Spin–Spin Interaction between Molybdenum and One of the Iron–Sulphur Systems of Xanthine Oxidase and its Relevance to the Enzymic Mechanism

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1. Electron-paramagnetic-resonance (e.p.r.) studies at 9 and 35 GHz at helium temperatures have given new information relating to the structure and mechanism of action of xanthine oxidase. 2. As reported by others, the enzyme gives two types of e.p.r. signal attributed to iron–sulphur systems. The first has $g_{av.} = 1.95$. Parameters of the second are determined as g_1 2.12, g_2 2.007 and g_3 1.91, with $g_{av.} = 2.01$. This species seems to have a slightly higher redox potential than the former one. 3. Temperature-dependent changes in the form of Mo^V e.p.r. signals from the enzyme, observed under certain conditions, are shown to be due to weak spin–spin interaction between Mo^V and $g_{av.} = 1.95$ Fe/S. The phenomenon has been studied most fully for the Slow Mo^V signal. Here, the spectral change takes the form of an additional approximately isotropic 11 G splitting, detected below about 45°K only. Samples without Fe/S reduced showed no such changes of spectrum. 4. Similar spectral changes were observed in the Rapid Mo^V signals, obtained in rapid-freezing experiments, but only in samples corresponding to relatively long reaction times with the substrate. It is suggested therefore that the phenomenon may provide a means of distinguishing enzyme centres with Mo only reduced from those in which both Mo and Fe/S are reduced. 5. Additional rapid-freezing data tending to support a two- rather than a one-electron transfer of reducing equivalents from substrates to xanthine oxidase are reported.

Despite extensive studies on the mechanism of action of xanthine oxidase many questions remain to be answered (see Bray & Swann, 1972). It is not yet fully established whether reducing equivalents are transferred singly or in pairs from the reducing substrate to the enzyme. Edmondson *et al.* (1972) recently proposed a hydride-transfer mechanism for this step, whereas Swann & Bray (1972) favoured a substrate radical mechanism. Evidence for the hydride mechanism rests mainly on the finding that borohydride behaves like a substrate in reducing active enzyme faster than the inactivated form with modified active centres (Edmondson *et al.*, 1972). However, direct hydrogen transfer from substrates to xanthine oxidase (though not necessarily in the form of a hydride ion) was clearly established in earlier work with deuterated substrates, by using electron paramagnetic resonance (e.p.r.) (Bray & Knowles, 1968; Bray & Swann, 1972). Evidence for a radical mechanism, on the other hand, is less direct. Bray *et al.* (1964) found that kinetics of molybdenum reduction differed from those of flavin and iron. More particularly, under the specific conditions of reduction with xanthine at pH 10, their results indicated substantial reduction of molybdenum from the hexa- to the penta-valent form, with apparently only very slight concomitant reduction of other constituents. This, taken with other findings, implied to Swann &

Bray (1972) and to Bray & Swann (1972) a one-electron transfer from substrates to the metal, with the substrate radical so produced lost from the system by rapid disproportionation.

There is further uncertainty concerning the mechanism of the oxidation–reduction reactions among the molybdenum, flavin and iron–sulphur systems of the xanthine oxidase molecule, which must follow the primary reaction with the substrate. Though it has been suggested that these reactions are remote inner-sphere electron-transfer reactions (see Mildvan, 1970), much remains to be established concerning them. The reactions might be of particular significance as model ones for the respiratory chain.

The present studies were undertaken with the aim of obtaining new information relating to the above problems.

Materials and Methods

Enzyme samples and anaerobic and rapid-freezing techniques

Samples and procedures were generally as described in earlier papers (Hart *et al.*, 1970; Swann & Bray, 1972; Palmer *et al.*, 1964). The xanthine oxidase samples used generally had activity/ E_{450} ratios in the range 80–130. Concentrations are expressed per mol

of enzyme (containing 2 atoms of Mo, 2 mol of FAD and 8 atoms of Fe). Results of tests (cf. Ballou, 1971) have confirmed the efficiency of the rapid-freezing procedure (C. Capillère-Blandin, D. J. Lowe & R. C. Bray, unpublished work). Reaction times when using this method have not been corrected for the freezing time.

Measurements of electron paramagnetic resonance

These were carried out on a Varian E9 spectrometer operating at 9 or 35 GHz (Varian Associates Ltd., Walton-on-Thames, Surrey, U.K.). For low-temperature work, cold helium gas was either passed through the variable-temperature quartz Dewar insert of the 9 GHz cavity or was passed over the outside of the 35 GHz cavity. Coarse control of temperature was achieved by varying the helium flow rate, with fine control via a small heater regulated from a gold-iron/Chromel thermocouple and a suitable feedback circuit (Harwell temperature-controller, from Oxford Instruments, Oxford, U.K.). Sample temperatures were estimated from intensity of e.p.r. signals from a Cu^{2+} -EDTA standard, run at a non-saturating power in a similar sample tube to the unknown. Integrated intensity of the Cu^{2+} -EDTA signal was taken to be proportional to the reciprocal of absolute temperature. At least for 9 GHz work, it was found that the controller enabled sample temperatures from 250°K down to 10°K or below to be reset to $\pm 1^\circ\text{K}$ over the entire range (cold nitrogen gas being used at the higher temperatures). Temperature stability during a given experiment was always better than this.

Results and Discussion

Iron-sulphur systems in xanthine oxidase

Reduced xanthine oxidase gives two types of e.p.r. signal, which are attributed to its iron-sulphur systems. The first, detectable at nitrogen temperatures (Bray *et al.*, 1961), sharpens up at helium temperatures and has parameters (Gibson & Bray, 1968) not unlike those of spinach ferredoxin. The second signal was recognized more recently (Orme-Johnson & Beinert, 1969; Palmer & Massey, 1969). It becomes apparent as a bump at low field, when the temperature is decreased below about 35°K, but its parameters have not hitherto been reported. The two signals have been referred to respectively as ' $g_{1.94}$ ' and ' $g_{2.11}$ ' Fe/S. However, this nomenclature is not systematic, as will become apparent below, since in the first case the g value referred to is g_2 , whereas in the second case it is g_1 . We prefer to name the signals according to g_{av} values. The first has $g_{av} = 1.95$ (Gibson & Bray, 1968), and the second, as shown below, has $g_{av} = 2.01$. The data of Palmer & Massey (1969) indicate

that when xanthine oxidase (in either the active or the inactivated form) is fully reduced with dithionite, then integrated intensity of the $g_{av} = 1.95$ and $g_{av} = 2.01$ signals each accounts for close to one electron/FAD, i.e. two electrons/mol of enzyme (containing 8 atoms of Fe/mol).

Fig. 1 shows spectra from a sample of xanthine oxidase reduced with excess of dithionite. Fig. 1(b) was obtained under non-saturating conditions at a temperature sufficiently low that both Fe/S signals had sharpened fully. At higher temperatures, the

Fig. 1. Iron-sulphur signals from a sample of xanthine oxidase reduced with sodium dithionite

Signals were recorded under various conditions: (a) 55°K and 100 mW microwave power; (b) 15°K, 0.1 mW; (c) 15°K, 100 mW. The sample (final concn. 0.10 mM) was treated with 100 mol of dithionite/mol of enzyme at pH 6.2 and 22°C for 30 min. The field scale corresponds to 9.110 GHz.

g_{av} . 2.01 signal was not detected (Fig. 1a). Conversely, at 15°K with a high klystron power, it was possible to observe the g_{av} . 2.01 signal in a fairly pure form (Fig. 1c), since this was found to saturate less readily than did the g_{av} . 1.95 signal. Saturation studies at a low temperature (21°K) indicated that power for 50% saturation (i.e. for normalized signal amplitude equal to 50% of maximum) was about 50mW for the g_{av} . 1.95 signal, whereas it was probably 1000mW or greater for the g_{av} . 2.01 signal.

Figs. 2 and 3 show spectra of samples containing increased proportions of the g_{av} . 2.01 signal relative to that with g_{av} . = 1.95, recorded so as to show the former species. Fig. 2 is from a sample prepared by partial reduction with dithionite (cf. Orme-Johnson & Beinert, 1969), showing spectra both at 9GHz and at 35GHz. Measurements of g values at the two frequencies were in satisfactory agreement with one another and we obtained g_1 2.12, g_2 2.007 and g_3 1.91 as the parameters of the signal, with g_{av} . = 2.01. Fig. 3 shows the purest g_{av} . 2.01 spectra so far obtained. Fig. 3(a) was from another dithionite-treated sample, and Fig. 3(b) was obtained in rapid freezing, by using

xanthine at high pH and a short reaction time, and is considered further below. It may be noted that D. Edmondson, D. P. Ballou, A. Vanheuvelen, G. Palmer & V. Massey (personal communication: results presented at Flavoprotein Workshop, Konstanz, March 1972) also found preferential reduction of g_{av} . 2.01 Fe/S relative to g_{av} . 1.95 Fe/S, at high pH, with xanthine. We found additionally that reduction with salicylaldehyde at lower pH values (e.g. 30min reaction time with 100mol of salicylaldehyde at pH 6.2) gave preferential reduction of g_{av} . 2.01 Fe/S.

The nature of the two types of iron-sulphur centres in xanthine oxidase is of interest. Presumably the structure of the one giving the g_{av} . 1.95 signal is closely similar to that in spinach ferredoxin. The centre giving the g_{av} . 2.01 signal must be substantially different. The unusually large line-width (about 70G at 21°K) indicates fast relaxation and the species seems unique among reduced iron-sulphur proteins in having two g values above the free electron value

Fig. 2. Iron-sulphur signal with g_{av} . = 2.01 from a sample of xanthine oxidase partially reduced with sodium dithionite

The signal was recorded at (a) 9.088GHz and (b) 34.860GHz. Recording temperatures were respectively 18°K and about 25°K, both with maximum microwave power. Some g_{av} . 1.95 Fe/S signal is also visible. The sample (0.27mm, in a tube with 2.8mm outside diam.) was reduced at pH 7.0 and 25°C with a limited amount of dithionite.

Fig. 3. Iron-sulphur signal with g_{av} . = 2.01 from samples of xanthine oxidase

Enzyme samples were obtained (a) by partial reduction of the enzyme (0.11 mM) with an excess of dithionite at pH 6.2 and 20°C for 3 days (partial re-oxidation appeared to have occurred; cf. Swann & Bray, 1972) and (b) by brief reduction of the enzyme (0.14 mM) with 7 mol of xanthine/mol of enzyme at pH 10.1 and 0°C for 4ms. Traces, only, of the g_{av} . 1.95 Fe/S signal are also visible. Recording conditions were 21°K, with maximum microwave power. The field scale corresponds to 9.086GHz.

(cf. Tsibris & Woody, 1970). The structural implications of this, however, are not clear. The indications are that the redox potential of the g_{av} . 2.01 species must be slightly higher than that of the other species. pH changes in the range 6–10 seem to have little effect on the behaviour of the two systems, since preferential reduction of the g_{av} . 2.01 species was found at both ends of this range.

Electron-paramagnetic-resonance signals at the short-reaction times with xanthine

It seemed desirable to repeat the work of Bray *et al.* (1964) on the early stages of reduction of xanthine oxidase by xanthine, at about pH 10. We were able to confirm, for samples prepared at reaction times of a few milliseconds at 0°C, that the only signal observable at about 120°K was the Very Rapid Mo^V signal [see Bray & Swann (1972) for Mo^V signal nomenclature]. We could not detect the Rapid Mo^V or free-radical signals in such samples. However, when the e.p.r. spectra were observed at helium temperatures, as noted above it was apparent that there was also much reduction of both Fe/S systems. Integrated signal intensities for a typical sample are summarized in Table 1. These results are possibly not inconsistent with the original work by Bray *et al.* (1964). However, g_{av} . 1.95 Fe/S reduction on such samples was not emphasized in this publication and it was indeed probably somewhat obscured by the use of signal amplitudes rather than of integrated intensities. The g_{av} . 2.01 Fe/S signal was, of course, not detected by Bray *et al.* (1964) at nitrogen temperatures.

The finding (Table 1) that, at the earliest time accessible to us in rapid-freezing experiments, the Very Rapid signal accounted for only 30% of the unpaired electrons detectable in the sample, with the remaining 70% distributed between the two Fe/S systems, destroys an important piece of the evidence appar-

ently favouring a one-electron mechanism for the transfer of reducing equivalents from xanthine to xanthine oxidase, and, conversely, must strengthen evidence for the hydride mechanism of Edmondson *et al.* (1972).

Reduction sequences in the enzyme in its reaction with 1-methylxanthine

The work of Bray *et al.* (1964) on intramolecular electron-transfer sequences in xanthine oxidase was based mainly on experiments with xanthine of the single turnover type, i.e. with xanthine concentrations comparable with those of the enzyme and with an excess of oxygen. These showed that the various e.p.r. signals reached their maximum intensities at different times. Thus the Very Rapid Mo^V signal reached a peak first, at a time quite short compared with the turnover time, followed by the Rapid Mo^V signal and the radical signal, with the g_{av} . 1.95 Fe/S signal following slightly after these. This work (subsequently confirmed by Bray *et al.*, 1967) provided very clear evidence for a different time-sequence of the signals, at least between Mo as the Very Rapid signal and Fe/S. Nevertheless, interpretation is complicated when xanthine is used as substrate by the presence of the two types of Mo^V signal. The relationship between these is only just beginning to be understood (Bray & Swann, 1972; D. Edmondson, D. P. Ballou, A. Vanheuvelen, G. Palmer & V. Massey, personal communication: results presented at Flavoprotein Workshop, Konstanz, March 1972). Another substrate reported to show clear time-sequences in appearance of its e.p.r. signals is 1-methylxanthine (Bray *et al.*, 1967; Bray, 1971). Interpretation in this case is simplified by the absence of Very Rapid signals. However, since the earlier work on this substrate failed to distinguish between O₂⁻ and FADH radical signals (Bray & Swann, 1972), we thought it desirable to repeat the single turnover experiment with 1-methylxanthine. Results are presented in Table 2. Superoxide dismutase was added to the system to remove O₂⁻, so that the only radical seen was the symmetrical FADH signal. It is clear from the changes in signal amplitude ratios with time that the Rapid signal is appearing fastest, with the FADH and the two Fe/S signals coming up later and essentially simultaneously to one another. This result thus provides the clearest confirmation of the original conclusion that the various e.p.r. signals do not appear and disappear simultaneously, even in the absence of the Very Rapid signal.

Table 1. *Electron-paramagnetic-resonance signal intensities after brief reaction between xanthine oxidase and xanthine at a high pH value*

Integrated signal intensities are given as percentages of the maximum possible for complete conversion into each species, based on reduction of active enzyme in the sample only. Reaction conditions were: 4 ms at 0°C and pH 10.1, anaerobically, with these final concentrations: xanthine oxidase, 0.14 mM; xanthine, 1.0 mM.

Signal	Intensity (% of theory)
Radical	<0.1
Rapid Mo ^V	<0.2
Very Rapid Mo ^V	6
Fe/S (g_{av} . = 1.95)	4
Fe/S (g_{av} . = 2.01)	10

Spin-spin interaction between the g_{av} . 1.95 Fe/S and Mo^V systems in xanthine oxidase: changes in the Slow signal with temperature.

During the course of ENDOR studies on xanthine

Table 2. *Changes in e.p.r. signals during a single turnover of xanthine oxidase in the presence of 1-methylxanthine and oxygen*

Signal amplitude ratios are expressed relative to those observed at the shortest reaction time. Some changes in the form of the Rapid signal took place during the reaction. Conditions were: 23°C and pH 8.2, with these final concentrations: xanthine oxidase, 0.16 mM; 1-methylxanthine, 0.2 mM; oxygen, 0.75 mM. Superoxide dismutase (about 2 μ M) was included in the system to remove O_2^- .

Time (ms)	Relative signal amplitude ratios		
	Rapid Mo^V	Rapid Mo^V	Fe/S ($g_{av.} = 2.01$)
	FADH	Fe/S ($g_{av.} = 1.95$)	Fe/S ($g_{av.} = 1.95$)
8	100	100	100
25	57	56	70
52	43	30	80
76	29	18	90
95	25	20	90
143	25	15	100

oxidase, A. Ehrenberg, G. Anger & R. C. Bray (unpublished work) observed striking changes in the form of Mo^V Rapid signals from xanthine oxidase on lowering the temperature into the helium range. We decided to investigate this phenomenon, but we chose to work not with the Rapid but with the Slow signal, since in preliminary experiments this was found by A. Ehrenberg, G. Anger & R. C. Bray (unpublished work) to change in an analogous manner. Although the Slow signal comes not from active but from inactivated xanthine oxidase (McGartoll *et al.*, 1970; Bray & Swann, 1972), it was preferred for two reasons. First, it is a much simpler signal than is the Rapid, consisting apparently of one species only, rather than of a number of different species. Secondly, it is possible to obtain the Slow signal alone, with all the Fe/S systems of the enzyme oxidized, by taking advantage of the relatively low rate at which the Slow Mo^V species is oxidized by air (Pick & Bray, 1969). The importance of this will become apparent below.

Fig. 4 shows the Slow signal recorded at a non-saturating power at 95°K and at 40°K. Above and to at least somewhat below this temperature range, the signal remained unchanged from the forms in Fig. 4(a) and 4(b) respectively. At intermediate temperatures the spectra changed smoothly from the high- to the low-temperature form. Fig. 5 illustrates this point. At 3297 G, in the low-temperature limit, the derivative spectrum had zero amplitude, whereas in the high-temperature limit it was zero at 3287 G (see arrows on Fig. 4). Amplitudes measured at these two points and normalized for the Boltzmann effect by multiplying by the absolute temperature are plotted as a function of temperature in Fig. 5. The change from the high- to the low-temperature form was half-complete at about 56°K. Although Fig. 5 is plotted in terms of signal amplitudes, it is particularly noteworthy that the integrated intensity of the Slow signal remained

Fig. 4. *High- and low-temperature forms of the Slow Mo^V signal from xanthine oxidase*

Recording temperatures (at 0.1 mW) were (a) 95°K and (b) 40°K. The field scale corresponds to 9.086 GHz. The sample (0.10 mm) was reduced with 25 mol of dithionite/mol of enzyme at pH 8.2 and 25°C for 20 min. See Fig. 5 for significance of the arrows.

unchanged over the temperature range in question. Thus, for the sample used in Fig. 4, integration indicated 0.11 electron/ Mo atom at 82°K and 0.10 at 28°K. Fig. 5 also shows the effect of temperature on the $g_{av.} 1.95$ Fe/S signal of the sample. Normalized

Fig. 5. Normalized amplitudes of e.p.r. signals from a sample of xanthine oxidase giving the Slow signal, as a function of temperature

Amplitudes, under standard recording conditions and at 0.1 mW, were normalized for the Boltzmann effect by multiplying by the absolute temperatures. The low-temperature form of the Slow signal (Δ) was measured at 3287 G and the high-temperature form (o) at 3297 G (fields at 9.086 GHz; see arrows on Fig. 4). g_{av} . 1.95 Fe/S ($\blacksquare$) was measured at its g_2 feature. The sample (0.4 mM) had been inactivated by treatment with cyanide (Edmondson & Massey, 1970) to maximize the Slow signal. Reduction was with 20 mol of dithionite/mol of enzyme at pH 8.2 and 25°C for 20 min.

amplitude of the signal decreases on raising the temperature, and the temperature for half-maximum normalized amplitude was found to be 58°K. On the other hand, the g_{av} . 2.01 Fe/S signal was detectable only at the bottom of the temperature range in question. The parallelism between the change of form of the Slow signal and the sharpening of the g_{av} . 1.95 Fe/S signal, and in particular the closeness of their mid-way temperatures, at once suggested a close relationship between the two phenomena and opened the possibility that g_{av} . 1.95 Fe/S and Mo might be interacting in some way in the enzyme molecule.

It therefore became of particular interest to study the Slow signal in samples showing no Fe/S signals.

Fig. 6. Slow signal from xanthine oxidase, obtained under conditions such that iron-sulphur is not reduced

Recording temperatures (at 0.1 mW) were (a) 122°K and (b) 28°K. The field scale corresponds to 9.142 GHz. The sample (final concn. approx. 0.05 mM) was reduced with excess of dithionite at pH 8.2, and then separated by gel filtration and reoxidized with 0.6 mM-oxygen at pH 10.1 and 24°C for 1.0 s.

The normal method of obtaining the Slow signal is to treat xanthine oxidase, e.g. with 20 mol of dithionite/mol of enzyme for 20 min (Pick & Bray, 1969). Under these conditions both Fe/S signals appear to be fully developed. However, after removing excess of dithionite with anaerobic gel filtration, it was possible to re-oxidize the samples with oxygen in the rapid-freezing apparatus (Pick & Bray, 1969) and to find conditions such that all e.p.r.-detectable chromophores were re-oxidized, except for Mo^V of the inactivated enzyme giving the Slow signal. The spectrum from such a sample is shown in Fig. 6. There may be small amounts of additional species present (compare Fig. 6a with Fig. 4a), but clearly the high-temperature form of the Slow signal is the only one detected under all conditions, thus establishing that the change in this signal occurs only if iron also is reduced in the enzyme.

Nature of the change in the Slow signal

The Slow signal, in its high-temperature form, has been studied at both 9 and 35 GHz, and it has thereby

Fig. 7. High- and low-temperature forms of the Slow signal recorded at 34.560 GHz

Recording temperatures (35 decibels attenuation) were (a) about 75°K and (b) about 30°K. The sample was similar to that in Fig. 4.

Fig. 8. Deuterium form of the Slow signal as a function of temperature

been demonstrated that the spectrum results from coupling of a single exchangeable proton to the Mo to give an isotropic splitting of 16 G (Bray *et al.*, 1968). We have now repeated these experiments on the low-temperature form of the signal (with iron reduced in all cases). The high- and low-temperature forms of the signal in water at 35 GHz are shown in Figs. 7(a) and 7(b), and 9 GHz spectra in $^2\text{H}_2\text{O}$ over a range of temperatures are presented in Figs. 8(a)–(f).

Detailed examination of the spectra makes it clear that the difference between the high- and low-temperature forms of the Slow signal is due to an additional, nearly isotropic, splitting of about 11 G, detected only in the latter form. Though the 35 GHz low-temperature spectrum is not particularly well resolved (Fig. 7b), nevertheless a 1:2:1 triplet is clearly apparent at high field. This arises from the superimposition of the new splitting on to the existing 16 G proton-splitting at the g_3 feature. Another point of particular significance in comparing the 35 GHz spectra with those at 9 GHz is that the total increase in width of the spectrum on going from the high- to the low-temperature form is of the order of 10 G at either frequency (Figs. 4 and 7). Finally, to confirm

(a)–(f) Experimental spectra recorded at the following respective temperatures: 122, 95, 61, 48, 41 and 21°K. The field scale corresponds to 9.148 GHz. Microwave power was 5 mW for (a) and 0.1 mW for (b)–(f). The sample was prepared in $^2\text{H}_2\text{O}$ at pD 8.4 in a manner comparable with the one in Fig. 5. Spectrum (g) is a simulation of the low-temperature form, based on the high-temperature spectrum, with an added isotropic 11 G splitting to simulate that from Fe/S. The simulation was carried out by adding together two spectra, displaced relative to each other by 11 G. Spectrum (a) was used for this purpose.

our interpretation, we carried out a simple simulation of the effect of an 11 G isotropic splitting on the 9 GHz $^2\text{H}_2\text{O}$ high-temperature spectrum. The result is shown in Fig. 8(g) and gives a reasonable approximation to the observed low-temperature spectrum (Fig. 8f).

Nature of the species interacting with Mo^V in the low-temperature spectrum

We now have to consider the nature of the spin $\frac{1}{2}$ species which produces the new 11 G splitting in the Slow signal at low temperature. It is just conceivable that the effect could be due to a non-exchanging proton, which is brought into interaction with Mo as a result of some conformation change in the protein that might take place in the region of 60°K. However, the necessity to have $g_{av.}$ 1.95 Fe/S reduced and the parallelism between changes in this signal and the Slow signal make it virtually certain that it is this spin system that is coupling directly to the Mo, to give a splitting that is by coincidence rather similar in magnitude to the proton-splitting, and that the temperature variation of the spectrum arises from the changes in the relaxation times of the Fe/S electron, rather than from any conformational changes. In earlier work on the closely related enzyme aldehyde oxidase, Beinert and co-workers obtained evidence for analogous interactions (Rajagopalan *et al.*, 1968*a,b*). However, with only saturation studies on the e.p.r. signals at nitrogen temperatures, they were unable to interpret the phenomenon in detail.

Interpretation of the temperature variation of the spectrum

If the splitting in the low-temperature form of the Slow Mo^V signal is indeed due to coupling to the iron-sulphur, then the $g_{av.}$ 1.95 signal should also show a splitting of 11 G at these temperatures from its coupling with the Mo electron. However, the line-width of the $g_{av.}$ 1.95 signal is about 25 G (see Fig. 1*a*). Furthermore, in our samples only a relatively small proportion of the molecules that have Fe/S reduced also have Mo in the pentavalent form. Hence any splitting of the Fe/S signal would be effectively masked. Although recourse to ELDOR measurements is clearly desirable to confirm our interpretation directly, nevertheless the fact that the Fe/S electron is more efficiently relaxed than the Mo electron enables us to explain the temperature variation of both signals in a convincing way.

This type of situation, involving relatively weak coupling, seems to be encountered more frequently in n.m.r. than in e.p.r. work. The spectrum of a spin, A, coupled to a second spin, X, changes as the rate of the X spin relaxation increases. Initially two sharp lines are separated by the coupling constant, J ; these broaden, coalesce and then sharpen into a single averaged line (Abragam, 1961, p. 505). The sequence of spectra is identical with those from a spin exchanging between two equally populated sites (see, e.g., Lynden-Bell & Harris, 1969, p. 139). The effect is analogous to changes in the proton n.m.r. spectra of ammonia and amines as the nitrogen quadrupolar relaxation rate is varied (Carrington & McLachlan, 1967, p. 210). Here, the difference is, of course, that the observed signal is a superposition of spectra due to many pairs of Mo atoms (A spin) and Fe atoms (X spin) with different resonant frequencies corresponding to the variation of g with orientation.

The e.p.r. spectrum of a particular pair of Fe/S and Mo centres in the frozen sample depends on the orientation of the pair relative to the direction of the magnetic field in the spectrometer. For most orientations the g values are sufficiently different that the resonances from the two centres are separated by considerably more than the coupling (J) between them. The low-temperature spectrum is then an AX type (n.m.r. nomenclature; see, e.g., Lynden-Bell & Harris, 1969, p. 65), consisting of four lines with equal intensities, with both the Fe and Mo signals split by J . As the complete Fe and Mo signals overlap, there will be a few pairs that are orientated so that their g values are similar. In these the wave-functions become mixed and the spectrum changes to an AB or A₂ type (Abragam & Bleaney, 1970, p. 509). This will give a slight distortion to the low-temperature Mo signal; this is in fact too small to show in the present spectra. The A₂-type spectra arise when the g values in the two sites are identical and correspond to strong coupling of the two spins into a triplet. The AX spectra correspond to a biradical spin system where the sites retain their identity.

The line-widths in the more common case, i.e. in the AX limit, are given in Table 3. As the widths of the Mo lines (A spin) depend upon T_1 of the Fe, whereas

Table 3. Predicted line-widths of an AX spectrum with the X spin relaxing

Line-widths are expressed as the inverse of the effective T_2 , which is equal to the product of π and the absorption line-width in Hz at half-height. Where two lines are predicted their separation is J , the coupling constant.

Temp.	X spin (Fe/S electron)		A spin (Mo electron)	
	No. of lines	Width	No. of lines	Width
Low	2	$[T_2(X)]^{-1}$	2	$[T_2(A)]^{-1} + \frac{1}{2}[T_1(X)]^{-1}$
Intermediate	2 overlapping	$[T_2(X)]^{-1}$	1 broad	Shape complex
High	2 very broad overlapping	$[T_2(X)]^{-1}$	1 sharp	$[T_2(A)]^{-1} + J^2 T_1(X)$

the Fe (X spin) line-widths are determined by T_2 , which is shorter than T_1 in solids (Carrington & McLachlan, 1967, p. 193), the Mo signal should be sharper than the Fe signal in the low-temperature limit. This is in accordance with observation. At 40°K, the line-width of the g_{av} . 1.95 Fe/S signal is about 25 G, i.e. T_2 is of the order of 2×10^{-9} s, whereas the Mo line-width is about 6 G, implying that T_1 (Fe/S) is at least twice as long.

Fig. 8 shows a sequence of Mo spectra as the temperature is raised, for a sample in $2H_2O$ giving the Slow signal. In the absence of proton-splitting, the line-width changes were more readily observable than, for example, they were in the experiment of Fig. 5. The expected broadening of the spectrum and sharpening into the high-temperature form is observed in Fig. 8. The signal is broadest at 48°K, corresponding to the coalescence temperature at which T_1 (Fe/S) is approximately equal to J^{-1} , where J is the iron-molybdenum coupling constant expressed as rad/s. At higher temperatures J does not appear as a splitting in the spectrum, but this coupling provides a relaxation mechanism for the Mo electron giving a contribution to the line-width proportional to $J^2 T_1$ (Fe/S) (Abragam, 1961, p. 308). This contribution seems to be small at temperatures of the order of 100°K (compare Figs. 4a and 6a). If the coupling J were anisotropic, there would be considerable variation in the line-width with orientation in the region of broadening, giving rise to spectral distortions as discussed by Leigh (1970). In this system it appears that J is nearly isotropic and no such effects are seen. The goodness of fit between the experimental low-temperature spectrum (Fig. 8f) and the one computed (Fig. 8g) by superimposing an isotropic splitting on to the high-temperature form enables us to set an upper limit of about 1–2 G on the anisotropic component of J . From this value it might be possible to obtain information about the distance between the Mo and g_{av} . 1.95 Fe/S centres in the molecule. However, simple dipolar-coupling calcula-

tions indicate distances of the order of 40 Å (4 nm). Since this seems improbably high, we conclude that other factors such as spin-orbit coupling must be influencing the situation substantially (cf. Abragam & Bleaney, 1970, p. 495).

Spin-spin interaction between Fe/S and other Mo^V signals and its relevance to the enzymic mechanism

Data on the behaviour of the Mo^V signals of xanthine oxidase on cooling to helium temperatures are summarized for each of the known signal types and for various reaction conditions in Table 4. As noted above, A. Ehrenberg, G. Anger & R. C. Bray (unpublished work) found that the xanthine Rapid signal (obtained at a long reaction time and with Fe/S reduced) does change with temperature. Though they did not study the phenomenon in detail, they found that the effect was entirely analogous to that described above for the Slow signal. Thus the spectrum increased in width by about 10 G, and resolved peaks in the spectrum increased in number, when the temperature was lowered. Further, A. Ehrenberg, G. Anger & R. C. Bray (unpublished work) also examined the Inhibited Mo^V signal, which is obtained on inactivating xanthine oxidase with formaldehyde (Pick *et al.*, 1971) and which is normally obtained with Fe/S in the oxidized state. They found, here, that the spectrum did not change with temperature, in keeping with the result on the Slow signal with Fe/S oxidized.

Though the findings support the view that reduction of Fe/S is necessary for a change of spectrum to occur, it is clear from Table 4 that this cannot be the only factor controlling the phenomenon. With the Very Rapid signal no change in spectrum occurred, despite the fact that there was substantial reduction of Fe/S in the sample examined. Similarly there was no change in the Rapid signal from 1-methylxanthine at 46 ms reaction time. On the other hand, there was a change on an entirely comparable sample, obtained with the same substrate but at 920 ms reaction time.

Table 4. Changes in the form of Mo^V e.p.r. signals on cooling to helium temperatures

Substrate (mol/mol of enzyme)	Reaction conditions (at 22–25°C)		Fe/S signals	Mo signal	
	pH	Time		Type	He changes observed
Dithionite (20)	8.2	20 min	Yes	Slow	Yes
Dithionite then O ₂	8.2	—	No	Slow	No
—	7.0	—	No	Inhibited	No*
Xanthine (5)	9.8	9 ms	Yes	Very Rapid	No
Xanthine (9)	8.2	30 min	Yes	Rapid	Yes*
1-Methylxanthine (5)	8.2	46 ms	Yes	Rapid	No
1-Methylxanthine (5)	8.2	920 ms	Yes	Rapid	Yes

* A. Ehrenberg, G. Anger & R. C. Bray (unpublished work).

Fig. 9. Rapid signals from xanthine oxidase at relatively long and short reaction times recorded at a low temperature

Microwave power was $10\mu\text{W}$ and the recording temperature was 28°K . The field scale corresponds to 9.140GHz . The samples (0.2mm) were reduced anaerobically with 5mol of 1-methylxanthine/mol of enzyme at $\text{pH}8.2$ and 24°C for (a) 46ms and (b) 920ms .

Both samples showed substantial reduction of Fe/S. The two samples showed qualitatively identical Rapid Mo^{V} signals at nitrogen temperatures but became quite different at helium temperatures (Fig. 9). Although the spectrum of the 46ms sample was unchanged at the low temperature (Fig. 9a), the 920ms sample showed changes, again analogous to those on the Slow signal with Fe/S reduced.

It is important that all the samples in Table 4 in which Fe/S was reduced, but which nevertheless failed to show evidence of iron-molybdenum interaction, were obtained at relatively short reaction times. Two possible explanations may be envisaged. The first is that, e.g. in the time-interval between 46ms and 920ms reaction time with 1-methylxanthine, a change of conformation occurs in the enzyme molecule and that this change allows the two centres to interact. The second explanation is that, in the early stages of the reaction with substrates, some active centres have $g_{\text{av.}} 1.95$ Fe/S reduced, whereas others have Mo reduced but few have both metals reduced. That this may be the case is suggested by the rela-

tively weak integrated intensities of the Mo signals under all conditions (see Table 1; also Swann & Bray, 1972). This must be considered along with the fact that Fe/S signals are by no means maximally developed after reaction for 46ms with 1-methylxanthine (Bray, 1971). It seems possible therefore that iron-molybdenum interaction will prove to be a new tool capable of distinguishing xanthine oxidase active centres that have reacted with a single substrate molecule, from those that have taken up additional reducing equivalents from further substrate molecules. Such a tool could be of the greatest value in studying the internal electron-transfer sequences of xanthine oxidase further and in particular in helping to understand the true basis of the time-sequences of e.p.r. signal changes during the catalytic reaction.

The molybdenum-iron-sulphur interaction described here is clearly not confined to milk xanthine oxidase, nor to one particular variety of iron-sulphur. Thus, in work on xanthine dehydrogenase from *Viellonella alcalescens* (formerly known as *Micrococcus lactilyticus*), it has been found that there is only one Fe/S signal, which is of the axial rather than of the rhombic type. Here also, however, the Mo signals showed clear changes at helium temperatures (H. Dalton, D. J. Lowe & R. C. Bray, unpublished work).

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