Steps in the Oxidation of Xanthine to Uric Acid Catalysed by Milk Xanthine Oxidase

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(*Received* 26 *January* 1959)

The development of various rapid-reaction techniques to study a number of distinct steps involved in individual enzyme catalysed reactions has been very successful in application to haem enzymes (Chance, 1951), diphosphopyridine nucleotide-linked systems (Theorell & Chance, 1951) and hydrolytic enzymes (Gutfreund, 1955). Speculations and preliminary evidence for the existence of intermediates in flavoprotein systems (Beinert, 1957) have encouraged us to study one of these in detail by fast-reaction spectroscopy. Milk xanthine oxidase has recently been crystallized by Avis, Bergel & Bray (1955), and it was found by Avis, Bergel, Bray, James & Shooter (1956) that the enzyme has a molecular weight of approximately 300 000, that it contains 2 flavinadenine dinucleotide groups, 8 iron atoms and 1–2 molybdenum atoms/molecule. Previous detailed studies of the catalytic activity (Morell, 1952; Mackler, Mahler & Green, 1954) made this enzyme system a most suitable one for a first attempt to apply new methods to the study of flavoprotein-catalysed reactions. The studies reported here can be regarded as an extension of Morell's investigations to the kinetics of the anaerobic reduction of the enzymic flavinadenine dinucleotide by xanthine, to the reoxidation of substrate-reduced enzyme by molecular oxygen, and of the steady-state formation of uric acid catalysed by the enzyme in the presence of xanthine and oxygen. Furthermore, the kinetics of the reduction and reoxidation of the enzymic flavinadenine dinucleotide was followed at different wavelengths between 400 and 600 mμ in a search for spectroscopically observable intermediates in these reactions. From the results of these experiments a complete kinetic scheme can be written for the enzymic conversion of xanthine into uric acid and in conjunction with data reported by others, preliminary suggestions for a mechanism can be proposed for this flavoprotein-catalysed hydrogen transfer.

EXPERIMENTAL

Methods. All spectra and activity measurements were obtained by use of a Unicam Instrument Co. Ltd. (Cambridge) SP. 500 spectrophotometer. The rapid reaction measurements were started for exploratory purposes in a Gibson (1952) stopped-flow apparatus, slightly modified for enzyme work (Gutfreund, 1955). All the quantitative kinetic measurements were carried out in a stopped-flow attachment to the Unicam spectrophotometer described by Spencer & Sturtevant (1959). This was kept at 25°. The observation cell of the stopped-flow mixing device has a 1 cm. light-path and fits in the place of the usual cell-holder. The optical system and photocell of the Unicam instrument were used for measurements of extinction under static conditions, while only the monochromator of the Unicam instrument and a separately attached (Spencer & Sturtevant, 1959) photomultiplier, amplifier and Bush Electronic Co. (Cleveland, Ohio) Model BL-201 pen-and-ink recorder were used for recording rapid changes during the course of enzymic reactions. This latter arrangement gives a record of percentage transmission which is converted into change in extinction for the calculation of rate constants.

Anaerobic experiments were carried out with solutions which had been shaken up *in vacuo* and then under nitrogen, this procedure being repeated three times before transfer of the solutions under anaerobic conditions into the syringes of the fast-reaction apparatus. Experiments at different concentrations of oxygen were carried out with solutions which had been treated *in vacuo* and had then been equilibrated with appropriate pressures of oxygen at 25°. The molarity of oxygen was calculated from the oxygen pressure on the basis that the solubility of oxygen in water at 25° at 1 atm. of O_2 is 1·23 mM.

Materials. Xanthine was obtained from Roche Products Ltd. and solutions of required concentration were made up in 0·1 M-sodium pyrophosphate adjusted to pH 8 with HCl with a glass electrode.

All xanthine oxidase preparations used were obtained from Professor F. Bergel, Dr R. C. Bray and Mr D. A. Gilbert of the Chester Beatty Research Institute and had been prepared from cow's milk by the method of Avis *et al.* (1955). Various individual pilot experiments were carried out with small special samples of the enzyme, but all the fast-reaction measurements from which quantitative conclusions are drawn in this paper were carried out with one preparation. The enzyme was obtained in solutions which contained 0·8 M-sodium phosphate (pH 7·5) and 4 mg. of sodium salicylate/ml. and it retained constant activity if stored at 2° for several months. The principal sample of xanthine oxidase was at stage m6 (before crystallization) in the scheme of purification of Avis *et al.* (1955). The protein concentration of enzyme solutions was not measured, but the concentration of total flavinadenine dinucleotide (FAD) and enzymically active FAD was estimated from the $E_{450\ m\mu}$ of the solution and from the change in $E_{450\ m\mu}$ on anaerobic reduction by xanthine. The method of interpretation of the extinction data was based on

Morell's (1952) results. The extinction, $E^{1\,cm.}_{450\,m\mu}$, of the stock solution was 1·22. The amount of enzyme required for a day's experiments was dialysed overnight against 0·1 M-sodium pyrophosphate, pH 8·0. After dialysis and slight dilution during washing of the dialysis sack, the extinction was reduced to approximately 0·85. In the mixing chamber the enzyme concentration was halved; all concentrations and extinctions quoted in the descriptions of kinetic experiments are those of the reaction mixtures after mixing enzyme and substrate solutions. Precise data for the extinctions of reaction mixtures are given in appropriate sections of the paper.

RESULTS

The spectrum of the xanthine oxidase stock solution, as well as the difference spectrum between oxidized and reduced enzyme, were very similar to the figures shown by Morell (1952). We have found, in agreement with Morell (1952), that the specific enzyme activity of different samples of xanthine oxidase is proportional to the percentage reduction of $E_{450\,m\mu}$ on anaerobic addition of xanthine. Using Morell's method of calculating the percentage of enzymically active FAD, we found that this corresponded in our stock sample to 33 % of the total flavine present. Like Morell we found that anaerobic reduction by xanthine was biphasic, consisting of a rapid reduction to 33 % of the total reduction by sodium dithionite, and a subsequent, very slow reduction which we interpreted, following Morell, as the reduction of FAD bound to inactive enzyme. First, we investigated in some detail the kinetics of the anaerobic reduction of xanthine oxidase by sodium dithionite and by xanthine.

Reduction of xanthine oxidase by sodium dithionite. The reduction of the enzyme by sodium dithionite was followed anaerobically by observing the change in $E_{450\,m\mu}$. The reaction mixture contained, apart from xanthine oxidase, 0·05 M-sodium dithionite and 0·1 M-sodium pyrophosphate, pH 8·0. The initial $E_{450\,m\mu}$ was 0·457 and the final value 0·179. Using a simple plot of log $(E-E_t)$, which is referred to below as log ΔE, against t, we found that the reaction was pseudo-first-order through 70 % of its course (k = 0·12 sec.$^{-1}$).

Anaerobic reduction of xanthine oxidase by xanthine. The rapid decrease in extinction on mixing oxygen-free solutions of xanthine oxidase and xanthine was observed at 400, 450, 500 and 550 mμ. Only at 450 mμ was the change in extinction sufficiently large to make accurate calculations of the time course of the reduction possible. From observations at the other wavelengths one could only draw the conclusion that the half-life of the reaction described is the same. Fig. 1 *A* shows the change in $E_{450\,m\mu}$ calculated from the record of such an experiment. The slow drift following the rapid change corresponds to the slow reduction of enzymically inactive FAD, and this was corrected for before calculation of the rate constant of the rapid reduction of the enzyme. As can be seen from the plot of log ΔE against t recorded in Fig. 2, this reaction follows first-order kinetics (k = 10·4 sec.$^{-1}$). A number of experiments showed that for substrate concentrations ranging from 500 to 50 μM the rate of the anaerobic reduction is characterized by $k = 10{\cdot}5 \pm 1{\cdot}0$ (max. deviation) sec.$^{-1}$

Reduction of xanthine oxidase by xanthine in the presence of oxygen. When the course of the reduction of xanthine oxidase was followed after mixing enzyme and xanthine in the presence of oxygen, it was found that $E_{450\,m\mu}$ was decreased by approximately one-third of the anaerobic reduction caused by xanthine and that it remained at this level throughout a steady-state period. At the end of the

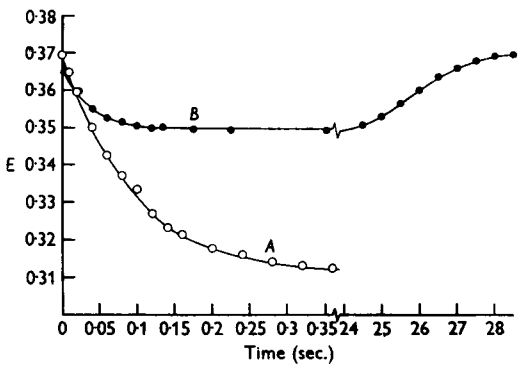


Fig. 1. *A*, Anaerobic reduction of xanthine oxidase ($E^{1\,cm.}_{450\,m\mu}$ 0·37) by xanthine (0·25 mM) in 0·1 M-sodium pyrophosphate, pH 8·0. The reactions were followed by recording the percentage transmission at 450 mμ and the data were converted in E units. *B*, Reduction of xanthine oxidase ($E^{1\,cm.}_{450\,m\mu}$ 0·37) by xanthine (0·25 mM) in the presence of oxygen (0·6 mM). (The concentrations are those after mixing of enzyme and substrate solutions.)

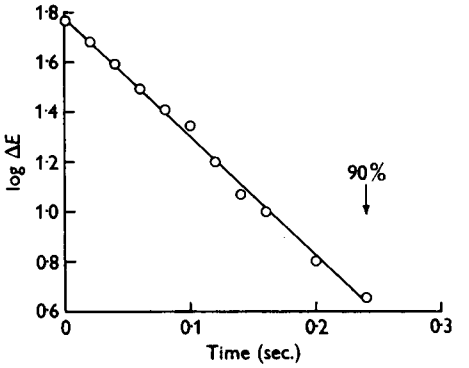


Fig. 2. A first-orderp lot of log $(E-E_\infty)$ against t for the experiment in Fig. 1 *A*.

reaction the extinction either dropped to the xanthine-reduced level, if the concentration of xanthine was greater than that of oxygen, or rose to the fully oxidized level, if oxygen was in excess of xanthine. Fig. 1 shows the record of two experiments, one with oxygen-free solutions and the other containing 0·6 mM-oxygen in the reaction mixture, the xanthine concentration being 0·25 mM in both reaction mixtures. We studied the rate of reduction of the enzyme to the steady-state level over a range of initial oxygen concentrations from 1 to 0·1 mM. From six measurements of the rate of reduction to the steady state, at initial oxygen concentrations greater than 0·2 mM we calculated a first-order rate constant 32 ± 2 (max. deviation) sec.$^{-1}$. At lower concentrations of oxygen this rate constant decreased, but since under those conditions no steady-state period is reached it becomes too complicated to evaluate individual rate constants. Approximate values for the half-optimum concentration of oxygen were obtained from measurements of the overall velocity of the enzyme reaction as described in the next section.

Rate of appearance of uric acid and the Michaelis parameters for the overall reaction. The kinetics of uric acid formation under steady-state conditions can be calculated from observations of the change in $E_{310\,m\mu}$. We used this wavelength, at which the change of ϵ on oxidation of xanthine to uric acid is $2{\cdot}1 \times 10^3$ l. M^{-1} cm.$^{-1}$, rather than 290 mμ, where the change in ϵ of this reaction is at its maximum, $9{\cdot}6 \times 10^2$ l. M^{-1} cm.$^{-1}$ (Kalckar, 1947). The observation of the rate of formation of uric acid under identical conditions of enzyme and substrate concentrations as used in our measurements of the reduction and reoxidation of the enzyme involves the presence of relatively large amounts of enzyme and products during the course of the experiment, and the extinction at 290 mμ became too great, while at 310 mμ satisfactory observations could be made and Beer's law was obeyed.

Fig. 3*A* gives the plot of $E_{310\,m\mu}$ against time for a typical experiment. The initial substrate and oxygen concentrations in the reaction mixture were 0·25 and 0·6 mM respectively. Fig. 3*B* is from a similar experiment in 50 % (v/v) ^2H_2O, the results of which are discussed below. The reaction was zero-order through approximately 80 % of its course and the xanthine concentration at half-optimum velocity was estimated to be 20 μM. This is not regarded as an accurate value for the Michaelis constant, since measurements in the fast-reaction apparatus are not suitable for the precise evaluation of this constant; it is, however, in good agreement with the detailed kinetic investigation of Hofstee (1955), who obtained $K_m = 20\ \mu$M.

From the record of the appearance of uric acid in one experiment at a low initial concentration of oxygen we have estimated an approximate value for half-optimum oxygen concentration. Fig. 4 shows the rate of appearance of uric acid for an experiment at initial xanthine and oxygen concentrations of 0·25 and 0·09 mM respectively. The scale on the left-hand side gives the change in extinction due to the appearance of uric acid and the scale on the right-hand side gives the calculated oxygen concentration. The dotted line gives the calculated limiting velocity at high oxygen concentration for

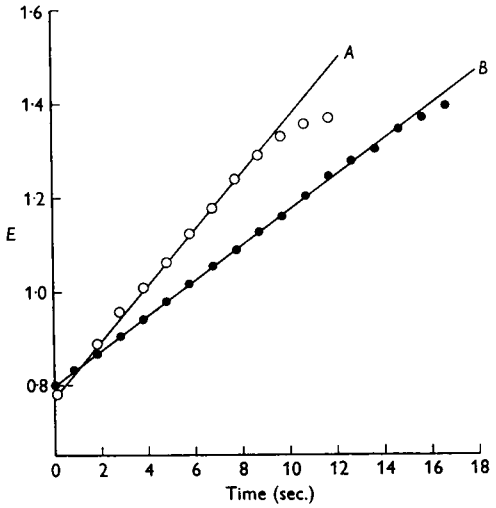


Fig. 3. Change in $E_{310\,m\mu}$ during the xanthine oxidase-catalysed formation of uric acid from xanthine in 0·1 M-sodium pyrophosphate, pH 8; initial concentrations of xanthine and oxygen 0·25 and 0·6 mM respectively; xanthine oxidase, $E^{1\,cm.}_{450\,m\mu}$ 0·40. Curve *A* is from an experiment in aqueous solution, curve *B* from an experiment in 50 % (v/v) ^2H_2O. (The concentrations are those after mixing of enzyme and substrate solutions.)

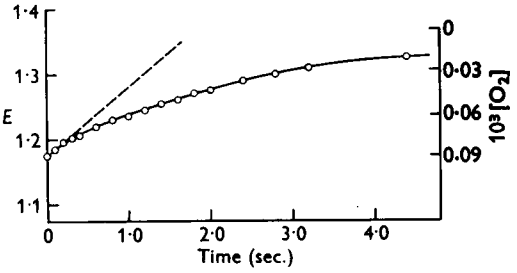


Fig. 4. Change in $E_{310\,m\mu}$ during the xanthine oxidase-catalysed formation of uric acid from xanthine in 0·1 M-sodium pyrophosphate, pH 8·0; initial concentrations of xanthine and oxygen 0·25 and 0·09 mM respectively; xanthine oxidase, $E^{1\,cm.}_{450\,m\mu}$ 0·251. The broken line gives the calculated initial velocity at optimum oxygen concentration. (The concentrations are those after mixing of enzyme and substrate solutions.)

the enzyme concentration ($E_{450\,m\mu}$ 0·388) and the half-optimum concentration can be estimated as 50 μM.

The overall rate constant of the enzyme for optimum concentrations of xanthine and oxygen has been calculated from the molarity of active enzyme and the rate of appearance of uric acid. The method for this calculation is described in detail by Morell (1952). The assumptions involved in the calculations of the molarity of active enzyme FAD sites from the molar extinction coefficient of enzymic FAD, the percentage reduction and the initial $E_{450\,m\mu}$ of the enzyme solution make estimates of moles of uric acid formed/mole of enzyme/sec. rather inaccurate. The value of 8 ($\pm$0·5) sec.$^{-1}$ represents the range of values calculated from six experiments.

Effect of D_2O *on different steps of the hydrogen-transport process.* Both the rate of anaerobic reduction of xanthine oxidase by xanthine and the overall rate of the steady-state formation of uric acid in the presence of oxygen have been determined in solutions which contained 50% (v/v) of ^2H_2O. The enzyme solution was made up in water as above, but the substrate was dissolved in D_2O which contained phosphate equivalent to that in the aqueous buffers used. As can be seen in Fig. 5, the two comparative kinetic measurements of the rate of anaerobic reduction at identical substrate concentration, $[S_0]$ = 0·25 mM, in H_2O and 50% (v/v) H_2O–^2H_2O mixtures respectively gave indistinguishable results (k = 10·5 sec.$^{-1}$).

Measurements of the rate of appearance of uric acid were carried out with solutions of the same concentrations of xanthine oxidase and xanthine as in the above anaerobic experiments but containing in addition 0·6 mM-oxygen. Two parallel experiments under identical conditions, except that in one reaction-mixture H_2O was the solvent while in the other a 50% (v/v) mixture of H_2O and ^2H_2O was the solvent, are recorded in Fig. 3. The rate of the overall enzyme reaction in 50% ^2H_2O is only 64% that of the rate in H_2O.

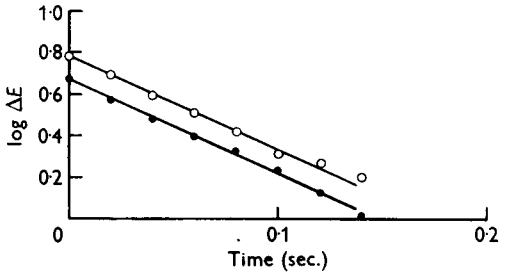
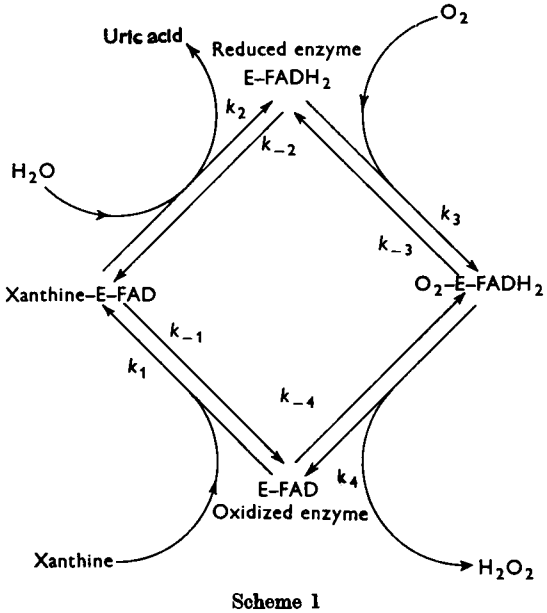


Fig. 5. Comparison of first-order plot of rate of anaerobic reduction of xanthine oxidase by xanthine in water (○) and in 50% (v/v) ^2H_2O (●); 0·1 M-sodium pyrophosphate, pH 8·0; initial xanthine concentration, 0·25 mM; xanthine oxidase, $E^{1\,cm.}_{450\,m\mu}$ 0·251. (The concentrations are those after mixing of enzyme and substrate solutions.)

DISCUSSION

The experiments with xanthine oxidase reported here were carried out primarily to explore the potentialities of spectroscopic fast-reaction techniques in the analysis of individual steps of hydrogen transfer catalysed by a readily available flavoprotein. We have concentrated on those features of the enzyme reaction which are common to all flavoproteins. We have neglected the possible role of metals, which has received very wide attention recently (Nicholas, 1957; Bergel & Bray, 1958), because many flavoproteins can participate in similar hydrogen-transport processes without the help of metals. It is our intention to use the experience gained here to select and study much simpler flavoproteins in greater detail.

Our most important conclusions are that both xanthine and oxygen combine rapidly with enzyme to form Michaelis–Menten compounds, before the reduction and reoxidation of the FAD moiety of the enzyme and that the steady-state ratio of $FADH_2$ (reduced flavinadenine dinucleotide) to FAD during turnover of the enzyme at optimum concentrations of xanthine and oxygen corresponds to the ratio of the first-order rate constants for reduction and reoxidation. To arrive at these conclusions we had to determine a sufficient number of the rate constants of the cyclic reaction (Scheme 1).



Scheme 1

When we studied the anaerobic reduction of xanthine oxidase by xanthine we found that the reduction of FAD, as observed by changes in $E_{450\,m\mu}$, followed first-order kinetics and was independent of xanthine concentration over a wide range. We concluded from this that the value of 10·5 sec.$^{-1}$ found as rate constant for the reduction process can be assigned to the step characterized by k_2; an initial second-order xanthine–enzyme combination, characterized by k_1, must precede this step. From our own and Hofstee's (1955) data we estimate the xanthine K_m to be 20 μM; since $K_m = (k_2 + k_{-1})/k_1$ one can give a minimum value for k_1 of 50 μM^{-1} sec.$^{-1}$.

Our findings, that the reduction of enzymic FAD to the steady-state level in the presence of oxygen is also a first-order process and is characterized by a rate constant three times that of k_2, can be explained as follows. Since the steady-state ratio of oxidized to reduced enzyme is independent of the oxygen concentration above 0·5 mM, one can conclude that oxygen too forms a compound with the enzyme before its oxidation of the enzymic FAD. From the dependence of the rate of the overall appearance of uric acid on the concentration of oxygen an estimation of the oxygen K_m (50 μM) was made and k_3 (>40 μM^{-1} sec.$^{-1}$) can be calculated in a manner analogous to the calculation of k_1 above. If one accepts that the second-order combination of enzyme with both xanthine and oxygen is rapid compared with the first-order reduction and reoxidation of the enzymic FAD, then one can regard the steady-state level as an equilibrium between two opposing reactions of the xanthine–enzyme compound with the oxygen–reduced-enzyme compound

$$\text{Xanthine–E–FAD} \underset{k_4}{\overset{k_2}{\rightleftharpoons}} \text{O}_2\text{–E–FADH}_2.$$

The rate constant of the reaction to the equilibrium position of two first-order opposing reactions, k_e, is equal to $k_2 + k_4$ and is itself a first-order constant. We have obtained a value for k_e of 32 sec.$^{-1}$ from our experiments on the reduction of enzyme by xanthine in the presence of oxygen, and from this value and that for k_2 (10·5 sec.$^{-1}$) we can conclude that reoxidation of the enzyme is approximately twice as fast (k_4 = 21·5 sec.$^{-1}$). The ratio of the rates of reduction and reoxidation is in reasonable agreement with the steady-state ratio of reduced and oxidized enzymic FAD, as shown in Fig. 1.

The overall turnover of 8 (± 0·5) sec.$^{-1}$ at 25°, calculated from our experiments on the xanthine oxidase-catalysed formation of uric acid under optimum conditions, is compatible with Morell's (1952) value of 5·22 sec.$^{-1}$ at 19°. If the overall rate of hydrogen transfer from xanthine to enzymic FAD and from enzymic FAD to oxygen is determined by the two steps characterized by k_2 and k_4, then the rate constant k_0 for the overall reaction is given by $1/k_0 = 1/k_2 + 1/k_4$. Substituting the values given above for k_2 and k_4 we obtain k_0 = 7·88 sec.$^{-1}$. This is in agreement with the hypothesis that the overall reaction of the enzyme is determined by the two successive steps of reduction and reoxidation observed by the techniques described.

We can conclude, from the good agreement between the values calculated from the constants assigned to individual steps and those found experimentally for the steady-state position and the turnover rate of the enzyme, that the scheme suggested for the reaction is correct and that the individual steps postulated are necessary and sufficient to describe the process. The determinations of the Michaelis constants for both xanthine and oxygen are not intended to be more than approximations, but the finding that both these substrates form an enzyme–substrate compound before their respective oxidation and reduction is substantiated by the fact that these oxidation and reduction steps are first-order and independent of xanthine and oxygen concentrations over a wide range. The rather primitive method used to determine K_m in the fast-reaction apparatus could give erroneous results due to inhibition by reaction products. We have, however, ascertained that these errors cannot be serious. We have also found that substrate inhibition investigated by Hofstee (1955) did not affect our experiments. Hydrogen peroxide has an inhibitory effect on the enzyme, but only if the enzyme and hydrogen peroxide are in contact for some minutes. This phenomenon prevented us from interpreting the results of measurements of the rate of reoxidation of xanthine-reduced enzyme.

Results of various experiments with isotopes have given interesting information about the mechanism of xanthine oxidase reactions. Mason (1957) has reported that molecular oxygen is used in the formation of hydrogen peroxide during the reoxidation of the enzymic $FADH_2$ and that xanthine oxidase behaves in this respect like uricase (Bentley & Neuberger, 1952) and notatin (Bentley & Neuberger, 1949). The investigations into the stereospecificity of hydrogen transfer by xanthine oxidase did not produce such decisive results as those carried out with DPN-linked enzymes. This is due to the rapid exchange of the hydrogen atoms of reduced $FADH_2$ with the medium. Vennesland (1956) did, however, get some evidence that during the oxidation of acetaldehyde by xanthine oxidase hydrogen is transferred directly from aldehyde to the electron acceptor. Our measurements of the anaerobic reduction (k_2) and the overall rate (k_0) in 50 % (v/v) ^2H_2O showed

that the rate of reduction was not changed by deuterium in the medium, while k_0 was smaller by a factor 0·643. We have drawn the conclusion that the decrease of k_0 in the presence of D_2O is due to a decrease of k_4, the rate constant for the reoxidation. The most plausible explanation for this effect is the rapid exchange of $FADH_2$ with the medium and a slower rate of reoxidation for FAD^2H_2. This point, as well as the rate of exchange of hydrogen of reduced $FADH_2$ with the medium, is being investigated further. A more detailed analysis of the behaviour of flavoproteins may throw some light on the effect of the presence of deuterium on the respiratory chain. Experiments with free FAD should also prove useful. The differences between the reactivity and physical properties of free FAD and enzyme-bound FAD should be investigated; deuterium exchange may give further information on this point. In this connexion our measurements of the reduction of xanthine oxidase by 0·025 M-sodium dithionite are of interest. The reduction of the enzyme was rather slow, giving a pseudo-first-order reaction ($k = 0{\cdot}12$ sec.$^{-1}$). Some preliminary experiments (Gutfreund, unpublished observations) have shown that the reduction of free FAD by sodium dithionite under identical conditions is considerably faster ($k = 100$ sec.$^{-1}$).

Under the conditions of our experiments no semiquinone intermediates were detected. From the intensities of the absorption bonds reported for such intermediates by Beinert (1957) one would not expect to find them under the conditions used by us. It will require a good deal of further experimental work to determine the molar extinction coefficients of the semiquinone bands in flavoprotein systems, and a subsequent calculation of the concentrations and rate of formation and decomposition of such intermediates, before one can draw any conclusions about their role on the direct path of flavoprotein-catalysed hydrogen transfer. The presence of a free-radical intermediate during some reactions of xanthine oxidase has been observed by electron-spin resonance as described by Bray, Malmström & Vänngard (1959). At this stage we should like to abstain from any discussion of the detailed reaction mechanism. Further experiments to be carried out in collaboration with Dr Bray and his colleagues will show whether a hydride ion transfers two electrons from xanthine to enzymic FAD or whether the two electrons are transferred in distinguishable steps.

SUMMARY

1. The individual steps in the hydrogen transfer from xanthine to xanthine oxidase and from xanthine oxidase to oxygen were studied by fast-reaction spectrophotometric techniques. The reduction and reoxidation of the enzymic flavinadenine dinucleotide was followed by observation of extinction value changes at 450 mμ and the appearance of uric acid at 310 mμ.

2. From studies over a wide range of concentrations of xanthine and oxygen we found that a very rapid combination of xanthine with enzyme is followed by the reduction of enzymic flavinadenine dinucleotide which is characterized by a first-order constant ($k_2 = 10{\cdot}5 \pm 0{\cdot}5$ sec.$^{-1}$). The reoxidation of the flavinadenine dinucleotide, which is preceded by a rapid combination with oxygen, is characterized by $k_4 = 21{\cdot}5 \pm 2$ sec.$^{-1}$.

3. Approximate values for the Michaelis constants for xanthine and oxygen, as well as the turnover number of the enzyme, have been determined from measurements of the rate of formation of uric acid, and a cyclic reaction scheme for xanthine oxidase is presented.

4. The effect of deuterium on the reactions of xanthine oxidase has been studied and has been provisionally interpreted.

This work has been generously supported by a grant from the U.S. Public Health Service, RG4725. We are very grateful to Professor F. Bergel, F.R.S., Dr R. C. Bray and Mr D. A. Gilbert for the supply of xanthine oxidase and for much useful advice about the properties of this enzyme. Discussions with Dr Britton Chance have been very helpful at various stages of this work.

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