The Nature and Catalytic Activities of Milk Xanthine Oxidase

By D. B. MORELL*
Molteno Institute, University of Cambridge

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The widely accepted view that xanthine oxidase is a flavoprotein whose flavine prosthetic group is alternately reduced by the substrate and oxidized by the hydrogen acceptor has not been firmly established experimentally. It rests almost entirely on the observations of Ball (1939*a*) and Corran, Dewan, Gordon & Green (1939) that preparations of the enzyme from milk contain flavin-adenine dinucleotide (FAD). A number of investigators (Ball, 1939*a*; Corran *et al.* 1939; Horecker & Heppel, 1949; Kalckar, Kjeldgaard & Klenow, 1950) have found that the FAD in xanthine oxidase preparations is reduced when xanthine or hypoxanthine is added under anaerobic conditions, but the reduction is incomplete and, in the only study in which the rate was measured, the authors (Corran *et al.* 1939) concluded that the reduction was too slow to account for the catalytic activity of the enzyme.

Ball (1939*a*, *b*) suggested that xanthine oxidase contained a second prosthetic group in addition to FAD. He claimed to have split the enzyme reversibly into its apo-enzyme and prosthetic group moieties by prolonged dialysis. The apo-enzyme was reactivated by the supernatant obtained by heating and centrifuging an enzyme preparation but not by pure FAD. Since the supernatant solution contained FAD, Ball suggested that the active enzyme contained as prosthetic groups both FAD and an additional component present in the heated enzyme preparation. However, he pointed out that it was possible that this additional component would be active on its own. The significance of Ball's findings has been questioned by Kalckar *et al.* (1950) who found that the reactivation obtained was due to the presence in the heated supernatant of sulphydryl compounds which removed metal ions acquired by the intact enzyme during dialysis. Thus, the reversible splitting of xanthine oxidase into apo-enzyme and prosthetic group has not yet been achieved.

Corran *et al.* (1939) believed that the typical reddish tinge of the enzyme was due to a second prosthetic group. This view, however, has since been criticized by Lowry, Bessey & Crawford (1949), who have suggested that since the red tinge disappears entirely when the enzyme is denatured, it may be due to an effect of the specific protein upon the light absorption of the enzymic FAD.

More recently, Horecker & Heppel (1949) have also suggested that xanthine oxidase contains a component which is reduced by dithionite ($Na_2S_2O_4$) but not by hypoxanthine.

The present paper shows that FAD does act as a hydrogen-carrying prosthetic group of xanthine oxidase and that there is no real evidence for the existence of other prosthetic group components.

MATERIALS AND METHODS

Xanthine. A stock solution ($6{\cdot}6 \times 10^{-4}$ M) of the Roche product, free from uric acid, was prepared every week. A final concentration of $6{\cdot}6 \times 10^{-5}$ M (which Hofstee (1949) found to be optimal) was obtained by using 0·3 ml. of this solution in a final volume of 3·0 ml.

Hydrochloric acid. This reagent was adjusted by titration to the same normality as the NaOH in which the xanthine was dissolved.

Albumin. A 1% (w/v) solution of partially purified egg albumin was used.

Reduced cozymase (Co I. H_2) was prepared by reduction of 35% pure cozymase by glutamate and glutamic dehydrogenase (Slater, 1950).

Reduced coenzyme II, prepared by reduction with the stoicheiometric amount of *iso*citric acid in the presence of *iso*citric dehydrogenase, was supplied by Dr E. C. Slater.

Salicylaldehyde was freshly distilled and a suitable dilution prepared immediately before use.

Xanthopterin was a pure sample of the monohydrate kindly supplied by Prof. A. Albert.

D-*Amino-acid oxidase* was prepared according to the method of Negelein & Brömel (1939) and purified to step 1. An O_2 uptake of 157 μl./10 min. at 38° was obtained with 0·2 ml. of this preparation, 0·042 M-DL-alanine and 0·088 M-glycine buffer, pH 8·3.

Measurement of enzyme activity

The spectrophotometric method of Kalckar (1947) was used. The rate of increase of the optical density at 290 mμ. caused by the oxidation of xanthine to uric acid is measured in the spectrophotometer.

Xanthine solution (0·3 ml.), HCl (0·3 ml.) and albumin (0·1 ml.) were added to two 1 cm. silica cells. To one (the reference cell) 2·3 ml. 0·1 M-glycine or pyrophosphate buffer, pH 8·3, were added; the other (the test cell) received 2·2 ml. buffer. The enzyme was so diluted in ice-cold buffer that an addition of 0·1 ml. to the above reagents in the test cell gave a change in optical density/min, at 290 mμ.

* Present address: Medical Research Institute, Royal North Shore Hospital, St Leonards, N.S.W., Australia.

(ΔD_{290}/min.), of less than 0·2. Readings of optical density were made against time in the Hilger 'Uvispek' spectrophotometer. All measurements were made in duplicate. A uniform rate was found for at least 80 sec. when ΔD_{290}/min. was less than 0·2. Catalase is not required to prevent inactivation of the enzyme by H_2O_2 under these conditions. The reaction rates are proportional to the enzyme concentration when albumin is present in the concentration given above. The reaction rate is optimal at pH 8·3.

Determination of Q_{O_2}. Under the conditions used in the activity test the oxidation of 1 μg./ml. xanthine to uric acid was found to give an increase of optical density of 0·048. The concentration of xanthine oxidized per min. is thus (ΔD_{290}/min./0·048 μg.)/ml. The Q_{O_2} (μl. O_2/hr./mg. dry wt.) of the preparation $= \frac{(\Delta D_{290}/\text{min.}) \times 0{\cdot}147 \times 60}{0{\cdot}048 \times W}$, where 0·147 is the factor used to convert μg. xanthine to μl. O_2 (1 mole xanthine is oxidized by 1 mole oxygen) and 'W' mg. is the dry weight of the preparation used in the activity measurement.

Method of measuring the reduction of the enzyme

The effect of reducing agents on the spectrum of the enzyme was studied with the use of a glass cell (internal optical path, 5 mm.) sealed to the bottom of a Thunberg tube. The cell fitted into a special holder designed for the compartment of the Beckman Model DU spectrophotometer. Stray light was excluded from the compartment by a metal hood. The enzyme and buffer were added directly to the cell and the reducing agent to the stopper. Anaerobic conditions were achieved by alternately evacuating the tube at the water pump and flushing with N_2 freed from O_2 by passing it over heated copper. It was sufficient to flush the tube twice with N_2. The readings were made against air, the cell blank being determined separately and subsequently subtracted from the original readings.

EXPERIMENTS

Purification of xanthine oxidase from milk

The method of purification is essentially that of Horecker & Heppel's (1949) modification of Ball's (1939*a*) method.

Stage 1. The casein was precipitated by calcium phosphate from trypsin-digested buttermilk according to the procedure of Ball (1939*a*). The original volume of buttermilk was 4 l. The resultant pale-yellow, slightly opalescent, supernatant was cooled to 4° and fractionated with solid ammonium sulphate. All subsequent operations were carried out at 4–8°. The protein fraction precipitated between the limits of 30 and 65 % saturation was dissolved in 0·01 M-pyrophosphate buffer, pH 8·3, and dialysed against a large volume of the same buffer. $Q_{O_2} = 333$ at 19°; the ratio of the absorption at 280 mμ. (protein absorption) to the absorption at 450 mμ. (flavine absorption) = 17·5. Yield = approx. 35 % of the enzyme activity of the original buttermilk.

Stage 2. Stage 1 preparation was dialysed against distilled water and fractionated with solid ammonium sulphate. The precipitate appearing between 33 and 40 % saturation was collected by centrifugation and dialysed against distilled water (three changes of 2 l.) for 12 hr. The dialysed preparation was then treated with calcium phosphate gel (prepared according to Keilin & Hartree, 1951) at pH 5·6 until almost all the enzyme was adsorbed (judged by disappearance of yellow colour). The enzyme was then eluted from the gel with 0·01 M-pyrophosphate buffer, pH 8·3, until the ratio $D_{280\,m\mu}/D_{450\,m\mu}$ rose above 9·0. After dialysis against distilled water for 3 hr. the enzyme solution was fractionated by adding saturated ammonium sulphate solution (adjusted to pH 7·5 with ammonia). Most of the enzyme was collected between 46 and 53 % saturation. This fraction was dialysed against 0·01 M-pyrophosphate buffer, pH 8·3, for 6 hr. $Q_{O_2} = 694$ at 19°; the ratio $D_{280\,m\mu}/D_{450\,m\mu} = 6{\cdot}15$; percentage flavine (as FAD) = 0·435. Yield = approx. 10 % of the original activity of the buttermilk.

Reduction of xanthine oxidase

The reduction of the flavine in the xanthine oxidase preparation was studied by following the disappearance of the flavine absorption band at 450 mμ. When an excess of hypoxanthine or xanthine was added to the active enzyme preparation under the conditions described in the Methods section there was always an immediate decrease in the absorption at 450 mμ. (rapid reaction). The extent of this rapid decrease varied from one preparation to another, but in all cases studied the initial rapid decrease was followed by a much slower decrease (slow reaction). The final optical density reading was approximately the same as that obtained by the addition of sodium dithionite. With highly purified preparations of the enzyme, sodium dithionite causes a decrease of about 60 % in the total absorption at 450 mμ. (Ball, 1939*a*; Corran *et al.* 1939; Horecker & Heppel, 1949). Because it was found difficult to make accurate readings of optical density before 20–30 sec. after adding the substrate, the first reading was taken at 30 sec. The reduction of the FAD which occurred in 30 sec. is referred to as 'immediate' reduction. The percentage of the total FAD which was reduced in time t was calculated from the expression

$$\frac{D_0 - D_t}{D_0 - D_{\text{dith.}}} \times 100,$$

where D_0 is the initial optical density at 450 mμ., D_t that at time t and $D_{\text{dith.}}$ the optical density after treatment with dithionite ($Na_2S_2O_4$). Reasons are given later for assuming that the sodium dithionite-reducible optical density is due to FAD.

In Fig. 1 the percentage reduction of FAD by xanthine is plotted against time for three enzyme preparations of different activity. Curves *A* and *B* describe the reduction of the FAD of two highly active preparations which gave 72 and 50 %

'immediate' reduction respectively. Curve *C* shows a preparation with a negligible xanthine oxidase activity which shows scarcely any 'immediate' reduction. This preparation had been stored in ammonium sulphate solution (10 % saturation) at approximately 5° for 8 months. When freshly prepared, 50 % of the FAD was reduced 'immediately' by xanthine.

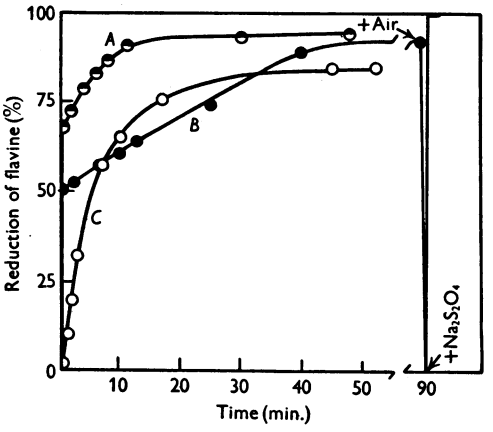

Fig. 1. The anaerobic reduction of the FAD of xanthine oxidase preparations by xanthine, showing rapid and slow reductions. Curves *A* and *B*, freshly prepared active preparations; curve *C*, preparation stored at 5° in $(NH_4)_2SO_4$ solution for 8 months. See text, p. 658 for calculation of percentage reduction.

Corran *et al.* (1939) calculated that if the function of the FAD of xanthine oxidase was that of a hydrogen carrier then, considering the activity of their enzyme preparation, the whole of the FAD should be reduced by excess hypoxanthine within 0·2 sec. of adding the substrate. The 'slow reaction' shown in Fig. 1 is very much slower than this and can contribute to only a very small fraction of the overall enzyme activity. The 'rapid reaction' might, however, be sufficiently fast to account for the enzyme activity. If this is the case, one would expect to find proportionality between the percentage 'immediate' reduction of the FAD and the activity per unit total FAD. This is tested in Fig. 2 which shows a straight-line relationship for four preparations of widely different activity. Thus that fraction of the FAD which is 'immediately' reduced can be considered to be attached to active enzyme. This enables a determination of the concentration of active enzyme in any spectroscopically clear preparation.

The secondary slow reduction of FAD observed in all xanthine oxidase preparations could be explained by any of the following hypotheses. (1) The FAD not attached to active apo-enzyme is reduced directly by xanthine in a non-enzymic reaction. (2) The FAD diffuses from inactive apo-protein to free, active apo-protein and is there reduced by xanthine. (3) FAD bound to an inactive apo-protein replaces the reduced FAD bound to active apo-protein and is there reduced by xanthine. (4) FAD which is either in the free state or is bound to inactive apo-protein is reduced by reduced FAD attached to active apo-protein.

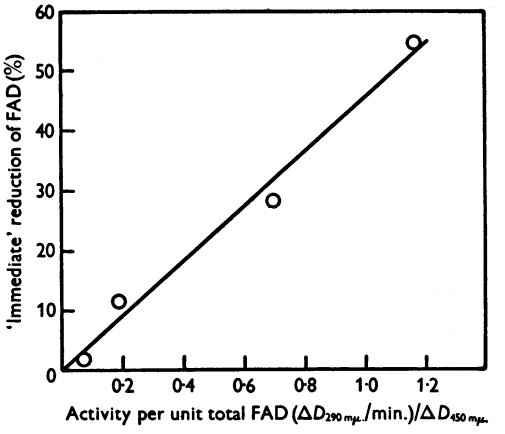

Fig. 2. Proportional relationship between the percentage of FAD 'immediately' reduced anaerobically by xanthine and the activity per unit total FAD for four xanthine oxidase preparations. Abscissa: $\Delta D_{450\ m\mu}$ is the decrease of absorption at 450 mμ. caused by addition of $Na_2S_2O_4$.

The first of these hypotheses was dismissed by direct experimentation. The FAD attached to enzyme which had been completely inactivated by treatment with cyanide (Dixon & Keilin, 1936) was not reduced when xanthine was added anaerobically. The second hypothesis was excluded by showing that when free FAD was added to the preparations there was no increase in enzyme activity. There was thus no active apo-enzyme unattached to FAD. The third hypothesis was considered unlikely because it is believed that the dissociation constant of the FAD-apo-protein complex is very small. That the fourth hypothesis is probably correct was shown by the following experiments.

In two Thunberg tubes were placed xanthine oxidase (final concentration, $0{\cdot}6 \times 10^{-5}$ M 'immediately' reducible FAD) and 1·0 ml. 0·1 M-pyrophosphate buffer, pH 8·3, and 0·1 ml. $6{\cdot}6 \times 10^{-3}$ M-xanthine added to each of the stoppers. Riboflavin (final concentration, $2{\cdot}1 \times 10^{-5}$ M) was added to one tube and pure synthetic riboflavin phosphate (final concentration, $2{\cdot}9 \times 10^{-5}$ M) to the other. After evacuation, the fluorescence of the flavines was examined visually, using a suitable blue exciting light, and the contents of the stoppers added to the tubes. The fluorescence of the riboflavin almost entirely disappeared after 15 min. and that of the monophosphate in about 25 min.

Since it is extremely unlikely that riboflavin can replace FAD on the apo-enzyme it must be assumed that, in the above experiment, the riboflavin was reduced by FAD which had itself been reduced at the apo-protein 'surface' by xanthine. A similar experiment in which the respective final concentrations of enzyme FAD (attached to active apo-protein) and added FAD were $3{\cdot}7 \times 10^{-6}$ M and $7{\cdot}7 \times 10^{-6}$ M was followed in the spectrophotometer at 450 mμ. Allowing for the known rate of reduction of the enzyme FAD it was calculated that all the added FAD was reduced in about 5 min. A further experiment of the same type in which cyanide-inactivated xanthine oxidase approximately equal in concentration to that of active enzyme was added showed that the FAD of the inactivated enzyme was reduced in about 40 min. It is, therefore, clear that the slow reductions demonstrated in Fig. 1 are due to the reduction of FAD attached to inactive apo-protein by that attached to active apo-protein.

It was of interest to determine the rate with which other flavine enzymes, in the presence of their substrates, would reduce free FAD. An active solution of notatin with glucose as the reducing substrate, at pH 5·8, required approximately 12 hr. for complete reduction of a stoicheiometric amount of free FAD. When excess glucose was added to the buffered notatin solution alone, 61 % of the total FAD was reduced in 20 sec. A secondary slow reduction, apparently complete in 2 min., of only 2 % of the FAD was thereafter recorded.

The ability of the D-amino-acid oxidase system to reduce free FAD was also investigated. A significant reduction of the FAD of this preparation by DL-alanine under anaerobic conditions could not be demonstrated. This is not surprising because the preparation was heavily contaminated with diaphorase and other pigments. However, in the presence of a small concentration of the enzyme and its substrate the reduction of added free FAD would have been detected if the reduction had occurred. No reduction of the free FAD could be demonstrated in 90 min. This agrees with the findings of Negelein & Brömel (1939) who found that the apo-enzyme of D-amino-acid oxidase, when saturated with FAD and in the presence of D-alanine under anaerobic conditions, did not appreciably reduce added FAD.

It was also of interest to find that xanthine, in the presence of xanthine oxidase, was able to reduce the FAD of notatin. This experiment was carried out similarly to those just described. Suitable concentrations of notatin and xanthine oxidase were placed in the optical cell fused to the Thunberg tube and xanthine solution added anaerobically. The reduction of FAD as measured by the decrease in absorption proceeded past the theoretical value for complete reduction of the FAD of the xanthine oxidase preparation, and it became apparent that there was a steady but slow reduction of the FAD of the notatin.

The linkage between FAD and the apo-enzyme of xanthine oxidase

During a study of possible procedures for the purification of xanthine oxidase, it was found that saturation of the enzyme preparation with magnesium sulphate, followed by dialysis to remove the salt, greatly decreased the activity of the enzyme and altered its spectrum in the visible region. The main alteration of spectrum lay in the partial removal of the flavine peak at 450 mμ. It was also noticed that when the enzyme preparation was saturated with magnesium sulphate and allowed to stand for 30 min. at 10°, the preparation lost some of its reddish tinge and was yellower. Examination of the dialysis water under ultraviolet light showed a strong yellow fluorescence.

A study of this effect of magnesium sulphate revealed that this salt and some other bivalent metal salts were able to detach the FAD from the enzyme protein. This removal could be followed in the spectrophotometer by the decrease in absorption at 450 mμ. In high concentration, magnesium sulphate or calcium chloride do not affect the absorption spectrum of free FAD. By saturating an enzyme preparation with calcium chloride most of the FAD was detached in 30 min. at room temperature or in a few minutes at 38°. When this reaction was carried out at room temperature, 75 % or more of the FAD could be detached without precipitating any of the protein. The whole of the enzyme FAD could be detached without protein precipitation if the reaction was carried out at 0°. The preparation was bright yellow after this treatment and had lost all traces of the reddish tinge associated with the intact enzyme. It was also found that merely by holding the salt-free preparation at 38° some FAD was detached before any protein was precipitated. Not only could the FAD detached from the apo-protein in the above experiments be dialysed from the preparations, but the increase in fluorescence due to the increased concentration of the free FAD was most marked. When bound in the intact enzyme the FAD of xanthine oxidase is not fluorescent (see p. 662).

Treatment of the enzyme preparation with divalent metal salts such as calcium chloride thus provided a convenient means of studying the effect of the FAD-protein linkage on the spectrum of the enzyme FAD without the danger of adsorption of some of the liberated FAD on precipitated protein. In Fig. 3 the spectra of the enzyme plotted before and after treatment with calcium chloride are given together with the difference spectrum.

The difference spectrum is obviously not that of FAD. The spectrum of FAD has maxima at 375 and at 450 mμ. whereas the above difference spectrum shows no maximum at 375 mμ. and a maximum in the green at 480 mμ. The only known reaction occurring when the enzyme is treated with calcium chloride is the breaking of the protein-FAD linkage. This suggests that the difference spectrum in Fig. 3 is partly or wholly derived from absorption which is due to an effect of this linkage upon the absorption of the enzyme FAD. Thus it seems clear that the combination of FAD with the enzyme leads to considerable spectral changes.

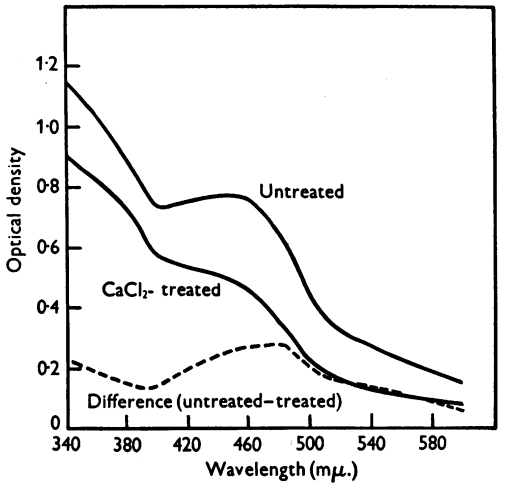

Fig. 3. The effect of $CaCl_2$ on the spectrum of xanthine oxidase. $CaCl_2$-treated, 2·9 ml. active enzyme solution + 0·4 ml. saturated $CaCl_2$, incubated at 15° for 90 min. Untreated, 2·9 ml. same enzyme solution + 0·4 ml. water.

In Fig. 4 the linearity of the relationship between decreasing absorption at 520, 480 and 450 mμ. on the one hand, and decreasing activity of the enzyme on the other, is demonstrated for an enzyme preparation treated with calcium chloride. If it can be assumed that at zero activity of the enzyme calcium chloride has no further effect upon the spectrum of the preparation we can, by extrapolating to zero activity at each wavelength, plot a portion of the spectrum of the calcium chloride-treated preparation. This agrees closely with that shown in Fig. 3. Thus the curve for calcium chloride-treated enzyme in Fig. 3 corresponds to complete inactivation.

Fig. 5 shows the spectrum of intact xanthine oxidase before and after complete reduction with xanthine. Above 400 mμ., reduction with sodium dithionite gives a similar curve (sodium dithionite absorbs below 400 mμ.). The effect of reduction on the spectrum of the enzyme preparation is shown by the difference curve. The fraction which is rapidly reduced shows the same type of difference spectrum as that which is slowly reduced (Fig. 6). Also shown in Fig. 5 is the difference spectrum (oxidized –

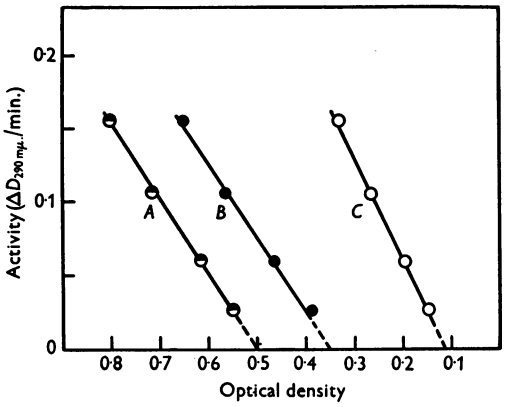

Fig. 4. The linear relationship between the activity of a xanthine oxidase preparation and the decrease in absorption at 450 (curve *A*), 480 (curve *B*) and 520 mμ. (curve *C*) caused by treatment with $CaCl_2$ (12% saturation). The $CaCl_2$-enzyme mixture was incubated in the spectrophotometer at room temperature. Samples (0·1 ml.) were removed at appropriate intervals and, after dilution in 0·4 ml. 0·1 M-glycine buffer, pH 8·3, the activity measured.

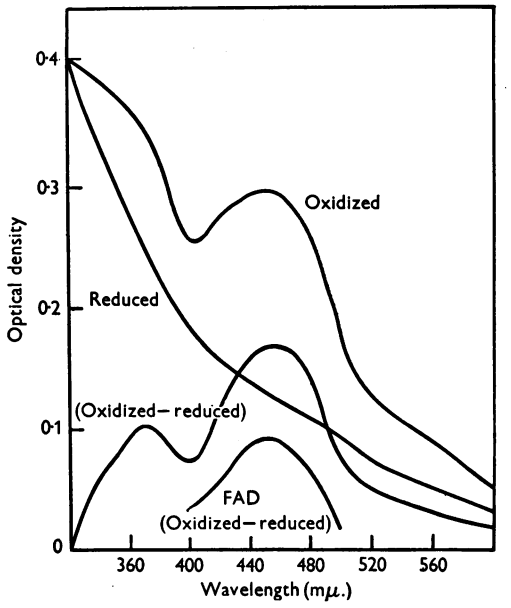

Fig. 5. The spectra of oxidized and reduced (with xanthine) xanthine oxidase plus the difference spectrum (oxidized – reduced). The difference spectrum (oxidized – reduced) of FAD equal in concentration to that in the enzyme preparation is shown for comparison.

reduced with sodium dithionite) for that concentration of FAD present in the enzyme preparation. The amount of FAD was determined by two methods:

(1) spectrophotometric determination of the optical density at 450 mμ. of the supernatant obtained by heating and centrifuging the enzyme; (2) enzymic determination of FAD on this same solution, using the D-amino-acid oxidase apo-enzyme of Negelein & Brömel (1939). The two methods gave figures within 12% of one another, thus confirming Horecker & Heppel (1949) and Lowry *et al.* (1949).

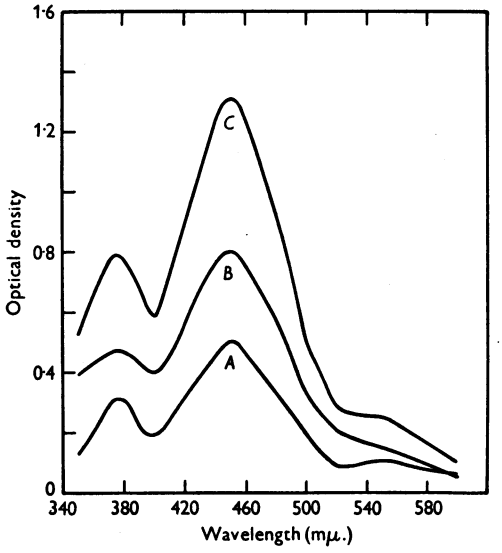

Fig. 6. Difference spectra (oxidized – reduced) obtained during the anaerobic reduction of the FAD of xanthine oxidase preparation by xanthine. (*A*) 58–100% reduction of FAD; (*B*) 0–58% reduction; (*C*) 0–100% reduction. 46% of the FAD of the preparation was anaerobically reduced by xanthine in 30 sec.

The difference spectrum for the enzyme differs from that of FAD in two important respects: (1) the intensity of the absorption is considerably greater, e.g. the absorption at 450 mμ. is 1·71 times that of the same concentration of FAD; (2) the shape of the curve is significantly different, the enzyme showing relatively much greater absorption than free FAD above 480 mμ. (cf. Ball, 1939*a*). Thus, Fig. 5 confirms the conclusion drawn from the measurements with calcium chloride-treated enzyme that combination of the FAD with the apo-enzyme causes changes in the spectrum of the former.

The non-reducible absorption of xanthine oxidase preparations in the visible spectral region

The enzyme preparation shows considerable absorption after reduction (Fig. 5). This 'residual' absorption has no sharp bands, but increases steeply towards the ultraviolet. It has been noted by previous workers (Ball, 1939*a*; Horecker & Heppel, 1949), who found that it largely disappeared when the enzyme was heated. This has been confirmed in the present study by the observation that the enzymic and spectrophotometric methods of determining FAD in the heated enzyme were in good agreement. Fig. 3 suggests that this residual absorption is not appreciably affected by calcium chloride treatment, but further work is necessary to clarify this point. It seems possible that the substance responsible for the residual absorption might be removed by precipitation at the same time as the proteins are denatured by heat. It remains to be seen whether the 'residual' absorption is a property of the enzyme or is due to an impurity.

Inhibition of D-amino-acid oxidase and notatin by calcium chloride

Calcium chloride was also found to inactivate the flavine enzymes, notatin and D-amino-acid oxidase, presumably by splitting off the FAD. Treatment of active solutions of these enzymes (buffered in glycine buffer) with equal volumes of saturated calcium chloride for 45 min. at 22° inactivated the notatin and D-amino-acid oxidase solutions by 73 and 89% respectively.

Fluorescence of xanthine oxidase preparations

All preparations of xanthine oxidase examined in these studies exhibited a distinct fluorescence when irradiated with blue light. Examination of these preparations by Dr G. Weber showed that the fluorescence was completely unpolarized (p is less than 0·01). Thus the fluorescence was emitted by molecules having relaxation times of the rotation much shorter than 10^{-8} sec. (the lifetime of the excited state of the fluorescence of riboflavin and FAD; Weber, 1950). Moreover, the appearance of polarized fluorescence when 1 vol. of the enzyme preparation was added to 3 vol. of glycerol showed that the absence of polarization was due to rapid rotation. It seems certain, therefore, that FAD bound to protein was not concerned in the emission of fluorescence from the enzyme preparation.

The relationship between xanthine, aldehyde and pterin oxidases

The experiments described earlier in which the anaerobic reduction of xanthine oxidase FAD was followed spectrophotometrically have been repeated using salicylaldehyde and xanthopterin as well as xanthine.

An active enzyme preparation ($Q_{O_2} = 333$ at 19°) was dissolved in 0·2M-phosphate buffer, pH 6·8. Concentrations of xanthine and salicylaldehyde approximately five times the molar concentration of enzyme FAD and 0·1 ml. of a saturated solution of xanthopterin (in 0·01N-sodium hydroxide) were added anaerobically in separate Thunberg tubes to the buffered enzyme solution. The reduction of the

enzyme FAD at 450 mμ. was followed as described earlier in this paper.

The reductions obtained within 30 sec. of adding xanthine, salicylaldehyde or xanthopterin were 35·8, 34·7 and 32·6 % respectively of the total FAD; eventually 97·5, 98·2 and 94·4 % respectively of the total FAD was reduced. Further experiments were made in which mixtures of the same amounts of the above substrates were added anaerobically to a similar sample of the enzyme. A mixture of salicylaldehyde and xanthine reduced 33·3 % of the total FAD in 30 sec., proceeding to a final reduction of 100·6 %, and a mixture of salicylaldehyde and xanthopterin gave corresponding reductions of 36·7 and 97·4 %.

The fact that all three substrates, both independently and in mixtures, reduced the enzyme flavine prosthetic group to the same extent indicates that the same flavine molecules take part as hydrogen carriers in the oxidation of these substrates.

The relationship between xanthine oxidase and milk diaphorase

An experiment similar to that described above was made with reduced cozymase as substrate. The molar concentration of Co I.H_2 used was approximately 16 times that of the enzyme FAD. In Table 1 the rate and extent of reduction by Co I.H_2 and by xanthine are compared.

Table 1. *Comparison of the reduction of FAD of xanthine oxidase by reduced cozymase and by xanthine*

Time after adding substrate (min.)	Reduction of enzyme FAD at 450 mμ. (%)	
	Co I.H_2	Xanthine
0·4	1	43·3
3·0	8·3	48·3
7·0	20·0	53·3
13·0	33·3	65·0
22·0	45·0	76·7
30·0	60·0	88·4
69·0	60·0	93·4

The slow reduction of the enzyme FAD by Co I.H_2 solutions, demonstrated in Table 1, was repeated with several Co I.H_2 preparations. Reduced coenzyme II (Co II.H_2), also considerably in excess of the enzyme FAD, reduced the enzyme flavine under anaerobic conditions even more slowly than Co I.H_2. The oxidation of Co II.H_2 by the enzyme preparation, in the presence of methylene blue, was negligible.

The slow reduction of xanthine oxidase flavine by Co I.H_2 may be explained in two ways: (1) the FAD attached to a very small concentration of diaphorase in the preparation was reduced by Co I.H_2 and in turn slowly reduced the FAD of xanthine oxidase, (2) by direct reduction of the FAD by Co I.H_2. Singer & Kearney (1950) have demonstrated that free flavines are reduced by Co I.H_2 and it is therefore likely that the bound FAD may be slowly reduced. There is no doubt, however, that the diaphorase activity of milk preparations is enzymic.

DISCUSSION

Reduction of xanthine oxidase

When hypoxanthine or xanthine is added to a solution of active xanthine oxidase under anaerobic conditions the reactions may be written as in equations (1) and (2) respectively:

hypoxanthine + oxidized enzyme
$\rightleftharpoons$ xanthine + reduced enzyme, (1)

xanthine + oxidized enzyme
$\rightleftharpoons$ uric acid + reduced enzyme. (2)

The potentials of the hypoxanthine-xanthine and xanthine-uric acid systems have been given as $E_0' = -407$ and -355 mV. respectively at pH 7·0, whereas that of the flavoprotein system is -80 mV., or higher (Kalckar, 1941). Thus, when the purines are in excess over the enzyme, the equilibria in the above reactions would be expected to be greatly in favour of the reduction of the enzyme flavine.

Experiments described in this paper have shown that the FAD of active xanthine oxidase is very rapidly reduced anaerobically by substrates of the enzyme. Together with the finding that the activity per unit total FAD for several preparations is proportional to the percentage of rapidly reducible FAD this is strong experimental evidence, hitherto lacking, that the FAD of xanthine oxidase does indeed act as a hydrogen carrier.

The rapid reduction of part of the FAD in the preparation is followed by a slow reduction of the remainder. This slow reduction has been shown to be due to the reduction of FAD attached to inactive protein by FAD attached to active apo-protein. It might be thought that the inactive flavoprotein was merely a contaminant of the preparations. However, the following evidence indicates that it is probably inactivated xanthine oxidase. (1) The enzyme preparations were highly purified. (2) Fresh preparations in which 72 % of the total FAD was reduced within 30 sec. of adding xanthine anaerobically showed, after ageing or other treatment, relatively little 'immediate' reduction and a correspondingly greater slow reduction. (3) The difference absorption spectrum (oxidized − reduced) of the slowly reduced flavine was very similar to that of the rapidly reduced flavine. This difference spectrum is not that of a typical flavoprotein which does not absorb so far into the longer wavelengths.

Previous reports (Corran *et al.* 1939; Horecker & Heppel, 1949) of the slow or incomplete reduction of the enzyme at 450 mμ. were thus due to the presence of inactivated enzyme in their preparations. It was found, in opposition to Horecker & Heppel (1949) but in agreement with Ball (1939*a*), that eventually hypoxanthine decreased the absorption of the preparation at 450 mμ. to the same extent as sodium dithionite.

The finding that a considerable percentage of the FAD of carefully prepared xanthine oxidase preparations is attached to inactive enzyme protein explains the experiments of Lowry *et al.* (1949) on the inhibition of pterin oxidase (which is identical with xanthine oxidase; see p. 662) by 2-amino-6-formyl-4-hydroxypteridine which is a remarkably powerful competitive inhibitor of the enzyme. Lowry *et al.* found that the dissociation constant of the enzyme-inhibitor complex is so small that it 'permits a virtual titration of the enzyme'. A study of the results of kinetic experiments using the above inhibitor suggested to Lowry *et al.* that only 60% of the FAD in their preparation was associated with active enzyme centres. The alternative explanation given by Lowry *et al.*, namely that 2 moles of flavin coenzyme were associated with each active centre, is made unnecessary by the present work.

Turnover number

Since the percentage of rapidly reducible FAD in any preparation is known, it is possible to calculate the concentration of FAD combined with active enzyme present from the total FAD concentration. This has enabled the turnover number of the enzyme to be computed more accurately than hitherto. For the enzyme purified to stage 1 (see Methods) the concentration of active enzyme in the activity test (see Methods) was found to be

$$\frac{1{\cdot}68 \times 10^{-5} \times 0{\cdot}383}{120} = 5{\cdot}37 \times 10^{-8}\text{M},$$

where $1{\cdot}68 \times 10^{-5}$ is the concentration of total FAD in the undiluted enzyme, 120 is the dilution factor in the activity test and the factor 0·383 is introduced because only 38·3% of the FAD of the preparation was 'immediately' reducible anaerobically by xanthine.

At 19° and under optimal conditions of pH (8·3) and substrate concentration ($6{\cdot}6 \times 10^{-5}$M), ΔD_{290}/min. in the activity test was 0·129. Since the oxidation of 1 μg./ml. xanthine to uric acid was found to give an increase of optical density of 0·048, the concentration of the xanthine oxidized per minute by the stage 1 enzyme was

$$\frac{0{\cdot}129}{0{\cdot}048 \times 152{\cdot}1 \times 10^{3}} = 1{\cdot}765 \times 10^{-5}\text{M},$$

where 152·1 is the molecular weight of xanthine. Thus the turnover number, which is defined as the number of molecules of substrate oxidized/molecule of enzyme/min., equals

$$\frac{1{\cdot}765 \times 10^{-5}}{5{\cdot}37 \times 10^{-8}} = 329 \text{ min.}^{-1}$$

(at 19°). The turnover number for the stage 2 enzyme preparation was found to be 298 min.$^{-1}$ so that the average turnover number = 313 min.$^{-1}$ at 19°. Assuming the Q_{10} for the enzyme to be 1·5 (Lowry *et al.* using 2-amino-4-hydroxypteridine as substrate) the turnover number at 38° would be $313 \times 2{\cdot}11 = 660$.

Does FAD act as a carrier between different flavoproteins?

It was shown earlier in this paper that xanthine oxidase, in the presence of xanthine and under anaerobic conditions, required approximately 5 min. to reduce completely a concentration of free FAD double that of the enzyme FAD. The D-amino-acid oxidase and notatin systems required very much longer time. The experiment with the xanthine oxidase system shows that the free FAD would have a turnover number of only 0·4 min.$^{-1}$ when acting as a hydrogen carrier linking this system with other flavine dehydrogenase systems. Such a slow reaction must be insignificant physiologically. It therefore seems that free FAD is not able to act as a carrier linking any of the above systems with other flavine dehydrogenase systems in the same way that coenzyme I can, *in vitro*, link the triosephosphate and lactic dehydrogenases in the reconstructed glycolytic cycle.

Substrate specificity

The finding that the same molecules of FAD which act as the hydrogen-transferring prosthetic group in xanthine oxidase also have the same function in the oxidation of salicylaldehyde and xanthopterin lends strong support to the belief that one and the same enzyme is concerned in the oxidation of the three compounds. There is already considerable evidence in the literature that the protein 'active centres' of xanthine, aldehyde and pterin oxidases are identical (see review by Dixon (1938) and papers by Lowry *et al.* (1949), Hofstee (1949) and Krebs & Norris (1949*a*, *b*).

It is significant that Co I.H_2, in a molar concentration of 16 times that of enzyme FAD, failed to reduce the enzyme FAD significantly within 1 min. Lowry *et al.* found that reduced coenzyme I was oxidized by a xanthine oxidase preparation with a velocity only 3 or 4% that of xanthine. These authors calculated the turnover number of their enzyme with xanthine as substrate to be about 210. 3% of this value would give a turnover number of

their enzyme with Co I.H_2 as substrate of 6·3, that is the enzyme FAD would be reduced, under anaerobic conditions, in approximately 10 sec. As the diaphorase activity of the preparation used in the present experiments was at least as great as that used by Lowry *et al.* these experiments must be considered as definite evidence that the FAD molecules acting as hydrogen carriers for the xanthine oxidase activity of the enzyme do not also take part in the oxidation of Co I.H_2.

Corran *et al.* (1939) were the first to show that the xanthine oxidase activity of milk preparations could be destroyed without affecting their diaphorase activity. Moreover, Lowry *et al.* (1949) have shown that 2-amino-6-formyl-4-hydroxypteridine, which is a powerful competitive inhibitor of xanthine, aldehyde and pterin oxidase, does not inhibit milk diaphorase. Since both prosthetic groups and protein 'active centres' for xanthine oxidase and milk diaphorase are different we must now regard these enzymes as separable entities.

Prosthetic group of xanthine oxidase

The red colour of xanthine oxidase solutions has been shown to be due to the effect of the FAD-protein linkage upon the spectrum of the enzyme FAD and not to the presence of a second, coloured, component of the prosthetic group of the enzyme. Little is known about the protein-FAD linkage and the cause of its rupture by calcium chloride.

Ball (1946), in an examination of the yellow flavoprotein prepared from yeast by Kunitz & McDonald (1946), found that it had an absorption spectrum in the visible region similar to that of xanthine oxidase except that the absorption at wavelengths greater than 500 mμ. was virtually absent. An acidified chloroform extraction of this yellow flavoprotein, after denaturation, produced a solution having absorption maxima in acid solution at 375, 285 and 265 mμ. Ball considers that this almost colourless substance may also be a component of other flavoprotein enzymes which have similar atypical flavoprotein absorption spectra and that these enzymes contain two or more prosthetic groups. These enzymes were stated to be: (1) the yellow flavoprotein of Kunitz & McDonald (1946); (2) the yeast flavoprotein described by Green, Knox & Stumpf (1941); (3) xanthine oxidase; (4) liver aldehyde oxidase; and (5) glucose oxidase. It should be pointed out that neither of these yeast flavoproteins has yet been found to possess catalytic activity. Extraction of the supernatant from a heat-denatured xanthine oxidase preparation of high purity in the present studies did not yield a compound with absorption maxima as given by Ball. The only extractable material showed, in aqueous solution of pH 5·5, a general absorption without maxima in the visible region of the spectrum. In view of: (*a*) the disappearance of the absorption not reducible by xanthine when the enzyme is denatured by heat; (*b*) the complete lack of evidence to show that a substance giving this absorption has a catalytic function (e.g. it is not reduced by substrates of the enzyme) and (*c*) the fact that FAD does act as a hydrogen carrier as shown by its 'immediate' reduction by xanthine, there seems no real evidence to suggest that xanthine oxidase contains any such second prosthetic group.

SUMMARY

1. Xanthine or hypoxanthine anaerobically reduce the flavin-adenine dinucleotide (FAD) of all xanthine oxidase preparations studied in two ways: (*a*) a rapid reduction, occurring within 30 sec.; (*b*) a slow reduction which may take up to 60 min. or more.

2. The activity per unit total FAD of four enzyme preparations is proportional to the percentage of FAD which is rapidly reducible by xanthine. This confirms the generally held belief that the FAD of active xanthine oxidase acts as a hydrogen carrier.

3. The slow reaction is due to the reduction of FAD attached to inactivated apo-enzyme by reduced FAD attached to active apo-enzyme.

4. Xanthine oxidase, D-amino-acid oxidase and notatin, in the presence of their substrates, anaerobically reduce free FAD so slowly that free FAD cannot act significantly *in vivo* as a carrier linking these systems with other flavine dehydrogenases.

5. Calcium chloride, magnesium sulphate and some other bivalent metal salts are able to detach the FAD of xanthine oxidase from the apo-protein without precipitating the latter. Spectrophotometric analysis of this reaction shows that the linkage between FAD and the apo-enzyme increases the absorption due to FAD by a factor of 1·71 at 450 mμ. It is also responsible for the reddish colour of xanthine oxidase preparations. There is no evidence for an additional coloured component of the prosthetic group of this enzyme, first postulated by Corran *et al.* (1939).

6. The same FAD molecules in a xanthine oxidase preparation act as hydrogen carriers in the oxidation of xanthine or hypoxanthine, salicylaldehyde and xanthopterin. This is further very strong evidence that the enzymes xanthine oxidase, aldehyde oxidase and pterin oxidase, occurring in milk, are one and the same enzyme.

7. The same molecules in xanthine oxidase preparation do not act as hydrogen carriers in the oxidation of xanthine and reduced cozymase. Since other workers have shown that the active centres for these reactions are different these enzymes must now be regarded as separate entities.

8. The minimum value for the turnover number of xanthine oxidase is 313 min.$^{-1}$ at 19°.

9. Xanthine oxidase has virtually no fluorescence when irradiated with blue light, compared to free FAD.

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The Reduction of Cytochrome *c* by Hypoxanthine and Xanthine Oxidase

By D. B. MORELL*
Molteno Institute, University of Cambridge

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The work by Bigwood, Thomas & Wolfers (1935) and Horecker & Heppel (1949) showing that xanthine oxidase, in the presence of its substrate, is able to reduce oxidized cytochrome *c* is of interest since it suggests the possibility that the reduced flavin prosthetic group of the enzyme may be re-oxidized via a cytochrome system rather than directly by oxygen. A puzzling finding by Horecker & Heppel was that the reduction of cytochrome *c* was very slow anaerobically and that the rate of the reduction increased markedly with increasing oxygen tension.

The present paper deals with a further study of the reduction of cytochrome *c* by hypoxanthine in the presence of xanthine oxidase. The reduction of cytochrome *c* under both anaerobic and aerobic conditions has been confirmed, but no evidence that oxygen increases the rate of this reduction has been found.

METHODS

The reduction of cytochrome *c* was followed at 550 mμ. in the Beckman spectrophotometer using a slit width of 0·1 mm. For anaerobic experiments a Thunberg tube fused to a 5 mm. optical glass cell contained the reaction mixture (see below). The reaction was started by adding the hypoxanthine from the hollow stopper. The readings were made against air. Anaerobic conditions were achieved by exhausting the tube at the pump and by flushing twice with N_2 freed of O_2.

Aerobic experiments were made in open 1 cm. cells. The readings were made against a reference cell containing water. In plotting the results of anaerobic experiments the cell 'blank' (determined separately) was subtracted from the optical density reading and the resultant figure multiplied by two. This facilitated comparison between data for aerobic and anaerobic experiments.

Unless otherwise stated, the reaction mixture, in both aerobic and anaerobic experiments, contained $1{\cdot}45 \times 10^{-5}$M-cytochrome *c*, $3{\cdot}2 \times 10^{-4}$M-hypoxanthine, 0·38 mg./ml. albumin, 0·05 ml. of catalase and 0·1 ml. xanthine oxidase preparation in a final volume of 3·2 ml.

Cytochrome c. Preparations containing 0·34% Fe, prepared by the method of Keilin & Hartree (1945), were kindly supplied by Dr C. L. Tsou. The preparations were thoroughly dialysed against distilled water.

Albumin. A highly purified horse serum albumin was kindly supplied by Dr E. F. Hartree.

Catalase. This preparation was made according to the method of Keilin & Hartree (1937), omitting the ultrafiltration step; it contained some ferritin. It was thoroughly dialysed against distilled water.

Hypoxanthine solutions. ($7{\cdot}35 \times 10^{-4}$M). These solutions were prepared in distilled water every 3 days from the pure Roche product and kept at 5° when not in use.

* Present address: Institute of Medical Research, Royal North Shore Hospital, St Leonards, N.S.W., Australia.