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The Chemistry of Xanthine Oxidase

7. THE ANAEROBIC REDUCTION OF XANTHINE OXIDASE STUDIED BY ELECTRON-SPIN RESONANCE AND MAGNETIC SUSCEPTIBILITY*

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The reduction of xanthine oxidase by substrates and other reducing agents is accompanied by complex changes in the visible absorption spectrum. Part of the change occurs rapidly and can be correlated with the overall kinetics of the enzyme-catalysed reaction (Gutfreund & Sturtevant, 1959), whereas part occurs much more slowly and appears to be due to the reduction of 'inactive' xanthine oxidase (Morell, 1952). The stoicheiometry of the optical changes was studied by Totter & Comar (1956). Bray, Malmström & Vänngård (1959) showed that electron-spin-resonance signals arise when the enzyme is treated with xanthine or sodium dithionite, and the present work consists of a quantitative extension of these studies. It is shown that the development of the signals on reduction of the enzyme consists of rapid and slow phases, whose rate and extent can be correlated with the nature and concentration of the substrate or other reducing agent and the composition of the xanthine-oxidase sample. The results provide information on the chemical nature of the reduced forms of the enzyme that may participate in the xanthine-oxidase reaction and in differences between the 'active' and 'inactive' modifications. Electron-spin-resonance and magnetic-susceptibility measurements have provided additional information on the state and function of the iron of the enzyme.

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EXPERIMENTAL

Reagents and buffers. The purity of the substrates, xanthine (from F. Hoffmann–La Roche and Co. Ltd., Basle, Switzerland), purine (from L. Light and Co. Ltd.) and salicylaldehyde, was checked by absorption measurements before and after complete oxidation by xanthine oxidase. The concentration of reduced diphosphopyridine nucleotide (DPNH; from Sigma Chemical Co., U.S.A.) was determined from the absorption at 340 mμ. Sodium dithionite solutions (1–10 mM) were prepared by dissolving the solid in pyrophosphate buffer, while bubbling nitrogen. The solution was immediately drawn into an all-glass micrometer syringe and standardized by titration with approx. 30 μm-moles of riboflavin phosphate (F. Hoffmann–La Roche and Co. Ltd.) in 2 ml. total volume, the end point being detected by the sharp disappearance of fluorescence under an ultraviolet lamp. Nitrogen was bubbled during the titration and a drifting end point, due to reoxidation, was corrected for by extrapolating to zero time. The concentration of riboflavin phosphate was determined from $\epsilon_{450} = 12\,200$ (Whitby, 1953). Molybdenum pentachloride and molybdenum trichloride dibenzoate were obtained from the Climax Molybdenum Co., New York. A solution of potassium octacyanomolybdenum (v) $[K_3Mo(CN)_8]$ was prepared by oxidizing potassium octacyanomolybdenum (IV) $[K_4Mo(CN)_8]$ with ceric sulphate (Weissman & Cohn, 1957) and a solution of oxotrithiocyanatomolybdenum $[MoO(SCN)_3]$ by dissolving $MoCl_5$ in a mixture of hydrochloric acid, acetone and KSCN (Perrin, 1958). Pyrophosphate buffer (0·05M, pH 8·0–8·2) was prepared by adjusting the pH of the tetrasodium salt with HCl.

Xanthine-oxidase solutions. These were prepared by the ammonium sulphate–butanol procedure, followed by calcium phosphate chromatography and in some cases by electrophoresis or low-temperature ethanol fractionation, as outlined by Haddow, de Lamirande, Bergel, Bray &

Gilbert (1958), Bray *et al.* (1959) and Avis, Bergel & Bray (1955). The solutions were dialysed against pyrophosphate buffer before use; where necessary, concentration was achieved by vacuum dialysis against the same buffer, by using a collodion shell (Membranfiltergesellschaft, Göttingen, Germany). The overall purity of the samples was checked roughly in the ultracentrifuge; the main peak accounted for at least 85% of the sedimenting material in all experiments. The total concentration of xanthine oxidase was calculated from $\epsilon_{450} = 70\,000$ (Bray *et al.* 1959).

Measurements of xanthine-oxidase activity. These were carried out at 23·5° under the conditions used by Avis *et al.* (1955). The effect of temperature on the reaction velocity in the range 5–30° was also studied. Q_{10} was found to be 3·0 below 20°, but fell off somewhat above this temperature; the mean value between 20° and 25° was 2·6. Activity measurements on samples at the end of electron-spin-resonance measurements indicated that no inactivation was caused by freezing, by substrates or by dithionite.

Analyses of flavin–adenine dinucleotide, iron and molybdenum. FAD was measured fluorimetrically, and iron with $\alpha\alpha'$-dipyridyl (Avis, Bergel & Bray, 1956*b*); Mo was measured with toluene-3:4-dithiol (Bray *et al.* 1959). The composition of the samples was essentially as reported by Avis *et al.* (1956*b*) and Bergel & Bray (1958).

Electron-spin-resonance measurements. Samples (generally 0·1–0·2 ml. of about 0·1 mM-xanthine oxidase) were placed in silica tubes (3 mm. internal diameter × 100 mm. overall length); the tubes were fitted with aluminium screw caps which pressed Neoprene gaskets on to the ground tops of the tubes, the metal fittings being joined to the silica by means of epoxide cement. Samples were frozen and measured in a Varian V 4500 spectrometer, with or without 100 kcyc./sec. modulation. Most experiments were carried out at 77° K or at about 120° K. When the Varian low-temperature fitting was used, the screw-top sample tubes were immersed completely in liquid nitrogen.

For quantitative measurement of signal intensities, a standard reference solution, consisting of 2 mM-$Cu(NO_3)_2$ in 25 mM-EDTA (disodium salt) (Malmström & Vänngård, 1960), was run at intervals and the settings of the instrument were kept as constant as possible throughout the series of measurements. Intensities were measured as the maximum deflexion of the trace from the base-line for the peak in question, and where necessary were corrected for variations in the sensitivity setting, in the signal intensity of the reference solution and in the diameter of the sample tube, assuming that the signal was proportional to the square of the internal diameter of the tube. To calculate the absolute concentrations of the unpaired electrons responsible for peaks *A* and *B*, the areas under the electron-spin-resonance absorption curves were compared with those from the copper–EDTA complex and factors for converting peak heights into absolute concentrations were deduced, assuming $s = \frac{1}{2}$, as would be expected for Cu(II), Mo(V) and the flavin–adenine dinucleotide semiquinone free radical (FADH). To check the procedure, the integrated areas for the copper standard and for an equimolar solution of $MoCl_5$ in conc. HCl were compared and found to agree almost exactly. Molybdenum solutions were not used for reference purposes owing to their low stability.

Magnetic-susceptibility measurements. The susceptibility balance of Theorell & Ehrenberg (1951), standardized with nickel chloride, was employed at temperatures of 8°, 10° or 20°. (Only the resting enzyme was measured at 20°.) Deoxygenated solutions of the enzyme were measured against buffer, generally 5–10 min. after anaerobic addition of the substrate or other reducing agent to the cold enzyme solution.

Anaerobic techniques. For the electron-spin-resonance measurements, solutions of the enzyme were deoxygenated by allowing them to stand in a flat open dish, in a layer 1–2 mm. deep, in a glove cabinet. Anaerobic conditions were achieved in the cabinet at 5°, by flushing continuously with moist commercial nitrogen. The flushing was started when the enzyme was placed in the cabinet and was continued for about 15 hr. before the tubes were filled. Glass syringes with polythene jets were employed for introducing enzyme and substrate solutions into the electron-spin-resonance tubes inside the cabinet, while this was still being flushed. The contents of the tubes were mixed, the tubes were fitted with the screw caps, removed from the cabinet via an air-lock and cooled in liquid air. For some critical experiments anaerobiosis was further improved by using argon in place of nitrogen and by recirculating the gas in the cabinet through pyrogallol wash-bottles by means of a pump. In one experiment of the type (Fig. 7) the efficiency of anaerobiosis was checked by returning a sample tube to the glove cabinet and thawing, stirring and refreezing the contents under conditions approximating to those used when the tube was filled; thus the sample was given a double exposure to any residual oxygen in the cabinet. Since this procedure had little effect on the signal intensities, it was considered that oxygen had been sufficiently excluded.

For the susceptibility measurements, the enzyme solution was deoxygenated, and, if necessary, mixed with a substrate, in Thunberg tubes filled with argon. The contents were then rapidly transferred to the apparatus, after flushing both the pipette used for the transfer and the tube of the apparatus, with argon.

RESULTS

Electron-spin resonance of Mo(V) *model compounds*

A series of well-characterized Mo(V) compounds were examined to determine g values (g, spectroscopic splitting factor) and to find out what linewidths and how much hyperfine structure were to be expected at the temperatures at which the xanthine-oxidase measurements were made (Table 1). The height of the hyperfine peaks on the traces was generally only about one-twentieth of that of the main peaks. The g value for $K_3Mo(CN)_8$ agreed with that of Weissman & Cohn (1957).

Types of signal from xanthine oxidase

Four signals have been obtained from xanthine-oxidase samples and these are designated *A*, *B*, *C* and *D* (Table 3). Signals *A* and *B* were described by Bray *et al.* (1959). We have not described signals *C* and *D* before; *C* is shown in Fig. 1. Its shape and position were similar to a part of the unidentified

Table 1. *Electron-spin resonance of* Mo(V) *compounds at* 77° K *or* 120° K

Compound	Solvent	g	Shape of main peak	Line width (gauss)	Hyperfine splitting ($cm.^{-1}$)
(*a*) $MoCl_5$	10 N-HCl	$1{\cdot}948 \pm 0{\cdot}005$	Asymmetric	Approx. 50	7×10^{-3}
(*b*) $K_3Mo(CN)_8$	N-HCl	$1{\cdot}99 \pm 0{\cdot}01$	Symmetrical	26	$4{\cdot}5 \times 10^{-3}$
(*c*) $MoO(SCN)_3$	Aq. acetone containing HCl and KSCN	$1{\cdot}938 \pm 0{\cdot}003$	Asymmetric	22	7×10^{-3}
(*d*) $Mo(C_6H_5 \cdot CO \cdot O)_2Cl_3$	Ethyl acetate	$1{\cdot}95 \pm 0{\cdot}01$	Symmetrical	Approx. 50	7×10^{-3}

References: (*a*) Sacconi & Cini (1954); (*b*) Weissman & Cohn (1957); (*c*) Perrin (1958); (*d*) Larson (1960).

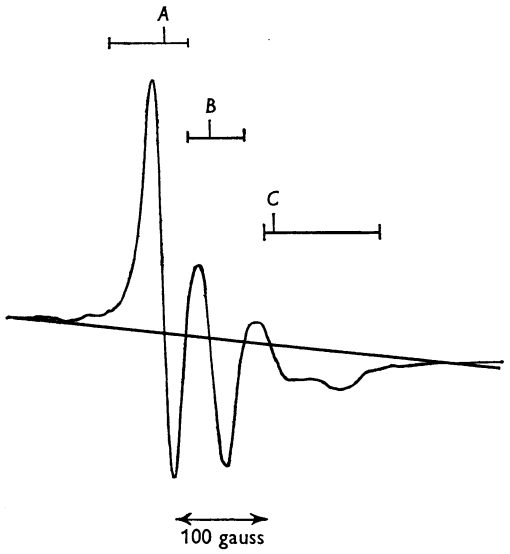

Fig. 1. Electron-spin-resonance absorption curve (first derivative) for a sample of xanthine oxidase reduced with xanthine. Temperature: 77° K; magnetic field increasing from left to right; modulation sweep approx. 12 gauss, peak to peak; the approximate position of the base-line is shown. The curve shows a strong signal *A*, a weaker signal *B* and one of the strongest signals *C* obtained in the present work.

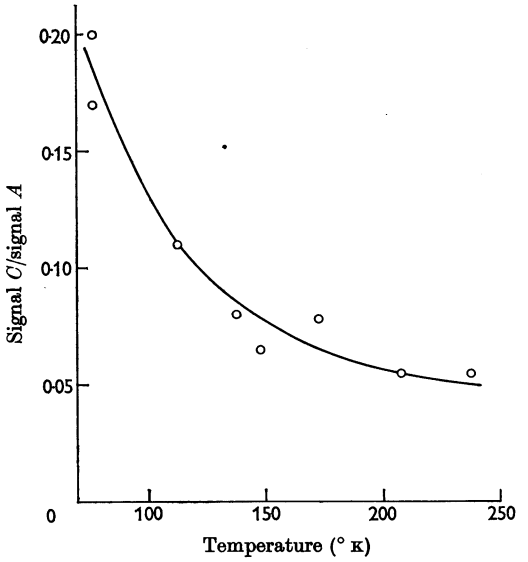

Fig. 2. Variation with temperature of the ratio of the height of signal *C* to the height of signal *A*. The sample of xanthine oxidase (about 0·2 mM) was reduced with xanthine. A constant microwave energy was used.

signal reported by Beinert & Sands (1960; cf. their Fig. 3*e*). On increasing the attenuation of the microwave energy, signal *C* decreased more than did *A* and *B*. Further, whereas signals *A* and *B* could be obtained at either liquid-air or room temperature (Vänngård, Bray, Malmström & Pettersson, 1961), signal *C* almost disappeared on warming samples from 77° K to 230–240° K, but reappeared on cooling (cf. Fig. 2). The signal of Beinert & Sands behaved similarly both with regard to temperature and to microwave-energy variations (Beinert & Sands, 1960; Sands & Beinert, 1960). The intensities of signals *A* and *B* relative to one another were little affected by temperature variations.

The shape of signal *D* was similar to that of the corresponding signal at this g value described by Beinert & Sands (1959).

Conditions affecting intensity of signals. Resting xanthine oxidase did not give signals *A*, *B* or *C*. However, a number of resting samples gave signal *D*. The intensity of this signal varied from one preparation to another and was least on the samples showing the highest purity in the ultracentrifuge. One sample which gave an abnormally intense signal *D* contained approximately 10 % of the fast-sedimenting impurity designated *fa* in previous publications (Avis, Bergel, Bray, James & Shooter, 1956*a*); samples which were about 98 % pure showed very weak signals. In one solution, which was contaminated with micro-organisms and which might therefore have been partially reduced, signal *D* increased on aeration. Signal *D* remained when the enzyme was reduced anaerobically with xanthine (3 mol.prop.); however, it disappeared on treatment with dithionite (10 mol.prop.).

Signal *C* appeared on anaerobic reduction of xanthine oxidase with xanthine ($\lessapprox$ 0·7mol.prop.);

the samples were frozen 2 min. after mixing at 5° and measurements were made at 77° K. The signal was always too weak for precise measurements to be made (Fig. 1). However, all samples examined under suitable conditions have shown the signal, and there was no tendency for purer samples to give less intense signals. The weakness of the signal and the fact that a twofold decrease in its relative intensity took place on changing the temperature from 77° K to 120° K (Fig. 2) explain why signal *C* was not observed by Bray *et al.* (1959). The temperature of measurement reported by these authors as about 100° K was, in fact, probably nearer to 120° K.

The effects on signals *A* and *B* of anaerobic additions of xanthine, purine and dithionite to a number of xanthine-oxidase samples are shown in Figs. 3–7. When samples were frozen 2 min. after the addition of xanthine, signals *A* and *B* were maximal with 1–2 moles of substrate/mole of total xanthine oxidase; with 3 or more moles of substrate, only very weak signals were obtained (Fig. 3*a*). Fig. 5 (*a*) shows the weak signal obtained in a similar experiment with a low-activity sample which had about the same molybdenum content as that in Fig. 3. (The composition of xanthine-oxidase samples is considered under Discussion.) Figs. 3 (*b*, *c*) and 5 (*b*, *c*) show the effect of allowing a longer period for the enzyme and substrate to interact. They indicate that there was a slow phase of the reaction in which both the samples gave signals, particularly with large amounts of substrate. Fig. 4 (*a*–*c*) shows similar curves for the 'active' enzyme sample employed in Fig. 3, but with purine as substrate. Here the maximum was initially in the region of 0·5 mole of purine/mole of enzyme, and the second phase of the

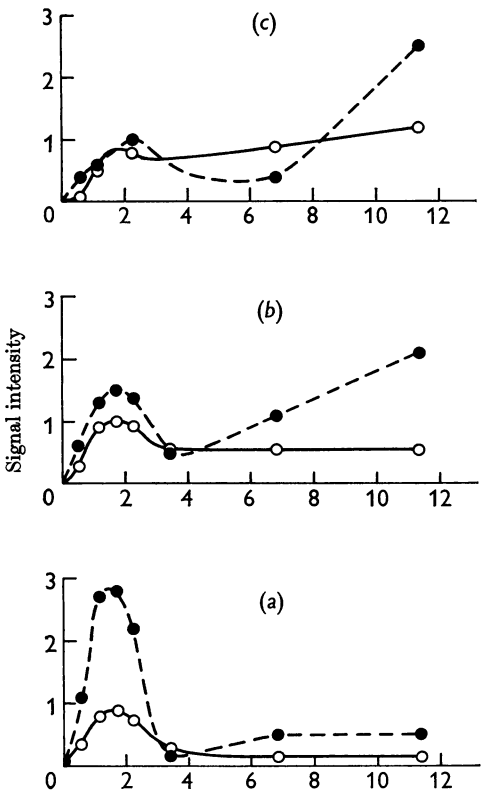

Fig. 3. Anaerobic titration of xanthine oxidase with xanthine. The intensities of signals *A* and *B* in arbitrary units are plotted against the number of moles of xanthine added/mole of total xanthine oxidase. The signal intensity units are roughly the same, and refer to the same total xanthine-oxidase concentration in all of Figs. 3–7; the units for the two signals have been adjusted so that equal ordinates for signals *A* and *B* correspond to equal areas under the integrated absorption curves. The sample of xanthine oxidase used contained 60% of xanthine oxidase-*a*, 15% of xanthine oxidase-i_1 and 25% of xanthine oxidase-i_2 (see Discussion). (*a*) Samples were frozen with liquid air 2 min. after adding the substrate at 4°; each point represents a separate sample. Measurements were made at about 120° K. (*b*) Samples from (*a*) were thawed, incubated for 15 min. at 28°, then refrozen and re-run, without opening the tubes. (*c*) Samples from (*b*) were thawed, incubated for a further 45 min. at 28°, then refrozen and re-run. ○, Signal *A*; ●, signal *B*.

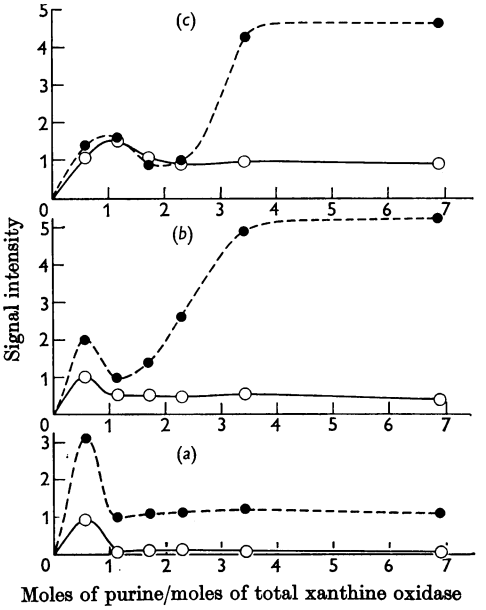

Fig. 4. Anaerobic titration of xanthine oxidase with purine. The enzyme sample and the conditions were the same as those in Fig. 3.

reaction, as far as signal *B* is concerned, was more rapid than with xanthine and had proceeded to a considerable extent even with only 2 min. incubation at 4° (Fig. 4*a*). In other experiments on this same enzyme sample, in which salicylaldehyde was the substrate, the second phase for signal *B* appeared to be more rapid still, so that, even at 2 min., high *B* signals were obtained with all except the smallest amounts of substrate; hence the peak in the curve could only just be distinguished. With DPNH as substrate the behaviour of the 'active' sample was similar to that of the 'inactive' sample with xanthine (Figs. 5*a* and 5*b*), in that there were no peaks in the curves and significant signals were obtained only after incubating for 15 min. at 28°.

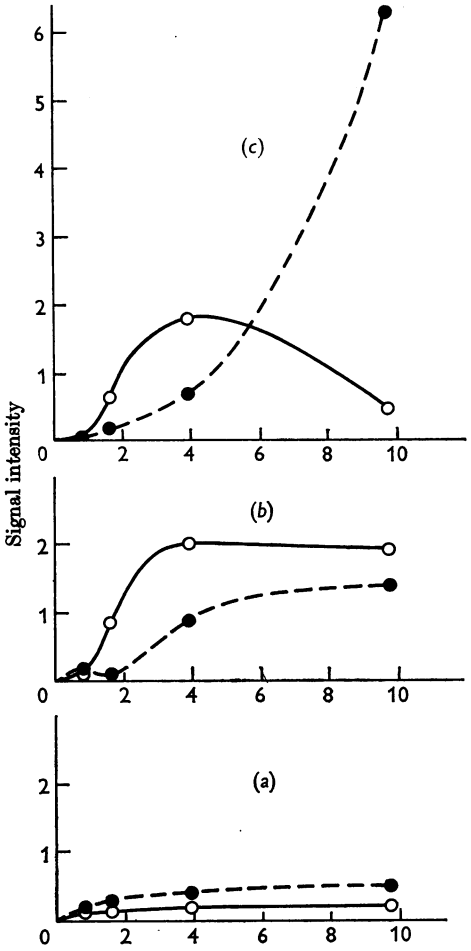

Fig. 5. Anaerobic titration of xanthine oxidase with xanthine. The sample contained 10% of xanthine oxidase-*a*, 70% of xanthine oxidase-i_1 and 20% of xanthine oxidase-i_2; the conditions were the same as those in Fig. 3.

Dithionite-titration curves for two enzyme samples are shown in Figs. 6 (*a*) and 6 (*b*) (cf. Vänngård *et al.* 1961). These curves were obtained by freezing for 2 min. after mixing at about 25°; substantial changes in the intensities, particularly of signal *B*, took place if longer incubation times were employed. In the 'active' sample (Fig. 6*a*) the change consisted mainly of a threefold intensification of signal *B* in the region of 7–9 mol.prop. of dithionite. For the 'inactive' sample (Fig. 6*b*) a similar intensification occurred and the *B* signals

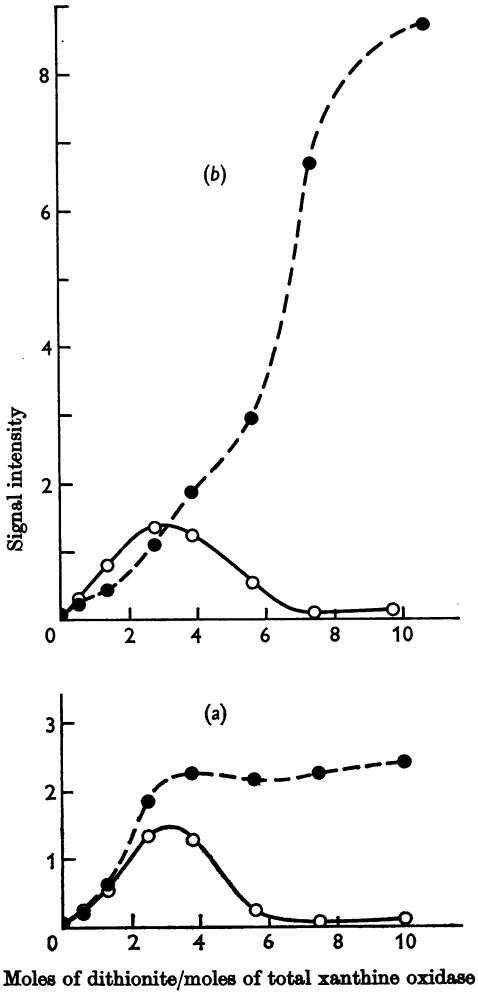

Fig. 6. Anaerobic titration of xanthine oxidase with dithionite. The samples were frozen with liquid air 2 min. after adding the reducing agent at about 25°. The samples contained: (*a*) 50% of xanthine oxidase-*a*, 15% of xanthine oxidase-i_1 and 35% of xanthine oxidase-i_2; (*b*) 10% of xanthine oxidase-*a*, 60% of xanthine oxidase-i_1 and 30% of xanthine-i_2. The other conditions were the same as those in Fig. 3.

obtained under these conditions were in fact the most intense that have been obtained from any xanthine-oxidase samples; the intensification was accompanied by a decrease in intensity of signal *B* in the region of 1–5 mol.prop. of reducing agent. For both the above samples, incubation gave rise to relatively small increases in the intensity of signal *A* in the region of 3 mol.prop. of dithionite. In another experiment, with a sample similar to that used in Fig. 6(*a*), more prolonged incubation at 28° was employed. With 5–7 mol.prop. of dithionite the intensity of signal *B* after 1 hr. or more was the same as that after 15 min. incubation. However, with 10 mol.prop., the signal decreased after its initial rise when the sample was incubated for more than 15 min.

The maximum in the curve obtained after 2 min. with xanthine (Fig. 3*a*) has been investigated in greater detail for a number of enzyme samples. Results obtained under fairly strict anaerobiosis are shown in Fig. 7. In other experiments the positions of the maxima for the two signals always coincided, but the ratios signal *A*:signal *B* were sometimes higher than those shown in Figs. 3(*a*) and 7. However, this may have been due to deterioration of the enzyme, since a number of freshly dialysed samples gave ratios similar to those in Fig. 7. When from a titration series an enzyme–xanthine mixture giving signals near to the peak was thawed, treated anaerobically with more xanthine and refrozen, the signals were markedly diminished. In a somewhat similar experiment, a controlled amount of oxygen (about 2 mol.prop. in the form of buffer saturated with the gas) was added to a sample containing 4 mol.prop. of xanthine. In this case the addition gave an increase in the signal intensities to about the values expected for the peak region.

Absolute intensity of signals. Calculations indicated that in Fig. 3(*a*) the maxima of signals *A* and *B* corresponded respectively to 0·07 and 0·23 equiv. of unpaired electrons/mole of total xanthine oxidase. Similarly, the intense signal *B* obtained on incubating an 'inactive' sample with about 10 mol.prop. of dithionite corresponded to about 1·9 equiv. of unpaired electrons/mole of xanthine oxidase.

Magnetic-susceptibility measurements

Preliminary measurements suggested that the resting enzyme was purely diamagnetic (Bray *et al.* 1959). The more accurate measurements now presented show, however, that the diamagnetism is only about half that of authentic diamagnetic proteins such as the carbon monoxide compounds of myoglobin and ferroperoxidase. This means that the resting enzyme has some paramagnetism; the value given in Table 2 has been calculated by assuming that the diamagnetic contribution is about the same as that of the above proteins. i.e. the volume-susceptibility increment for a 1% solution is $-54(\pm 9) \times 10^{-11}$ c.g.s. units (A. Ehrenberg, unpublished data). This assumption seems justified, since the partial specific volume of xanthine oxidase, 0·74 ± 0·02 (Avis *et al.* 1956*a*), is close to the values for the haemoproteins. The increases in magnetic susceptibility that occurred on treatment with xanthine, purine or dithionite are also shown in Table 2, together with some theoretical values. Measurements were also made on the xanthine-oxidase sample containing 10% of the impurity *fa* which, as stated above, gave a rather high signal *D* in the electron-spin-resonance measurements. The susceptibility increase on

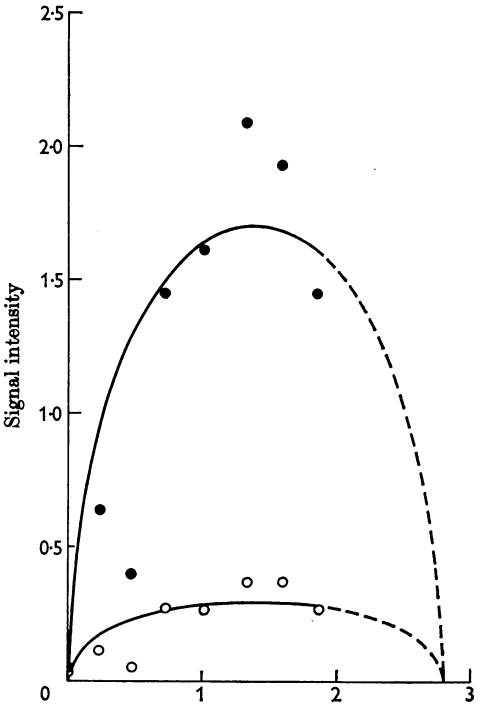

Fig. 7. Anaerobic titration of xanthine oxidase with xanthine. The samples were frozen with liquid air 2 min. after the substrate was added at 5° and measurements were made at 77° K; the preparation contained 50% of xanthine oxidase-*a*. Other conditions were as in Fig. 3. The lines are theoretical curves calculated from the equations of Michaelis & Schubert (1938) for the concentration of an intermediate in a reversible two-step reduction; they were adjusted to obtain the best fit to the data and it was assumed that the concentration of the intermediate did not exceed about 20% of that of the starting material, since in these circumstances the form is independent of the height of the maximum.

adding purine to this sample was only about one-third of the one in the Table. A rather high paramagnetism of the resting enzyme was also indicated, with χ(XO) in the region of 20×10^{-3} units, but this result needs confirmation, since the solution also contained some polyethylene glycol for which no correction could be made.

DISCUSSION

Molecular species responsible for signals

The general conclusions are summarized in Table 3.

It was concluded by Bray *et al.* (1959) and Vänngård *et al.* (1961) that signal *A* was due to the FAD semiquinone free radical (FADH) and signal *B* to Mo(v) or Mo(III). The present experiments have shown: (1) that under certain conditions (e.g. Fig. 3*a*) signal *B* disappears with excess of reducing agent and (2) that 'inactive' samples can give signal *B*. Thus some of the reasoning on which the identification of the signals was originally based has now become invalid. Nevertheless, there seems little doubt that the identification, which now rests primarily on the properties of the signals themselves (cf. Table 3), is correct. The fact that under conditions such as those in Fig. 3 (*a*) the species responsible for signal *B* can apparently be further reduced, so that the signal disappears, suggests very strongly that it is due to Mo(v) rather than Mo(III). In general, rather vigorous conditions are required to reduce molybdenum compounds below the tervalent level. The possibility that in some cases the rather complex signal *B* is due to Mo(v) and in others to Mo(III) is unlikely. It was shown by Bray *et al.* (1959) that, on decreasing the modulation field, the signal became resolved into four or more components, and during the present work this was confirmed. Although variations in the relative intensities of the components were occasionally noted, they were not apparent in those samples from the experiment in Fig. 3 which were examined at low modulation. Hence the same signal-giving species must have been involved throughout. The data on the Mo(v) model compounds (Table 1) are entirely consistent with the assumption that signal *B* is due to Mo(v).

Signal *C* cannot at present be identified with certainty, but the resemblance between it and the '*g*' signal of Beinert & Sands (1960) is striking and it seems probable that they are identical. These workers concluded that their signal probably appeared when iron was reduced. The appearance of signal *C* seems likewise to be associated with a reduction of iron (see last section of Discussion). The temperature-sensitivity of the signal (Bowers & Owen, 1955) lends some support to the hypothesis that *C* is due to resonance absorption by Fe(II) itself. However, as pointed out by Beinert & Sands (1960), no analogies to simple iron complexes

Table 2. *Molar magnetic susceptibility of xanthine oxidase under various conditions and possible contributions of the constituents*

The figure for the resting enzyme is the average of results on four preparations which contained 1·5–4% of *fa*; those for the xanthine-reduced enzyme are averages for two samples which contained 2 and 4% of *fa* respectively; the remaining figures refer to a preparation containing 4% of *fa*. All the preparations used contained about 60% of xanthine oxidase-*a*. The theoretical values (cf. Selwood, 1956) are based on 2FAD, 1·5Mo and 8Fe/mol. of xanthine oxidase (XO), assuming that all of each of the constituents considered is present in the stated form or reacts according to the equations. Susceptibilities are given in milli-c.g.s. units and the errors are estimated possible deviations. For conditions, see the Experimental section.

Experimental	Theoretical
Resting enzyme: χ(XO) = 8±4	Possible contribution to susceptibility of resting enzyme
Increase on reduction	Fe(III), low-spin: 10–20
χ(XO + xanthine) (1·5 mol.prop.) − χ(XO) = 6±2	Fe(II), high-spin: 80
χ(XO + xanthine) (3 mol.prop.) − χ(XO) = 9±2	Fe(III), high-spin: 120
χ(XO + purine) (8 mol.prop.) − χ(XO) = 11±3	Possible increase on reduction
χ(XO + purine) (8 mol.prop.); reoxidized − χ(XO) = 1±2	FAD→FADH: 2·6
χ(XO + dithionite) (7 mol.prop.) − χ(XO) = 15·5±2	Mo(VI)→Mo(V): 2·0
χ(XO + dithionite) (20 mol.prop.) − χ(XO) = 14·5±2	Mo(VI)→Mo(III): 10
	Fe(III), low-spin→Fe(II), high-spin: 65

Table 3. *Electron-spin-resonance signals from xanthine oxidase*

Signal	*g*	Probable source	Remarks
A	2·00	FADH	Cf. Bray, Malmström & Vänngård (1959)
B	1·97	Mo(v)	
C	Approx. 1·9	Fe(II) (high-spin)?	See Fig. 1; cf. Beinert & Sands (1960)
D	4·2±0·1	Fe(III) (high-spin; impurity)	Cf. Beinert & Sands (1959)

have so far been reported. Hence other alternatives, such as the reduction of part of the iron of an enzyme molecule interfering with the remaining tervalent iron, so that this gives absorption *C*, should perhaps also be considered.

There is little doubt that signal *D* is due to the high-spin form of Fe(III). This is consistent with the *g* value and its similarity to Beinert's ferric signals (Beinert & Sands, 1959, 1960). However, it seems clear that the signal is associated not with the enzyme but with an impurity. This impurity may, in fact, be the coloured, fast-sedimenting component described as *fa* by Avis *et al.* (1956*a*).

Forms of xanthine oxidase

Previous work (Avis *et al.* 1956*b*; Bergel & Bray, 1958) has indicated that xanthine oxidase as normally prepared is contaminated with several 'inactive' forms. These may represent degradation products or possibly incompletely formed xanthine-oxidase molecules; they cannot at present be separated from the 'active' form or from one another, nor can they be distinguished spectrophotometrically. The existence of the different forms has to be assumed to account for variations in the specific activity and molybdenum content. If certain simplifying assumptions are made the relative proportions of the species may be calculated, and values so obtained are used below to assist in the interpretation of electron-spin-resonance experiments. The fact that a consistent picture emerges indicates the usefulness of electron-spin resonance for problems of this kind and strengthens the evidence for the existence of the different form of xanthine oxidase.

Since both the specific activity (activity/E_{450}) and the molybdenum content vary, and since there is not a direct correlation between these variations, it is necessary to assume the presence of at least three forms of the enzyme (Table 4). In the present discussion, the possible existence of forms containing only one molybdenum atom/molecule is ignored, as is the possible presence of the inhibitor described by Bray (1959). For the forms in Table 4, it is obvious that the ratio of activity to molybdenum will be maximal for preparations devoid of the molybdenum-containing 'inactive' form, xanthine oxidase-i_1. Previous publications and unpublished results of Bergel & Bray indicate that this ratio does in fact tend towards a maximum: the highest value obtained was for a crystallized sample for which activity/E_{450} was 82 and Mo/FAD was 0·70, corresponding to an average molybdenum content of 1·40 g.atoms/mole. To deduce the activity/E_{450} value of pure xanthine oxidase-*a*, it is assumed that the above sample was completely devoid of xanthine oxidase-i_1. The activity/E_{450} value is then obtained by extrapolating to 2 atoms of molybdenum/enzyme molecule and is thus $82 \times 2/1{\cdot}40$ (= 117). In fact, samples having activity/E_{450} values of about 100 have been obtained on several occasions (Avis *et al.* 1955) and values in the region of 110 have been found by D. A. Gilbert & F. Bergel (unpublished experiments). Unfortunately no samples with such high activities have yet been analysed for molybdenum: the highest recorded Mo/FAD value is 0·87 (Avis *et al.* 1956*b*). Independent justification for pure xanthine oxidase-*a* having activity/E_{450} of about 117 is provided by the work of Morell (1952) and of Gutfreund & Sturtevant (1959), who calculated the turnover number of the pure active form by an entirely different type of extrapolation based on changes in the 450 mμ absorption band which occurred after anaerobic addition of substrate to the enzyme. Morell obtained a value of 313 min.$^{-1}$/mole of active FAD at 19° and Gutfreund & Sturtevant one of 8 ($\pm$0·5) sec.$^{-1}$ at 25°. These data may be converted into our activity/E_{450} values by taking ϵ_{450} as 70 000 for xanthine oxidase, $\Delta\epsilon$ as 9600 (Avis *et al.* 1956*b*) for the conversion of xanthine into uric acid, and Q_{10} as 2·6 and introducing a factor of 2 since there are 2 FAD/mol. The resulting activity/E_{450} values are respectively 132 and 114 ($\pm$7), and are thus in good agreement with our own extrapolated value.

The percentages of the various forms are then given by: xanthine oxidase-*a*: 100(activity/E_{450})/117; xanthine oxidase-i_1: 100[(g.atoms of Mo/moles of FAD) − (activity/E_{450})/117]; xanthine oxidase-i_2: 100[1 − (g.atoms of Mo/moles of FAD)]. On this basis the concentration of xanthine oxidase-i_1 is a measure of the excess of molybdenum over activity, and the concentration of xanthine oxidase-i_2 measures the excess of flavin over molybdenum.

Substrate-induced signal changes not related to enzymic activity

Only those changes in the signals that occur rapidly with xanthine, purine and salicylaldehyde can possibly be related to changes in the enzyme essential for its activity. On this basis, all changes that are not complete in the shortest interval between mixing enzyme with substrate and stopping the reaction by freezing must belong to a slow phase of the reaction, which is clearly irrelevant to

Table 4. *Forms of xanthine oxidase assumed to be present*

Form	Fe:FAD:Mo*	Activity/E_{450}
Xanthine oxidase-*a*	8:2:2	117
Xanthine oxidase-i_1	8:2:2	0
Xanthine oxidase-i_2	8:2:0	0

* g.atoms or moles/mole of enzyme.

the catalytic activity. The shortest time interval used in the present work (2 min. at 4°) is of the order of 100 times as long as the time required for the enzyme to be reduced by the substrate (Gutfreund & Sturtevant, 1959). The velocity of the secondary changes in the intensity of signals *A* and *B* (Figs. 3–5) is of the same order of magnitude as the slow phase in the reduction of the enzyme observed by Morell (1952). It seems probable that both phenomena are related to the reduction of 'inactive' xanthine-oxidase molecules, as was originally suggested by Morell. This view is strengthened by the fact that secondary signal changes were larger in a sample that contained excessive amounts of the 'inactive' xanthine oxidase-i_1 (Fig. 5) than in one which contained relatively little of this species (Fig. 3).

The velocity of the secondary changes in signal *B* depended on the amount of substrate present and increased in the order xanthine, purine, salicylaldehyde. This would be expected if the substrate with the lowest redox potential (Anderson & Plaut, 1949) gave the most rapid reduction. It follows that the reduction of xanthine oxidase-i_1 (or at least of the molybdenum of this species) proceeds by direct interaction with the substrate. On the other hand, the velocity of the secondary changes in signal *A* were independent of the substrate used and of its concentration (Figs. 3 and 4). Hence it seems that reduction of 'inactive' flavin proceeds predominantly by some secondary process which is not substrate-dependent. Thus, the suggestion of Morell (1952), that the slow reduction of 'inactive' enzyme is due to interaction with the reduced 'active' forms, is probably correct for xanthine oxidase-i_2 but incorrect for xanthine oxidase-i_1.

Substrate-induced signal changes probably related to enzymic activity

From the argument presented above only the peak in the signal *A* and signal *B* curves can possibly be related to the enzymic activity. Peaks were observed with three substrates known to be oxidized rapidly and for a number of preparations containing substantial proportions of xanthine oxidase-*a*, but not for one containing little of this (Figs. 3, 4, 5, 7). The possibility that even the appearance of the peaks does not occur rapidly enough to be related to enzymic activity seems to be excluded by experiments with fast-reaction techniques (Bray, 1961).

Since signal *C* appeared on treatment of 'active' enzyme samples with xanthine, within 2 min. at 5°, its appearance may also be related to the catalytic activity (see also Bray, 1961).

The oxidation of DPNH is very slow, and is brought about by 'inactive' as well as by 'active' xanthine oxidase (Bergel & Bray, 1958). This is consistent with the present observation that DPNH caused only a slow development of signals *A* and *B*.

Nature of the changes occurring on reduction of xanthine oxidase-a

The peak in the xanthine titration curves (Figs. 3*a* and 7) suggests very strongly that both the flavin and the molybdenum of xanthine oxidase-*a* can be reduced by the substrate to two different levels. Thus reduction of flavin to the first level (FADH) gives signal *A*, and reduction to the second level causes this signal to disappear. Similarly, reduction of molybdenum to Mo(v) gives signal *B* and this also disappears on further reduction. This interpretation is supported by the fact that partial reoxidation of the enzyme with oxygen from a point to the right of the peak caused signals *A* and *B* to increase, whereas further reduction brought about by the addition of more xanthine caused peak-region signals to diminish.

The proportions of flavin and molybdenum in xanthine oxidase-*a* that are present in signal-giving forms during titration with the substrate are of interest. As stated above in one experiment the maxima corresponded to 0·07 and 0·23 equiv. of unpaired electrons/mole of total enzyme for signals *A* and *B* respectively, and 60% of the enzyme was in the *a* form. Hence for the xanthine oxidase-*a* (which contains 2FAD and 2Mo/mol.) about 6% of the FAD was present as the free radical and about 19% of the molybdenum as Mo(v), during the titration.

The conclusion reached in previous publications (Vänngård *et al.* 1961) that the formation of signals *A* and *B* did not involve the participation of oxygen is strengthened by the present data, which showed: first, that the signals were still obtained under improved anaerobiosis; secondly, that the effect of introducing a controlled amount of oxygen could be explained by assuming that this merely diminished the amount of xanthine available.

Since xanthine and purine gave peaks of about the same intensity (Figs. 3*a* and 4*a*) it follows that equal numbers of active centres are involved. Further, the positions of the maxima expressed in terms of reducing equivalents/mole of enzyme (1 mole of xanthine $\equiv 2e$ and 1 mole of purine $\equiv 6e$) are about the same for the two substrates. Therefore each active centre reacts with three times as many xanthine as purine molecules. It is unlikely that as many as three xanthine molecules would react at a single centre, and hence each purine molecule must react successively with more than one active centre. Hence the signals cannot be derived from enzyme–substrate complexes of the type discussed by Hollocher & Commoner (1960).

The facts that the flavin of the enzyme can be reduced beyond the signal-giving level and that it is never all present in this form can be explained by equilibria between the semiquinone free radical (FADH) and the oxidized (FAD) and fully reduced ($FADH_2$) forms:

$$FAD \overset{\pm 1e}{\rightleftharpoons} FADH \overset{\pm 1e}{\rightleftharpoons} FADH_2$$

For molybdenum the most obvious explanation would be analogous equilibria such as:

$$Mo(VI) \overset{\pm 1e}{\rightleftharpoons} Mo(V) \overset{\pm 1e}{\rightleftharpoons} Mo(IV)$$

However, reduction to the quadrivalent level is not the only possible cause for a Mo(v) signal's disappearing. For instance, the two Mo atoms of the enzyme could conceivably interact as in the diamagnetic dimer of Mo(v) reported by Sacconi & Cini (1954).

Since the peaks of the flavin and molybdenum curves (Figs. 3*a* and 7) are both at the same xanthine concentration, the redox potentials of the flavin and molybdenum systems of the enzyme must be very close. A more generalized equation for the reduction of the enzyme may therefore be written:

$$E \overset{\pm ne}{\rightleftharpoons} EH_n \overset{\pm me}{\rightleftharpoons} EH_{n+m}$$

in which E represents the 'resting' enzyme, EH_n the form containing the signal-giving species and n and m the number of reducing equivalents required, per mole of xanthine oxidase-*a*, to bring about the transformations. Theoretical curves for the proportion of the species EH_n at any point in such a reversible two-step reduction process may be calculated, if $m = n$, by using the equations of Michaelis & Schubert (1938), and this has been done in Fig. 7. Despite scatter of the experimental points, the calculations provide some further justification for assuming that a two-step reduction does take place.

Evidence about the chemical nature of the reduced forms of the enzyme might in principle be deduced from the values of n and m. In Fig. 7, the maximum occurred at $1{\cdot}4 \pm 0{\cdot}4$ moles of xanthine/mole of total enzyme and the proportion of xanthine oxidase-*a* was 50%; therefore $n = 6 \pm 2$; less-precise data from Fig. 3 (*a*) support this value. Although it would have to be reduced somewhat if substrate binding to 'inactive' forms of the enzyme took place, the value is probably still high enough to suggest that there may be two independent active centres in each molecule of xanthine oxidase; this would, of course, be consistent with the content of two Mo atoms and 2FAD molecules/enzyme molecule. One possible interpretation of 6*e* reduction giving the highest signals would be by way of the following reaction in each molecule of xanthine oxidase-*a*:

$$2\,Mo(VI) \rightarrow 2Mo(V) \rightleftarrows Mo(VI) + Mo(IV)$$
$$2FAD \rightarrow 2FADH \rightleftarrows FAD + FADH_2$$
$$2Fe(III) \rightarrow 2Fe(II)$$

However, there are other possibilities and further speculation does not seem justified.

Our stoicheiometric results appear reasonably consistent with spectrophotometric titrations (Totter & Comar, 1956), especially if, as seems probable, the flavin figures of these workers are rather too high. However, since the spectra of the reduced forms of the enzyme described here have not so far been determined, detailed comparison is not possible.

Dithionite-induced signal changes

From the above, pure xanthine oxidase-*a* would be expected to behave in the same way in titration with dithionite as with xanthine, i.e. it should give signals only in the peak region (cf. Fig. 3*a*). It therefore seems that most of the signals obtained in the presence of dithionite must come from 'inactive' forms of the enzyme. It appears that reduction of the molybdenum of xanthine oxidase-i_1 to the quinquivalent level proceeds relatively slowly in the presence of either the substrate or dithionite; there is no evidence that substrates can reduce the molybdenum of 'inactive' enzyme beyond the signal-giving level, though excess of dithionite apparently did so very slowly. The slowness of this second stage explains the very intense molybdenum signals obtained under some conditions with low-activity samples. As stated above, we obtained the most intense signal *B* in a dithionite titration; the sample contained 60% of xanthine oxidase-i_1 and only 10% of xanthine oxidase-*a*, and the signal corresponded to about 1·9 equiv. of unpaired electrons/mole of total enzyme. Thus the signal was rather higher than would be required if all the molybdenum was in the quinquivalent state. Although the apparatus had been only roughly standardized, it is concluded that substantially all of the molybdenum of the xanthine oxidase-i_1 at least was in the signal-giving quinquivalent state.

The observation of striking differences between xanthine oxidase-*a* and -i_1 in their behaviour towards dithionite represents the first chemical, as opposed to enzymic, difference that has been observed. The two forms thus cannot differ merely in their ability to bind substrate molecules; pre-

sumably the bonding of the molybdenum must differ in the two species.

It is useful to re-examine the results of Bray *et al.* (1959). The surprising conclusion is that nearly all the signals in Fig. 1 of that paper must have come from 'inactive' rather than 'active' xanthine oxidase. The new results emphasize that extreme caution must be exercised in interpreting incomplete electron-spin-resonance data on an enzyme such as xanthine oxidase which, as normally prepared, consists of 'active' and 'inactive' forms.

The disappearance of signal *D*, which is due to impurities unrelated to any of the forms of xanthine oxidase, was brought about, not by xanthine, but by an excess of dithionite or by micro-organisms. Changes in this signal appear to be unrelated to the catalytic activity of xanthine oxidase.

Magnetic susceptibility; state of the iron

The resting enzyme gives no electron-spin-resonance signals from flavin or molybdenum, and its paramagnetism must therefore be due to the iron. Table 2 shows that the experimental value is compatible with 2–8 of the iron atoms' being tervalent, in the low-spin state. Some contribution from the high-spin state would be expected when the impurity responsible for signal *D* was present, since signals in the region of $g = 4$ would come from high-spin rather than low-spin forms of Fe(III). A single measurement on the sample showing a rather intense signal *D* and containing about 10% of *fa* indicated a more than doubled paramagnetic susceptibility of the resting enzyme, with diminished susceptibility increases on reduction. The resonance data give no evidence for low-spin Fe(III) in the resting enzyme. Bray *et al.* (1959) concluded that the iron of xanthine oxidase is in the ferrous state. However, the absorption by low-spin Fe(III) at $g = 2$ might easily be too broad to be detected by the present technique (cf. ferrihaemoglobin: Gibson, Ingram & Schonland, 1958; ferricyanide: Bleaney & Ingram, 1952).

The changes of susceptibility brought about by substrates or dithionite are generally too large to be accounted for by reduction to FADH and Mo(v) (Table 2), especially since, according to electron-spin-resonance data, only a portion of the flavin and molybdenum is ever in these paramagnetic forms. In the reduction by xanthine, which gave the smallest increase, the change could be explained by the formation of FADH, Mo(v) and Mo(III) in suitable proportions, if it is assumed that most of the metal is in the tervalent state. However, this is unlikely, and it is more probable that most of the increase in susceptibility with xanthine, purine or dithionite comes from reduction of low-spin ferric to high-spin ferrous atoms, this change being associated with the appearance of signal *C*. Thus at least part of the iron of the pure resting enzyme is in the ferric state and must be capable of reduction by xanthine to the ferrous state.

SUMMARY

1. Electron-spin-resonance and magnetic-susceptibility measurements have been carried out on xanthine oxidase alone and after the anaerobic addition of substrates or dithionite.

2. Four types of electron-spin-resonance signal have been observed. Two of these are the ones obtained previously and are attributed to the flavin–adenine dinucleotide semiquinone free radical (FADH) and Mo(v) respectively. The third signal has not been identified, but may be due to Fe(II) in the reduced enzyme; the fourth signal appears to be due to impurities.

3. An account is given of changes in the intensity of the FADH and Mo(v) signals that depend on the nature and concentration of the substrate and on the proportions of 'active' and 'inactive' xanthine oxidase. These take place only relatively slowly on adding the substrate. Differences between the 'active' and 'inactive' forms of xanthine oxidase are discussed.

4. It is concluded that 'active' xanthine oxidase can be reduced by the substrate to two different levels. The first of these shows the FADH and Mo(v) signals, but the second does not. The question of the nature of the reduced levels is discussed.

5. The strongest FADH and Mo(v) signals are, under some conditions, given by 'inactive' forms of the enzyme; it is concluded that in general incomplete electron-spin-resonance data on enzymes with variable specific activities should be interpreted with caution.

6. Susceptibility measurements indicate that, contrary to previous statements, some at least of the iron of resting xanthine oxidase is in the ferric state. Changes in susceptibility on reduction, together with the appearance of the unidentified signal, suggest that iron as well as flavin and molybdenum is reduced by the substrate.

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Sudden Freezing as a Technique for the Study of Rapid Reactions

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The kinetics of reactions in solution, having half-reaction times in the range of a few seconds down to 1 msec., are frequently studied by continuous-, stopped- or accelerated-flow methods. The 'quenching' method has been less widely used and differs from these methods in that the extent of reaction is measured at leisure on a series of samples taken from the streaming solution and 'fixed', by stopping the reaction after appropriate time intervals (Roughton & Chance, 1953). The essential requirement of this method is a quenching process which is rapid compared with the reaction being studied. Chemical quenching, in which the reaction is stopped by introducing a reagent that arrests the reaction at an appropriate point in the stream, has found limited application, but the principle of stopping a fast reaction by sudden cooling does not appear to have been considered. In this paper some of the conditions necesssary for the application of what may be termed the 'thermal quenching' method are discussed and a suitable apparatus is described. The usefulness of the method is limited by the availability of procedures for determining the extent to which the reaction has proceeded in the frozen samples. It was devised for use in conjunction with electron-spin-resonance measurements, which are in any case frequently made on frozen samples (see Addendum). The method has also proved suitable for the semiquantitative study of reactions involving colour changes.

Principle of the technique

Effect of cooling on reaction velocity. The technique depends on obtaining a series of samples of a mixture in which the reaction has been allowed to proceed for controlled periods and then stopped by suitable cooling. For a reaction dependent on molecular collisions and with a half-time of