentrations used in the rapid freeze experiments.

In spite of this discrepancy, the model does explain qualitatively steady state observations concerning the reduction of cytochrome c during catalysis. Fridovich (23) reported that more \( \text{O}_2^- \) is generated during catalysis when either the concentration of xanthine is lowered or the concentration of oxygen is increased. At high oxygen and low xanthine, the amount of reduced enzyme in the steady state is quite small (2). The reaction cycles principally between 2-electron-reduced and oxidized enzyme molecules. For these species, oxidation occurs only by 1-electron transfer steps so that \( \text{O}_2^- \) is the primary product, in agreement with the results of Fridovich (23). At low oxygen and high xanthine, large amounts of reduced enzyme are present in the steady state phase (2). In the limit, the catalytic reaction would cycle between fully reduced and 4-electron-reduced enzyme molecules, and under these conditions no \( \text{O}_2^- \) is generated.

Perhaps the most satisfying consequence of the agreement between the observed and calculated results shown in Figs. 14 to 16 is the fact that the electron distributions presented in Table II are capable of describing not only equilibrium results, but also the kinetics of oxidation. This, in conjunction with the results for reduction, supports strongly the supposition that
the acceptor groups are in rapid equilibrium with each other, and that the reactions of xanthine oxidase can be interpreted in terms of 6-electron affinity constants (Table I) and a minimum number of kinetic constants representing the formation and decay of enzyme substrate composites.

**Mechanism of Action of Xanthine Oxidase**—The ability of xanthine oxidase to catalyze efficiently the conversion of xanthine to uric acid is, in the main, a reflection of the relative reduction potentials of iron-sulfur centers, flavin, and molybdenum. Since xanthine must first react with oxidized molybdenum, the lack of substantial amounts of Mo(IV) and Mo(V) in 1- to 4-electron-reduced enzyme molecules allows the substrate reaction to occur at rapid rates for all three steps in reduction. Similarly, oxygen reacts primarily with FADH₂ which is the major reduced species in all intermediates except 1-electron-reduced enzyme molecules. The iron centers can then be considered as electron sinks which serve to connect the reductive and oxidative sites, as suggested by Komai and Massey (26). In the case of reduction they serve to remove electrons from Mo(V) and Mo(IV) to allow further reaction with substrate whereas, in the case of oxidation, they serve to regenerate fully reduced flavin for further reaction with oxygen.

Most previous interpretations of the mechanism of action of xanthine oxidase have described its reactions in terms of a linear sequence of intramolecular electron transfer steps in the order of Mo → FAD → Fe/S (1, 9, 16-18). This is clearly not correct. Intramolecular electron transfer is not rate limiting, as was previously shown by the pH jump experiments of Edmondson et al. (1). Rather, the appearance of reduced acceptor groups is simply a function of their relative reduction potentials. A better description of the mechanism of xanthine oxidase would be the order of the electron affinity constants which is roughly FAD > Fe/S > Mo. It is this order of relative reduction potentials which accounts for most of the properties of xanthine oxidase. Differences between reductive substrates can be interpreted in terms of differential perturbations of these reduction potentials when substrate is bound to the enzyme (viz. Table III). Similarly, the effects of pH presumably represent differential changes in the reduction potentials so that new electron distributions are formed. In all cases, the kinetic constants are probably also altered with changing conditions. However, these changes no longer seem intractable. At the very least, the formulations used here present a framework within which these effects of changing conditions can be examined more systematically.