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Complex-Formation between Reduced Xanthine Oxidase and Purine Substrates Demonstrated by Electron Paramagnetic Resonance

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The origin of the Rapid molybdenum electron-paramagnetic-resonance signals, which are obtained on reducing xanthine oxidase with purine or with xanthine, and whose parameters were measured by Bray & Vänngård (1969), was studied. It is concluded that these signals represent complexes of reduced enzyme with substrate molecules. Xanthine forms one complex at high concentrations and a different one at low concentrations. Purine forms a complex indistinguishable from the low-concentration xanthine complex. There are indications that some other substrates also form complexes, but uric acid, a reaction product, does not appear to do so. The possible significance of the complexes in the catalytic cycle of the enzyme is discussed and it is suggested that they represent substrate molecules bound at the reduced active site, waiting their turn to react there, when the enzyme has been reoxidized. Support for this role for the complexes was deduced from experiments in which frozen samples of enzyme–xanthine mixtures, prepared by the rapid-freezing method, were warmed until the signals began to change. Under these conditions an increase in amplitude of the Very Rapid signal took place. Data bearing on the origin of the Slow molybdenum signal are also discussed. This signal disappears only slowly in the presence of oxygen, and its appearance rate is unaffected by change in the concentration of dithionite. It is concluded that, like other signals from the enzyme, it is due to Mo^{V} but that a slow change of ligand takes place before it is seen. The Slow species, like the Rapid, seems capable of forming complexes with purines.

In the preceding paper (Bray & Vänngård, 1969) the classification of molybdenum e.p.r.* signals from reduced xanthine oxidase was discussed and the Rapidly appearing group of signals, which are believed to be significant in the catalytic reaction, were described in detail. Rapid signals obtained on reducing with xanthine or purine differ from those produced by salicylaldehyde. In the latter case it was concluded that the spectrum arises from reduced enzyme alone and does not depend on interaction with the reducing agent. This paper is concerned mainly with the origin of Rapid signals obtained with the purine substrates and with the significance of the species giving rise to them in the catalytic reaction of the enzyme.

Another group of signals from the enzyme are the Slow signals (Bray & Vänngård, 1969; Bray, Knowles, Pick & Vänngård, 1968*b*). These develop only in times much longer than the turnover time and are not thought to have any relevance to the catalytic cycle. We now present further studies bearing on the origin of these signals.

* Abbreviation: e.p.r., electron paramagnetic resonance.

MATERIALS AND METHODS

Xanthine oxidase and buffers were as used by Bray & Vänngård (1969). Samples of the enzyme frozen after anaerobic treatment with substrates were prepared by manual mixing under argon of solutions from syringes in special e.p.r. tubes, or alternatively were prepared by the rapid-freezing method of Bray (1961). These techniques have been described previously (Palmer, Bray & Beinert, 1964; Bray, Knowles & Meriwether, 1967). The samples were examined at 9GHz only, with a Varian V-4502 e.p.r. spectrometer with the V-4532 dual-sample cavity and V-4557 variable-temperature accessories. The latter was modified to operate from liquid nitrogen instead of from gaseous nitrogen. This was supplied from a 25l. liquid-nitrogen container [type LD25; Union Carbide (U.K.) Ltd., London W.1] in which a heating coil was suspended. The flow gauge of the instrument was by-passed and nitrogen gas generated was fed directly from the container to the

Varian heat-exchanger, via a Dewar tube. Spectra were generally recorded at about −150°. The procedure in the warming-up experiments was to scan the spectrum repetitively, while progressively increasing the sample temperature, usually in steps of 10° or 5°. Each temperature setting was held until signal changes became slow. In some cases the warming-up process was interrupted by recooling to −150°, for more detailed examination of spectra. No attempt was made to calibrate the temperature controller in this work so that the absolute values of the temperature may be somewhat approximate.

Anaerobic gel filtration of xanthine oxidase reduced with sodium dithionite was performed on a column of Sephadex G-25 (1·0cm.×15cm.). The column (Whatman) had minimum dead spaces at the ends and was modified by replacing thin-walled Teflon outlet and inlet tubes with heavy-wall polythene and stainless-steel. The column was washed thoroughly before use with buffer through which purified argon was bubbled continuously. The sample was introduced from a syringe via a three-way tap and the column effluent was passed directly into one of the syringes of the rapid-freezing apparatus via its connecting hose. The syringe plunger was inserted when the colour of the reduced enzyme could be seen in the syringe. The completeness of removal of dithionite was checked by experiments in which the reducing agent was applied on its own to the column in the absence of enzyme. The plunger was in this case inserted when an appropriate volume of effluent (as determined from the enzyme experiment) had been collected. After reaction with oxygenated buffer in the rapid-freezing apparatus no e.p.r. signals were observed from these blanks, indicating satisfactory removal of dithionite (cf. Knowles, Gibson, Pick & Bray, 1969).

RESULTS

Effects of varying the substrate and its concentration. With both purine and xanthine in H_2O, e.p.r. spectra at 9GHz varied profoundly according to the substrate concentration employed. These effects are summarized in Fig. 1. It is to be noted that effects occur in opposite directions with the two substrates. Thus the spectrum at high xanthine concentrations resembles that at low purine concentrations. Detailed examination of these spectra and comparison with data in the preceding paper (Bray & Vänngård, 1969) lead to the conclusion that at high purine concentrations (Fig. 1*b*, upper trace) the spectrum must arise almost exclusively from the signal that was designated 'Complex formed, type 1'. This signal also appears to be the dominant one at the lower concentration of xanthine (Fig. 1*a*, lower trace). In contrast, with higher concentrations of xanthine or lower concentrations of purine, the signals are of the very characteristic form that, as Bray & Vänngård (1969) showed, arises from mixtures of the 'Complex detected, type 1' and 'Complex formed, type 2' signals, with the latter species predominating slightly.

Fig. 2 shows the Rapid signals obtained with several other substrates. Although they all are generally quite similar to the 'No complex formed' type of spectrum, as given by salicylaldehyde (Bray & Vänngård, 1969), there are nevertheless slight but significant differences among some of them. In a further experiment, it was found that addition of uric acid (10mol.prop.) did not modify the spectrum obtained on reduction of the enzyme with salicylaldehyde (10mol.prop., 1min. at pH 8·2). It remained as in Fig. 2(*b*).

'Rapid' treatment with dithionite followed by xanthine. Fig. 3 shows experiments in which the three-syringe rapid-freezing technique (Palmer, *et al.* 1964) was employed to treat xanthine

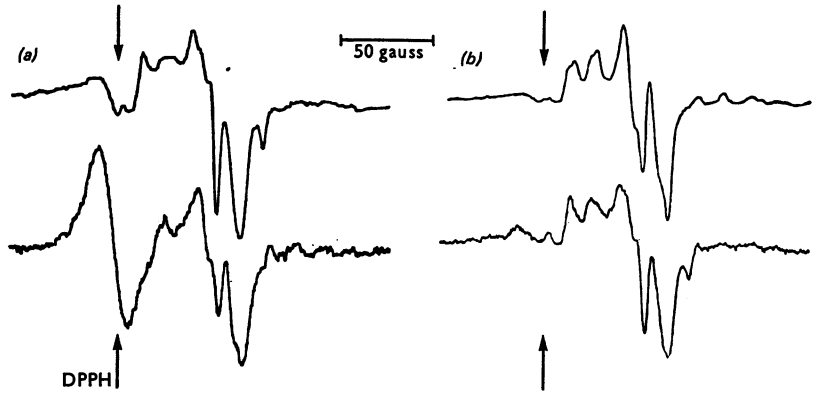

Fig. 1. Rapidly appearing molybdenum e.p.r. spectra at 9GHz obtained on reducing xanthine oxidase at pH 8·2 with two different concentrations of (*a*) xanthine and (*b*) purine. In (*a*) the enzyme (0·13mM) was reduced at about 20° for 135msec., by using the rapid-freezing technique, with 12mol.prop. (upper trace) and 1·5mol.prop. (lower trace) of xanthine. In (*b*) xanthine oxidase (0·36mM) was reduced for about 1min. at 20° with 20mol.prop. (upper trace) and 2·0mol.prop. (lower trace) of purine. Spectra were recorded at about −150°. The arrow here and in other Figures indicates the resonance of the diphenylpicrylhydrazyl (DPPH) standard.

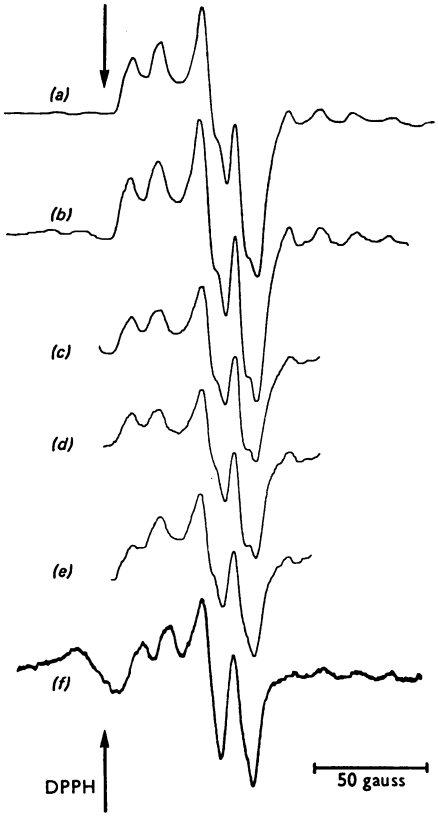

Fig. 2. Rapidly appearing molybdenum e.p.r. spectra at 9GHz obtained on reducing xanthine oxidase with various substrates at pH 8·2. Substrates used were (*a*) dithionite; (*b*) salicylaldehyde; (*c*) formaldehyde; (*d*) *N*-methylnicotinamide; (*e*) NADH; (*f*) 1-methylxanthine. Spectra were recorded at about −140° to −150°. Conditions of reduction were as follows: (*a*), (*b*) and (*f*): 10–30 mol.prop. of substrate; (*c*), (*d*) and (*e*): 200–600 mol.prop.; manual mixing with about 1 min. reaction time was employed in (*a*)–(*e*), at 0° in (*a*) and at 20° in (*b*)–(*e*); in (*f*) rapid freezing was used with a reaction time of about 200 msec. at 20°.

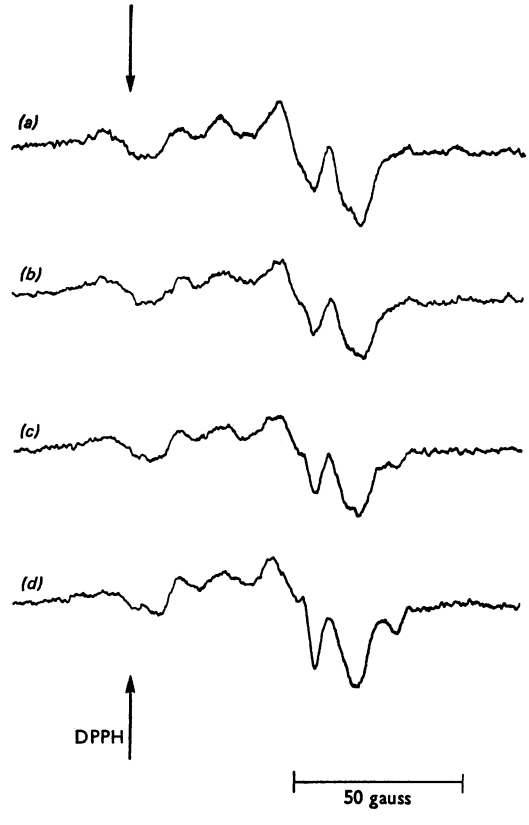

Fig. 3. E.p.r. spectra at 9GHz obtained with the three-syringe rapid-freezing method, with dithionite reduction followed by reaction with increasing concentrations of xanthine. Xanthine oxidase (1 vol., 0·38 mM) was treated at pH 8·2 and 25° with sodium dithionite (1 vol., approx. 200 mol.prop.) for 2·0 sec., then with xanthine (1 vol.) for 80 msec. The following quantities of xanthine were employed: (*a*) none; (*b*) 0·5 mol.prop.; (*c*) 1·0 mol.prop.; (*d*) 8·9 mol.prop. Spectra were recorded at about −150° with the same instrument settings for all samples.

oxidase at pH 8·2 first with dithionite (200 mol. prop. for 2 sec.) and then with xanthine (0–8·9 mol. prop. for 80 msec.). In the absence of xanthine, a spectrum of the 'No complex detected' type (Bray & Vänngård, 1969) was observed. As the xanthine concentration was increased it gradually changed over until at the highest value it was very similar to that in Fig. 1(*a*) (upper trace) produced in the absence of dithionite by high concentrations of xanthine, i.e. the 'Complex formed, type 2' spectrum clearly predominates. The changes in spectrum took place without any large changes in signal amplitude.

Development and disappearance of Slow signals. When xanthine oxidase is reduced with dithionite at pH 8·2 the Rapid signal is produced initially and may be observed either with the rapid-freezing technique (Fig. 3*a*), or by using manual mixing with the shortest reaction time and working at 0° (Fig. 2*a*). At longer times the Rapid signal is replaced by the Slow signal described by Bray *et al.* (1968*b*), and this in its turn disappears on prolonged incubation (Gibson & Bray, 1968). We have now studied the effect of dithionite concentration on the time-course of development and disappearance of the Slow signal (Fig. 4). Increasing the concentration of reducing agent resulted in decreased signal intensities and in the maximum intensity being

reached at an earlier time. As is shown by the theoretical curves through the experimental points, the transient nature of the Slow signal may be understood in terms of a first-order appearance process followed by its first-order disappearance. The results indicate that the effect of increasing the dithionite concentration tenfold is to increase the signal disappearance rate tenfold, without affecting its appearance rate. (Although our results may not be adequate to exclude some slight dependence of the appearance rate on dithionite concentration, any such dependence must clearly be much less than that of the disappearance rate, as may be seen from comparison of the experimental points in Fig. 4 with the light curve.) We conclude, since the formation of a Michaelis-type complex with dithionite seems unlikely, that the rate-limiting step in the appearance of the Slow signal cannot be a reductive reaction. In contrast, its disappearance at long times in the presence of excess of dithionite must be due to further reduction of the enzyme.

Bray *et al.* (1968*b*) found that dithionite was not the only reducing agent capable of giving rise to the Slow signal. They found that with purine or salicylaldehyde the Slow signal gradually took the place of the Rapid ones on prolonged incubation. We have attempted to extend this observation to xanthine also, but without success. Thus the Rapid signals obtained on treatment of the enzyme with xanthine (40mol.prop.) for 1min. at pH8·2 showed no signs of changing to the Slow signal after incubation for 90min. at 25°. The Slow signal was, however, obtained with xanthine in a 'warming-up' experiment (see below).

Reoxidation of the enzyme with molybdenum in the Slow signal-giving form was also studied. During experiments related to O_2^- production by xanthine oxidase (cf. Knowles *et al.* 1969) enzyme was reduced with dithionite at pH7·0 and excess of reducing agent was removed by anaerobic gel filtration on Sephadex G-25. On reoxidation of the effluent in the rapid-freezing apparatus with

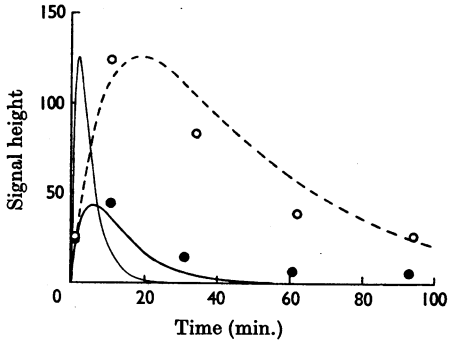

Fig. 4. Time-course of development of the Slow e.p.r. signal at two concentrations of dithionite. The enzyme (0·4mM) was treated at pH8·2 with dithionite with either about 10mol.prop. (○) or about 100mol.prop. (●). Samples were frozen after 1min. at about 25° and their e.p.r. spectra were recorded. They were then thawed and incubated for further periods before being refrozen and having their spectra re-run. Signal amplitude of the Slow signal (which was the only molybdenum signal observed) is plotted against total incubation time. The curves are theoretical ones calculated for signal appearance being by a first-order process, which is then followed by a first-order disappearance process (Frost & Pearson, 1961). The following values for the velocity constants were assumed: dashed curve: k_a 0·1, k_d 0·025; heavy curve: k_a 0·1, k_d 0·25; light curve: k_a 1; k_d 0·25. (k_a is appearance velocity constant and k_d the disappearance velocity constant, both in min.$^{-1}$).

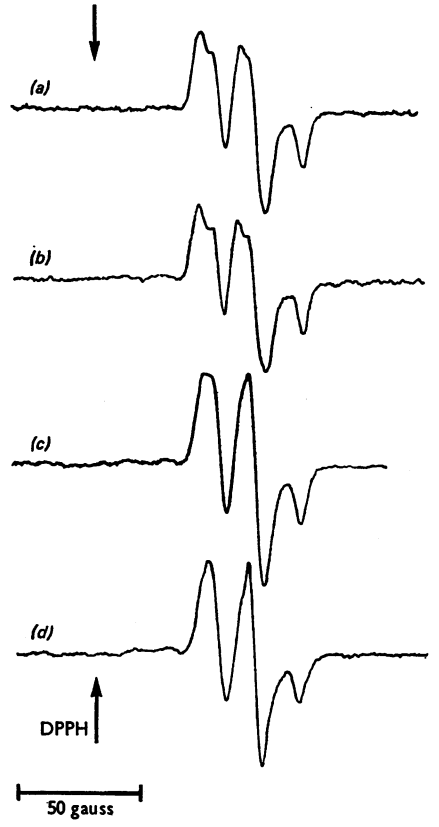

Fig. 5. Variations in the Slowly appearing molybdenum e.p.r. signal, obtained by reducing xanthine oxidase with dithionite, produced on subsequent addition of substrates. The enzyme (0·23mM final concn.) was treated at pH8·2 for 1min. with sodium dithionite (approx. 20mol.prop.), then with substrates (5mol.prop.) for 30min. at 25°, before freezing and recording the 9GHz spectra at about −150°. The substrates used were: (*a*) none; (*b*) salicylaldehyde; (*c*) purine; (*d*) xanthine.

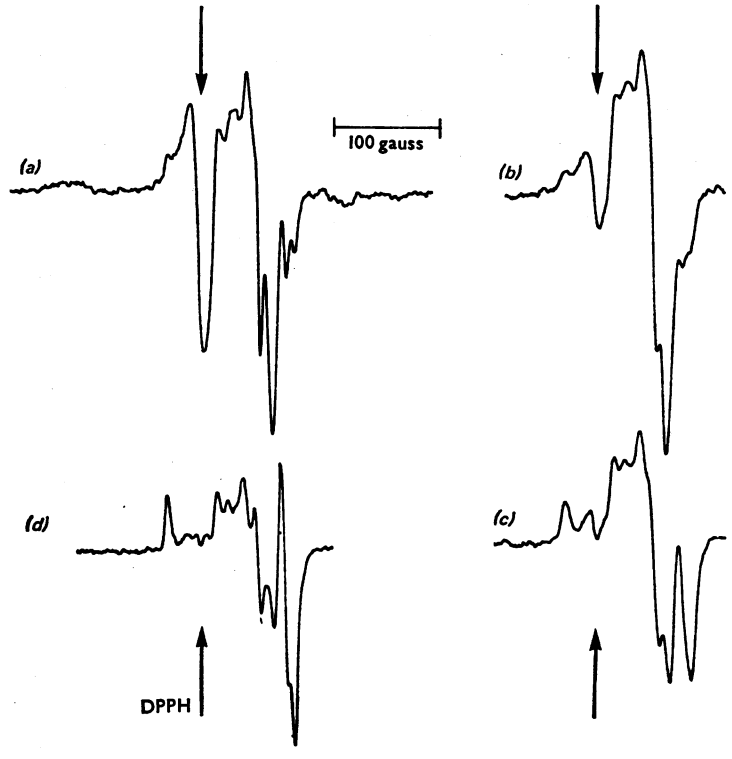

Fig. 6. Effect of warming a frozen sample of xanthine oxidase prepared by the rapid-freezing method. The enzyme (0·19 mM final concn.) was treated with xanthine (2 mM final concn.) in the presence of oxygen (0·76 mM final concn.) for 175 msec. at pH 10·0 and 21°, before freezing. (*a*) 9 GHz spectrum initially obtained at about −150°; (*b*) spectrum recorded at about −65° after gradual warming to this temperature; (*c*) spectrum recorded at about −50° after gradual warming to this temperature; (*d*) spectrum recorded at about −150° after gradual warming to about −40°. The modulation setting for (*b*) and (*c*) was twice that for (*a*) and (*d*), resulting in distinct over-modulation of the signals in the former case.

oxygenated glycine buffer (1·0 M) at pH 10·6 it was noticed that the Slow signal persisted for up to at least 1 sec. This slow reoxidation of molybdenum in the Slowly appearing form (which has been confirmed independently by H. Beinert & W. H. Orme-Johnson, personal communication) contrasts sharply with its rapid reoxidation in its Rapidly appearing form, as originally observed by Bray, Palmer & Beinert (1964).

'Slow' treatment with dithionite followed by substrates. As stated above, when the enzyme was incubated with dithionite at pH 8·2, under suitable conditions, the Slow signal developed. If substrates were subsequently added to the reaction mixture, before freezing, there were in some cases changes in the spectra. As shown in Fig. 5, salicylaldehyde had no effect whereas purine and xanthine both produced characteristic, though minor, changes. [In the experiment in Fig. 5 a relatively short incubation with dithionite followed by a long incubation in the presence of the substrate was used. Other experiments with xanthine showed that these incubation times could be reversed without changing the form of the signal from that in Fig. 5(*d*).] In another experiment a sample of enzyme in D_2O was treated with dithionite (20 mol.prop.) at pD 8·7 for 30 min., then xanthine (10 mol.prop.) was added shortly before freezing. The 35 GHz spectrum was obtained by Dr T. Vänngård and found to be a superposition of two signals. One was the normal Slow signal, as obtained with dithionite alone, whereas the other was closely related to the Rapid signals described by Bray & Vänngård (1969) but was not quite identical with any of them.

'Warming-up' experiments. As noted by Bray (1964) samples of reduced xanthine oxidase prepared by the rapid-freezing technique are stable at −196°. Indeed in the current work samples have been kept for periods of weeks under liquid nitrogen without any obvious changes in e.p.r. spectra taking

place. It was found, however, that on raising the temperature of samples changes in spectra began to occur and that, with careful and progressive temperature increases, a whole series of reactions could be followed in the frozen state. As found by Bray & Knowles (1968) the first change to take place on warming up samples, frozen during anaerobic oxidation of xanthine at pH 10, was the disappearance of the O_2^- signal (Knowles *et al.* 1969). At slightly higher temperatures it was found that the Rapid signals ('Complex formed, type 1 and type 2') decreased somewhat, while simultaneously the Very Rapid signals increased markedly. These effects are illustrated in Fig. 6.

In another similar experiment with a low xanthine concentration (1·0 mol.prop., 10 msec. reaction time at 10° and pH 8·2) changes in the spectrum also took place on warming up, and after warming to about $-35°$ the spectrum had the characteristic appearance of the Slow signal.

DISCUSSION

Complex-formation in the Rapid species. All the results seem most readily interpreted in terms of complex-formation between the enzyme and substrate molecules. This would lead to the small changes in the environment of the molybdenum atoms that are responsible for the differences in e.p.r. parameters, which in turn lead to dithionite and salicylaldehyde on the one hand giving distinctly different signals from xanthine and purine on the other, as noted by Bray & Vänngård (1969). It seems most likely that unchanged substrate molecules combine as such with the reduced enzyme and the evidence would point to affinities being in the following order: xanthine > purine ≫ salicylaldehyde. This would be in keeping with the fact that the K_m values for purine substrates are much lower than those for aldehydes (Booth, 1938).

The reality of complex-formation between xanthine and the dithionite-reduced enzyme is clearly shown by the three-syringe experiments (Fig. 3), in which the enzyme was first reduced, then allowed to react with the substrate. Bray & Vänngård (1969) noted at 35 GHz variations from one sample to another in the relative intensities of the two signals into which they analysed the Rapid spectra obtained with xanthine. Since neither of the signals they observed was identical with the 'No complex detected' signals, this suggests that this substrate forms two different complexes. The importance of xanthine concentration on the type of signal obtained is clearly shown in Fig. 1(*a*), and the results in Fig. 3 might be consistent with the 'Complex formed, type 1' signal arising from a 1:1 complex of xanthine and reduced enzyme and the 'Complex formed, type 2' signal from a 2:1 complex. However, systematic measurements on the stoicheiometry of complex-formation have not been made.

Bray & Vänngård (1969) found that the 'Complex formed, type 1' spectrum was obtained under their conditions both with xanthine and with purine. In the latter case it was seen as the dominant species, whereas with xanthine it was observed along with the 'Complex formed, type 2' species. The initially surprising observation (Fig. 1) that purine concentration effects are in the opposite direction from those with xanthine is understandable if it is assumed that purine forms a weaker complex with reduced enzyme than does xanthine. Anaerobic reduction with low concentrations of purine would be expected to lead to largely complete oxidation of this substrate to hypoxanthine, xanthine and uric acid, and under appropriate conditions the xanthine formed would form a complex with the reduced enzyme to give the characteristic observed spectrum in which the 'Complex formed, type 2' features can clearly be discerned. With larger concentrations of purine the unchanged substrate would be able to compete successfully for binding to the reduced enzyme, so giving the 'Complex formed, type 1' spectrum as the dominant species. Under comparable conditions Bray & Vänngård (1969) observed that with relatively high purine concentrations a minor component was also present in the 35 GHz spectrum. This would then be due to the xanthine present giving a little of the 'Complex formed, type 2' species (or possibly to a hypoxanthine complex).

The reasons for the small differences in the Rapid signals obtained at 9 GHz with certain substrates other than xanthine and purine have not been investigated in detail. It is noteworthy, however, that dithionite, which is presumably the least likely to form a complex with the enzyme, is extremely similar in its spectrum to salicylaldehyde, as indeed was noted for the more revealing 35 GHz spectra by Bray & Vänngård (1969). It is tempting to speculate that some of the other spectra in Fig. 2 (e.g. Figs. 2*e* and 2*f*) may be made up from the 'No complex detected, type A and type B' spectrum, as obtained with salicylaldehyde (Bray & Vänngård, 1969), with various additional amounts of the 'Complex formed, type 1' spectrum. However, this possibility has not been investigated in detail, and it is also possible that each complex of the enzyme with the various substrate molecules has its own characteristic e.p.r. spectrum. The conclusion (cf. Bray & Vänngård, 1969) that an apparently identical 'Complex formed, type 1' spectrum can arise when either xanthine or purine molecules are bound to the enzyme perhaps makes this latter possibility unlikely. Finally, it seems that uric acid, the ultimate reaction product with either xanthine or purine as substrate, does not form a

complex with the reduced enzyme detectable by e.p.r., since it did not affect the spectrum obtained with salicylaldehyde.

The source of the protons in the various complexes is discussed by Bray & Vänngård (1969).

Nature of the Slow species. V. Massey [results presented at the Third International Conference on Magnetic Resonance in Biological Systems, Warrenton, Va., 1968; see also Massey, Brumby, Komai & Palmer (1969)] has suggested that the Slow species may be due to Mo^{III} in the reduced enzyme, while accepting earlier assumptions that the Rapid and Very Rapid signals are due to Mo^{V}. We reject this suggestion in the light of the present results. The Slow signal disappears much more slowly than the Rapid on reoxidation of the reduced enzyme, which would probably not be consistent with Mo^{III}. [To the extent that different molybdenum species from the reduced enzyme differ greatly in autoxidizability, the behaviour of xanthine oxidase seems analogous to that of aldehyde oxidase (Rajagopalan, Handler, Palmer & Beinert, 1968).] Further, and more important, our results indicate that the rate-limiting step in the production of the Slow signal with dithionite is non-reductive in nature. This seems inconsistent with Mo^{III}, but is consistent with the suggestion of Bray *et al.* (1968*b*) that this signal is, like the other signals, due to Mo^{V} but that a change of ligand is involved in its development. Since the Slow signal develops faster at lower pH values, these workers suggested that hydrolysis of a molybdenum–sulphur bond might be involved. The disappearance of the Slow signal on long reduction with high dithionite concentrations is presumably due to reduction of Mo^{V} to Mo^{IV}.

Failure to develop the Slow signal when xanthine is the reducing agent, except at very low concentrations in a warming-up experiment, suggests that complex-formation in the initially formed Rapid species hinders changeover to the Slow species, and would be consistent with the conclusion above that xanthine forms tighter complexes than do other substrates.

Complex-formation in the Slow species. The small changes in the Slow signals that take place after these have been allowed to develop in the presence of dithionite suggest that complexes with xanthine and purine may be formed, analogous to those in the Rapid reduced forms of the enzyme.

Significance of the complexes in the enzymic reaction. The Slow signal-giving species and its complexes are not, of course, thought to play any part in the catalytic cycle, and the importance of the Rapid signal-giving species generally was summarized by Bray & Vänngård (1969). The significance of the complexes with substrate molecules in the Rapid species is not entirely certain. It is, of course, possible that they are related to inhibition of the enzymic reaction, which is well known at high substrate concentrations. However, it can also be postulated that they represent substrate molecules, bound to the reduced enzyme at the active site, waiting for their turn to react when the enzyme has been reoxidized via some other site. The results of the warming-up experiments with xanthine seem consistent with this view. When the enzyme turns over in the frozen state, diffusion of substrate molecules to and from the active site might be expected to become a limiting process. Under these conditions, relative to the reaction in solution, intramolecular reactions would be favoured over intermolecular ones. Thus the fact that when the enzyme became reoxidized it already had a new substrate molecule in the active site, ready to react, would be an advantage in the frozen state and would tend to favour the initial step of the reaction sequence, relative to the situation in free solution where this molecule would have a chance of dissociating rather than reacting. Bray *et al.* (1964) showed that kinetically, with xanthine at least, the Very Rapid signal is a precursor of the Rapid. Its development is the first observable step in the catalytic cycle. [Bray & Knowles (1968) and Bray, Knowles & Pick (1968*a*) suggested further that appearance of the Very Rapid signal may represent the initial one-electron step of the overall two-electron and one-proton substrate oxidation process. However, the exact nature of the Very Rapid species does not affect the present discussion.] The general situation envisaged is summarized in the following scheme:

$$\text{Enzyme}+\text{substrate} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{Michaelis complex} \xrightarrow{k_{+2}} \text{Very Rapid} \xrightarrow{k_{+3}} \text{Rapid} \rightarrow \ldots\ldots$$

Both in solution and in the frozen state, k_{+2} would be fast with k_{+3} rate-limiting. In the frozen state the local concentration of substrate molecules would effectively be increased as a result of the complex-formation, as on dissociation they would be unable to diffuse away. Hence formation of the Michaelis complex would be favoured, leading to the observed predominance of the Very Rapid signal over the Rapid signal (Fig. 6). Studies with an analogue computer have indicated that the scheme is consistent with all available data, and we conclude that our results are in keeping with a catalytic role for complexes of the Rapid species.

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