The Chemistry of Xanthine Oxidase

5. ELECTRON-SPIN RESONANCE OF XANTHINE OXIDASE SOLUTIONS*

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The participation of metals in the action of metallo-flavoproteins is the subject of much controversy, and it has recently been concluded by Singer & Massey (1957) that only for nitrate reductase has strong evidence been presented for valency changes in the metal in the course of the enzymic reactions.

Studies on the function of the molybdenum, iron and flavinadenine dinucleotide of xanthine oxidase are handicapped by the firmness of their binding to the apoenzyme. There is some suggestion of correlation between the molybdenum content of the purified enzyme and its purine/oxygen activity (Avis, Bergel & Bray, 1956; Avis, Bergel, Bray & Shooter, 1956; Bergel & Bray, 1958; Richert & Westerfeld, 1954; Westerfeld, Richert & Higgins, 1956). Reports that molybdenum can be removed reversibly from the enzyme (De Renzo, Heytler & Kaleita, 1954; Mackler, Mahler & Green, 1954) appear to rest on somewhat inadequate evidence (Avis, Bergel, Bray & Shooter, 1956; Westerfeld *et al.* 1956), and do not agree as to which enzyme-catalysed reactions are affected by loss of the metal. According to Totter & Comar (1956), iron is in the ferrous state whether the substrate is present or absent, but the colorimetric method used cannot provide conclusive evidence of this (cf. Singer & Massey, 1957). On the other hand, Fridovich & Handler (1958*a, b*) give indirect evidence for the presence of a metal-sulphur bond in the enzyme which is supposed to be split by the substrate to the reduced metal and a thiol group; they presume that the metal involved is iron, but most of their data could be explained equally well on the basis of a molybdenum-sulphur bond (P. Handler, personal communication, 1958). It appears that the flavinadenine dinucleotide analysis, on which much of their argument for implicating iron rather than molybdenum in this scheme depends, may be high by a factor of 1.71 (Morell, 1952; P. Handler, personal communication, 1958); if an appropriate correction is applied, it seems to the present authors that molybdenum should replace iron in this formulation before it could be accepted.

* Part 4: Bergel & Bray (1959).

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It is generally agreed that the flavinadenine dinucleotide of xanthine oxidase is alternately reduced by the substrate and oxidized by the electron acceptor (cf. Gutfreund & Sturtevant, 1959), though the spectral changes accompanying this reaction are complicated (Morell, 1952). Considerable evidence from electron-spin resonance studies (Ehrenberg & Ludwig, 1958; Commoner, Lippincott & Passonneau, 1958) and other experiments (Beinert, 1956, 1957) has recently suggested that flavin free radicals may be intermediates in catalysis by a number of flavoprotein enzymes. However, xanthine oxidase has not been included in any of these studies.

The technique of electron-spin resonance (Wertz, 1955; Ingram, 1958) allows the study of ions or molecules containing unpaired electrons, e.g. free radicals and ions of transition metals. The sample to be examined is placed in a static magnetic field which is varied continuously, while at right-angles to this field electromagnetic radiation of a fixed radio-frequency is applied; absorption of energy from the radio-frequency oscillation by the sample is measured while the magnetic field strength is varied. A simple explanation of the resonance phenomenon may be given by likening each unpaired electron in the sample to a spinning top. The rotating electron acts as a magnetic dipole and the effect of the static field is to cause precession of the 'gyroscope'. The radio-frequency oscillation may be considered as equivalent to a rotating magnetic field; when the frequency of the rotation coincides with that of the precession, the motion of the electron is affected and energy is absorbed. The condition for this 'resonance absorption' is given by $h\nu = g\beta B$, where h is Planck's constant, ν the frequency of the radio-frequency electromagnetic field (and of the associated rotating magnetic field), β is the Bohr magneton, B is the strength of the static magnetic field and g is a dimensionless quantity called the 'spectroscopic splitting factor'. For a completely free electron g has the value 2.0023, but deviations from this value, due to binding of the electron, are often observed and the g -value is thus an important factor in interpreting resonance absorption. Other characteristic features are line-width and the so-called 'hyperfine

structure' which results from a splitting of energy levels, owing to interaction with nuclear spin. The intensity of an electron-spin-resonance absorption signal is related to the number of unpaired electrons in the sample.

Electron-spin resonance thus detects free radicals and transition metals at certain valency levels; under favourable conditions it is capable of distinguishing between these and of estimating their concentrations. Biochemical applications of electron-sp' resonance are relatively new, but it has recently been used successfully in a study of the state of copper in laccase by Malmström, Mosbach & Vänngård (1959). In the present work xanthine oxidase solutions treated in various ways have been examined by the method in the hope that it might give reliable evidence on the valency of the metals and on the possible presence of free radicals.

MATERIALS AND METHODS

The xanthine oxidase used for most of the experiments was a sample (stage S4) from the procedure of Avis, Bergel & Bray (1955), further purified by zone electrophoresis on a cellulose column according to the technique of Porath (1954) and Flodin & Kupke (1956). The cellulose was obtained from Grycksbo Pappersbruk, Grycksbo, Sweden, and the buffer (total ionic strength approx. 0.05) for the electrophoresis was tris (2-amino-2-hydroxymethylpropane-1:3-diol)-maleic acid containing ethylenediaminetetraacetic acid and salicylic acid (Bergel & Bray, 1959). The xanthine oxidase obtained had a ratio of extinctions at 280 and 450 $m\mu$ (E_{280}/E_{450}) of 5.5 and appeared to be at least 90% pure in the ultracentrifuge; the ratio activity/ E_{450} was about 60. [The ratios E_{280}/E_{450} and activity/ E_{450} are referred to as 'PFR' and 'AFR' in previous publications (Avis *et al.* 1955).] The concentration of xanthine oxidase in the solution (0.2 mM) was calculated on the basis of a molar extinction coefficient of 70 000 at 450 $m\mu$ (Avis, Bergel, Bray, James & Shooter, 1956; Avis, Bergel & Bray, 1956). The contents of iron, molybdenum and flavinadenine dinucleotide were presumably similar to those reported previously (Avis, Bergel & Bray, 1956). Several of the experiments described below were also repeated with a crystallized sample (Avis *et al.* 1955) of the enzyme.

A sample obtained from a crystallized preparation which had lost almost all of its activity on storage was also used for certain experiments; this had activity/ $E_{450} = 1$ and its concentration was 0.1 mM. It was analysed (after dialysis) for molybdenum by a modification of the procedure of Clark & Axley (1955) by digesting with sulphuric acid and hydrogen peroxide, treating with potassium iodide in hydrochloric acid and removing liberated iodine with an excess of ascorbic acid, then forming the green complex with toluene-3:4-dithiol in the presence of tartaric acid, extracting it into amyl acetate, and measuring at 680 $m\mu$. Despite its low activity, the enzyme sample contained about one atom of molybdenum/mole of xanthine oxidase (cf. Bergel & Bray, 1958).

Solutions were dialysed against phosphate buffer, pH 6 (0.08 M- KH_2PO_4 and 0.02 M- Na_2HPO_4) containing mM-sali-

cylate and mM-ethylenediaminetetra-acetic acid. Samples (approximately 0.15 ml.) were placed in quartz tubes (2.5 mm. internal diameter; 5 mm. outside diameter). Additions of xanthine or dithionite were made if required and the solutions were frozen in liquid air before measurement in a Varian V 4500 3 cm. spectrometer. For anaerobic experiments, the tubes were filled and the contents frozen inside a glove cabinet containing nitrogen (cf. Bergel & Bray, 1959).

RESULTS

Electron-spin resonance results are generally plotted as a curve of the first derivative of the absorption against magnetic-field strength; a single absorption band thus gives rise to a peak above, followed by a peak below, the base line.

Fig. 1. First derivative of electron-spin-resonance absorption curves of solutions of xanthine oxidase under various conditions. Temperature about 100° K; microwave frequency approx. 9500 Mc/sec.; modulation sweep approx. 15 gauss peak-to-peak; magnetic field increasing from left to right. The arrows indicate the resonance field for free electrons. (a) Untreated 'active' xanthine oxidase; (b) xanthine oxidase shortly after adding xanthine aerobically; (c) xanthine oxidase in presence of excess of sodium dithionite; (d) 'inactive' xanthine oxidase with a limited amount of sodium dithionite; sensitivity in (d) four times that in (a)-(c).

Untreated xanthine oxidase gave no signal (Fig. 1*a*). When xanthine (approximately 10 moles/mole of enzyme) was added aerobically a signal consisting of two peaks (marked *A* and *B* respectively; Fig. 1*b*) appeared at once and remained unchanged after thawing, shaking to admit more oxygen, and re-freezing. This procedure could be repeated until, at a point presumably corresponding to exhaustion of the substrate, the signal disappeared completely. A similar double signal, though with a slightly different relative intensity of peaks *A* and *B*, appeared on adding xanthine to the enzyme under anaerobic conditions.

In another experiment a solution of the enzyme was titrated anaerobically with sodium dithionite, the solution being frozen for measurement of electron-spin resonance after each addition, then thawed out before the next portion was added. Peaks *A* and *B* appeared simultaneously and increased side by side with small additions of the reducing agent. When an excess of dithionite was present, peak *A* disappeared, leaving only *B* (Fig. 1*c*). If a smaller modulation field was used, peak *B* could be resolved into several components (Fig. 2).

When an 'inactive' sample of xanthine oxidase was treated with substrate, no signals appeared; however, on titration with sodium dithionite a single peak corresponding to *A* was obtained on partial reduction (Fig. 1*d*) and this disappeared, leaving no signal at all, on complete reduction.

A detailed examination of all the electron-spin resonance curves which have been obtained during the course of the work indicated that only two basic types of signal were observed, corresponding to the peaks marked *A* and *B* respectively in Fig. 1. The *g*-values and other features of these signals were accurately reproduced from one experiment to another and from one enzyme sample to another.

DISCUSSION

The most plausible explanation of the results seems to be that peak *A* is due to a flavinadenine dinucleotide semiquinone free radical and peak *B* to a reduced form of molybdenum. The absence of a signal from the resting enzyme indicates that this does not contain significant concentrations of free radicals, or of Fe^{III} , Mo^{V} or Mo^{III} . This conclusion is supported by a preliminary magnetic-susceptibility measurement (Theorell & Ehrenberg, 1951), carried out by Dr A. Ehrenberg, which indicated that the untreated enzyme was diamagnetic to about the extent expected on the basis of the protein content of the solution. These results are thus in agreement with those of Totter & Comar (1956) for the valency of the iron.

Peak *B* must be due to a paramagnetic form of

molybdenum or Fe^{II} , since it appears on partial reduction of the enzyme and is still present at full reduction, and hence cannot be due to a free radical or to Fe^{III} . Its *g*-value of 1.971 ± 0.002 at maximum absorption is in the range reported for 3- and 5-valent molybdenum compounds (Griffiths, Owen & Ward, 1953; Weissman & Cohn, 1957), while paramagnetic Fe^{II} compounds would give very broad and weak resonances. It is unfortunately not possible to decide from the available data whether peak *B* is caused by Mo^{III} or by Mo^{V} . However, by analogy with nitrate reductase (Nicholas & Stevens, 1955), Mo^{V} is perhaps more probable. If this is so, it is necessary, in order to explain the four components or more in peak *B* (Fig. 2) (which cannot be due to hyperfine structure), to assume the presence of chemically non-equivalent forms of molybdenum in the enzyme samples. The reason for the absence of peak *B* from the signal of the 'inactive' preparation reduced by sodium dithionite is not certain, as the sample contained molybdenum. Possibly inactivation of the enzyme results in steric changes or a change in the binding of the protein-bound metal, so that the molybdenum ceases to be affected, or is affected in a different manner, by the reducing agent.

Peak *A*, which appears on partial reduction and disappears on complete reduction of xanthine oxidase, has a *g*-value of 2.004 ± 0.001 , and a line-width of 18 gauss. These figures are identical with the values in the literature for free radicals from riboflavin compounds (Ehrenberg & Ludwig, 1958;

Fig. 2. Electron-spin resonance signal of the sample used for Fig. 1 (*c*), recorded with a smaller modulation field (approx. 5 gauss peak-to-peak).

cf. Commoner & Lippincott, 1958); similar signals were also obtained here from riboflavin reduced by means of zinc and hydrochloric acid. There has been one report of a Mo^V compound giving a g -value of 2.005 (Griffiths *et al.* 1953), and this might suggest that the data could be interpreted by assigning peak *A* to a reduced form of molybdenum. However, the appearance of this peak on partial reduction and its disappearance on complete reduction, particularly with the 'inactive' sample where there is no complication from the presence of peak *B*, makes it highly probable that peak *A* is in fact due to a free radical. Since the signal corresponding to peak *A* is the same whether reduction is carried out with xanthine or with sodium dithionite, the free radical seems to be associated with the enzyme rather than with the reducing agent, and it is most likely that it is due to a semiquinone flavinadenine dinucleotide free radical. All semiquinones do, in fact, have almost identical g -values (Adams, Blois & Sands, 1958).

Comparison of the intensity of the peak *A* signal with that from a solution of diphenylpicrylhydrazyl in benzene suggested that, in the presence of the substrate and of oxygen, about 15% of the total flavin was present as the free radical. However, as the enzyme sample contained a proportion of 'inactive' molecules (Avis, Bergel & Bray, 1956), the percentage of active enzyme flavinadenine dinucleotide in the free-radical form would be higher than this.

It has recently been claimed (Isenberg & Szent-Györgyi, 1958), that a riboflavin-tryptophan free-radical complex is produced on mixing the amino acid with riboflavin derivatives, and that this observation may make it necessary to reinterpret some past work on flavin-free radicals. Nevertheless, in agreement with A. Ehrenberg (1958, personal communication), we found no signal and hence no significant amounts of free radicals in the orange-red riboflavin-tryptophan complex in the absence of a reducing agent. Similarly, no signals were obtained on adding tryptophan to xanthine oxidase. Commoner & Lippincott (1958) obtained free radicals on irradiating a flavoprotein enzyme. Although we have not studied the effect of light on electron-spin resonance signals from xanthine oxidase, the early experiments of Bernheim & Dixon (1928), quoted by the above workers, in which it was claimed that xanthine oxidase was activated by light, could not be repeated by Siegel & Weintraub (1952); the latter found, as confirmed in Part 4 (Bergel & Bray, 1959), that light had an inactivating effect on the enzyme.

The experiments reported herein thus provide good evidence that iron is unaffected, molybdenum changes valency and free radicals are formed when the substrate is added to the enzyme. While it has

not actually been shown that these changes are an essential part of the enzymic reactions, they seem to provide the strongest presumptive evidence which is yet available that molybdenum and also flavinadenine dinucleotide free radicals participate in the oxidation-reduction sequences of xanthine oxidase action.

Since the work reported in this paper was completed, E. Ackerman & A. Brill (personal communication; paper presented at Pittsburgh, Pa., U.S.A., on 25 February, 1959) have reported on magnetic-susceptibility measurements which they carried out on dilute xanthine oxidase solutions. They concluded that their data suggested the presence of free radicals outside as well as inside the enzyme; further work will be needed to establish the relationship between their work and that presented above.

SUMMARY

1. Xanthine oxidase solutions have been examined in the frozen state by means of electron-spin resonance.

2. 'Active' and 'inactive' samples of xanthine oxidase were used and measurements were made both on the resting enzyme and after treatment with either xanthine or sodium dithionite.

3. Electron-spin resonance signals consisted of two separate peaks. Under various conditions these peaks could be obtained alone or in combination with each other.

4. It is concluded that the iron of the enzyme is always in the ferrous state, and that the molybdenum is reduced and free radicals are formed when the substrate is added to the active enzyme.

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Metabolism of Xylose by the Lens

2. RAT LENS *IN VIVO* AND *IN VITRO**

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When a diet containing 35% of D-xylose is fed to a weanling rat, complete opacity of the lens (cataract) ensues. The relevant literature has been summarized earlier (van Heyningen, 1958).

Because the young rat lens is very small, the calf lens was used for a preliminary investigation of the metabolism of xylose by the lens *in vitro* (van Heyningen, 1958). In the present work, the metabolism of xylose *in vivo* and *in vitro* by the lens of the weanling rat was investigated. The cataract, which closely resembles that due to feeding with galactose or experimental diabetes, does not appear in its initial stages to be due to precipitation of the proteins of the lens, but to swelling of the outermost lens fibres (von Sallmann, Caravaggio, Grimes & Collins, 1958; Friedenwald & Rytel, 1955).

EXPERIMENTAL

Methods

Diet and examination of eyes. In feeding experiments weanling Wistar albino rats 22–24 days old were used,

except in one experiment where the rats were 14 weeks old. One or more litters of the same age were divided into two groups: one group was given a xylose-containing diet and the other group an identical diet except that glucose was substituted for xylose. The diet (Darby & Day, 1939) consisted of glucose or xylose 35%, crude casein 18%, cornstarch 26%, dried baker's yeast 10%, butter 6%, salts 3% and cod-liver oil 2%. On a few occasions, indicated in the text, 25% of xylose and 10% of glucose were substituted for 35% of xylose. Examination of the eye by ophthalmoscope or slit lamp showed early signs of cataract in the xylose-fed rats after 5–6 days on the diet. After 7–8 days cataract was usually well advanced and visible to the naked eye. The animals were killed with ether, usually after 1–5 days on the diet, and the tissues removed immediately for analysis.

Preparation of tissue extracts. The lens was removed from the eye, still in the animal, by cutting the cornea with a sharp scalpel and then applying gentle pressure to the eyeball. Each lens weighed 14–18 mg. The lenses from a group of xylose-fed or glucose-fed rats were pooled, weighed and ground in ten times their weight of 5% (w/v) trichloroacetic acid; the protein precipitate was removed by centrifuging at 0° at 10 000 g for 20 min. The trichloroacetic acid extract was first treated with methyldiethylamine by the method of Hughes & Williamson (1951) to remove

* Part 1: van Heyningen (1958).