pH-Jump Studies at Subzero Temperatures on an Intermediate in the Reaction of Xanthine Oxidase with Xanthine

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Xanthine oxidase is stable and active in aqueous dimethyl sulphoxide solutions of up to at least 57% (w/w). Simple techniques are described for mixing the enzyme in this solvent at -82°C , with its substrate, xanthine. When working at high pH values under such conditions, no reaction occurred, as judged by the absence of e.p.r. signals. On warming to -60°C , for 10 min, however, the Very Rapid molybdenum(V) e.p.r. signal was obtained. This signal did not change on decreasing the pH, while maintaining the sample in liquid solution at -82°C . In contrast, a similar pH decrease, also at -82°C , for the enzyme nitrate reductase, caused its molybdenum(V) e.p.r. signal to change from the high-pH to the low-pH form. These findings are not compatible with the conclusions of Edmondson, Ballou, Van Heuvelen, Palmer & Massey [*J. Biol. Chem.* (1973) **248**, 6135–6144], that the Very Rapid signal is in prototropic equilibrium with the Rapid signal, and should be important in understanding the mechanism of action of the enzyme. They emphasize the unique nature of the intermediate represented by the Very Rapid e.p.r. signal. The possible value of the pK for loss of an exchangeable proton from the Rapid signal is discussed.

Studies on enzymes in aqueous/organic solvents at subzero temperatures are now well established in investigations of their reaction mechanisms (Fink, 1976; Douzou, 1977*a,b*). A particular advantage is that intermediates that, at ordinary temperatures, have lifetimes in the millisecond range can be preserved for long periods at low temperatures, so enabling detailed studies of their properties to be carried out. An early example involved stabilization of the peroxidase Complex-I intermediate in aqueous dimethylformamide at -65°C (Douzou *et al.*, 1970; Douzou & Leterrier, 1970). The application of such techniques to xanthine oxidase has particular attractions, since it is known that several intermediates in the catalytic reaction may be detected by observation, at liquid- N_2 temperatures, of e.p.r. signals from molybdenum(V) (Bray, 1975). Further work on these intermediates should provide useful insights into the mechanism of action of the enzyme.

The e.p.r. signal from xanthine oxidase known as Very Rapid is likely to be of particular importance in understanding the enzymic mechanism. It was first observed by Palmer *et al.* (1964), by the rapid-freezing method of Bray (1961), and was further studied by Bray & Meriwether (1966) and others [see Bray (1975) for references]. This signal predominates only when xanthine is the reducing substrate at high pH values. Furthermore, it is a transient under all conditions, appearing in a time quite short compared with, and disappearing in a time comparable with, the turnover time, this being about 80 ms under the

conditions of assay of Hart *et al.* (1970). Olson *et al.* (1974*b*) proposed that the Very Rapid signal represents an intermediate in which the xanthine molecule has lost the hydrogen atom from its 8-position and become bound covalently to the enzyme via its 8-carbon atom.

When xanthine oxidase is treated with xanthine at low pH values, the signal known as Rapid is obtained, whereas at high pH values the Very Rapid signal appears first, being replaced subsequently by the Rapid. Edmondson *et al.* (1973) carried out pH-jump rapid-freezing experiments. In these, the pH of xanthine/xanthine oxidase mixtures, prepared at different pH values, was changed by using a three-syringe flow-mixing system, and the samples were then frozen a few milliseconds later. These workers claimed that the two signals were related to one another by a prototropic equilibrium and this was widely accepted (Bray, 1975; Stiefel *et al.*, 1977). Such an equilibrium is now well established for two other molybdenum-containing enzymes, sulphite oxidase (Cohen *et al.*, 1971) and nitrate reductase (Vincent & Bray, 1978). For both these enzymes, a low-pH e.p.r. signal in which molybdenum interacts with a single exchangeable proton is related, by a simple pK of about 8.2, to a high-pH signal without proton coupling. For each enzyme, though, and in contrast with the situation in xanthine oxidase, both high-pH and low-pH signals are stable at 20°C for extended periods. Stability is even maintained, in the case of nitrate reductase, in the presence of air.

For xanthine oxidase, the proposal of Edmondson *et al.* (1973) raises further problems. Understanding of the Rapid signal has increased considerably (Bray *et al.*, 1978). This signal can originate from enzyme species with or without xanthine molecules bound. Furthermore, when xanthine is involved, it may be bound in different orientations within the active site of the enzyme. Involvement of the substrate molecule in certainly one, though not definitely in both, of the partners of the proposed pH-dependent equilibrium, thus makes the situation complicated, and we thought it important to reinvestigate.

It seemed that studies in liquid aqueous/organic solvents at subzero temperatures might be useful for this purpose. We sought to generate the Very Rapid signal on its own at high pH, then, by suitable lowering of the temperature, to prevent further reaction to other e.p.r.-detectable species. pH decrease, at this lower temperature, could then be carried out and its effects studied under conditions where interference from further reaction with substrate would be excluded.

Materials and Methods

Materials

Milk xanthine oxidase was prepared by the salicylate-denaturation method of Hart *et al.* (1970) and assayed at pH 8.2 as described by these workers; concentrations of functional active centres were calculated from the activity measurements (see Bray, 1975). Nitrate reductase, prepared from *Escherichia coli* K12 by the method of Vincent & Bray (1978), was kindly provided by Mr. S. P. Vincent. Dimethyl sulphoxide (AnalaR grade), obtained from BDH, Poole, Dorset, U.K., and stored under N₂, was used without further purification for all the work described. In additional experiments, some samples of the solvent caused serious inactivation of xanthine oxidase, that could, however, be prevented by redistillation under vacuum from calcium hydride. Buffers were Caps [3-(cyclohexylamino)propane-sulphonic acid], Ches [2-(cyclohexylamino)ethane-sulphonic acid] and Tris, all from Sigma, Kingston upon Thames, Surrey, U.K., and maleic acid (from Fisons, Loughborough, Leics., U.K.). EDTA was added to all buffers at a concentration (before addition of dimethyl sulphoxide) of 1 mM. Adjustments to the required pH values were with NaOH or HCl.

E.p.r. spectroscopy

Spectra were obtained on a Varian E9 spectrometer. Computer manipulation of spectra, if required, was as described by Bray *et al.* (1978). All spectra were recorded at about 9 GHz, at a temperature of -150°C , with 20 mW microwave power and 0.32 mT modulation.

Procedure for mixing xanthine oxidase, at low temperatures, with xanthine or with low-pH buffer

Simple procedures, which were to some extent novel, were used. No special equipment was required. For mixing the dimethyl sulphoxide (m.p. 18°C) with the enzyme, the latter was first cooled to 0°C . Addition of the solvent in five to ten portions as an aqueous 91% (w/w) solution (m.p. -7°C) was preferred, with careful mixing of the solution after each addition. After the first addition, the sample was cooled to -10°C and it was then maintained in a bath at this temperature.

For e.p.r. work, solvent addition was from a syringe with a long needle, into enzyme contained in a quartz e.p.r. sample tube of 4 mm internal diameter. When addition was complete, the solution was frozen in liquid N₂. Without warming the tube, the substrate solution, in aqueous dimethyl sulphoxide of the same concentration as that in the enzyme, was then added by means of a syringe, so that it froze as a separate layer immediately on top of the enzyme sample; some care was necessary to achieve this. The tube was raised a little above the surface of the liquid N₂ to make the addition, but too much warming had to be avoided, so as to prevent the sample from thawing. Also, to achieve rapid mixing subsequently, it was necessary to see that the two frozen layers were immediately in contact with one another, with no intervening air space.

When it was desired to mix the frozen layers, the tube was placed in a bath at a suitable temperature, such as -82°C , and shaken. At this temperature, the solution liquified and it was found possible to mix the two layers, by stirring with a rod that had been precooled in liquid N₂, within 30–60 s of placing the tube in the bath. Because of the high viscosity, air bubbles carried into the solution during too-vigorous stirring were slow to clear. The most satisfactory stirring was achieved by using a length of stainless-steel tubing, 1 mm outer diameter, closed at one end by flattening over a length of about 2 cm, with the flattened portion twisted to give a helix. Mixing was achieved by working this tube up and down in the sample in the e.p.r. tube.

After the layers had been mixed, the temperature of the sample could be raised to initiate or to speed up reaction between the enzyme and the substrate, by placing the tube in another bath at an appropriate temperature. Subsequently, the sample could be frozen again in liquid N₂, to stop the reaction for measurement of e.p.r. spectra.

A similar addition procedure, using two frozen layers, was used when it was desired to lower the pH at -82°C . In this case, a suitable low-pH buffer containing the same concentration of dimethyl sulphoxide was frozen on top of the enzyme/substrate mixture. The desired pH jump was achieved on mixing the layers at -82°C , as described above.

The low-temperature baths used for this work were simply Dewar flasks containing ethanol, which had been cooled to the required temperature by cautious addition of liquid N_2 . Warming rates, for $-80^\circ C$, were only about 0.10 – $0.15^\circ C/min$.

Control and measurement of pH at low temperatures in solutions containing dimethyl sulphoxide

We shall use the symbol $pH^* (T^\circ C)$ to denote the apparent pH, as defined by Hui Bon Hoa & Douzou (1973), of a sample containing dimethyl sulphoxide at a temperature $T^\circ C$. These workers established the linearity, for a number of buffer systems in aqueous/organic solvents, of plots of pH^* against T (see also Maurel *et al.*, 1975). It is therefore possible to estimate the value of pH^* at a low temperature, for a particular mixture, from measurements made on it with a glass electrode at temperatures above $0^\circ C$. The linear plot of pH^* against T so obtained is simply extrapolated to the desired low temperature (Fink, 1976).

We used mixtures of Ches and Caps buffers to control pH^* , at high pH^* , and these buffers with addition of excess maleic acid buffer, at low pH^* . In addition, low concentrations of EDTA were also present. Also, xanthine, when present, as well as the enzymes, would contribute somewhat to the buffering capacity of the systems. To estimate pH^* at low temperatures in a particular experiment we obtained a plot of pH^* against T for a comparable system, with all components present, except the enzyme, over the range 0 to $+50^\circ C$, and then carried out the extrapolation.

In the actual experiments with xanthine oxidase, we could not predict precisely in advance the value of $pH^* (20^\circ C)$ that would be obtained, either after addition of xanthine (which had to be in alkaline solution because of solubility problems), or after the subsequent addition of low- pH^* buffer. Values of $pH^* (20^\circ C)$ were therefore measured directly with a glass electrode at the end of the experiments, either on the main samples or on replicates. Because of the decrease in pH^* with increasing temperature, $pH^* (20^\circ C)$ for samples after addition of maleic acid were as low as 3.8 . This value is below the stability range for the enzyme. However, at $20^\circ C$, precipitation occurred only slowly and hence did not interfere with measurement of $pH^* (20^\circ C)$.

In all the experiments to be described, all components of the systems remained in solution throughout all the low-temperature work.

Results and Discussion

Effects of aqueous dimethyl sulphoxide on xanthine oxidase

In selecting an organic solvent for low-temperature work on xanthine oxidase we were restricted by

reactivity of the enzyme towards several alcohols. It has long been known that methanol can react with the enzyme (see Bray, 1975), and ethylene glycol has also been shown to have effects on it (Lowe *et al.*, 1976). Recently it has been established that the latter solvent is a very slow substrate for the enzyme (Tanner & Bray, 1978). Preliminary tests also showed that dimethylformamide caused enzyme inactivation. However, dimethyl sulphoxide proved a suitable solvent. Thus at $pH^* (20^\circ C)$ about 8.7 , when dilute Tris buffer was used, an enzyme solution (8 mg/ml) remained clear when dimethyl sulphoxide was added to a concentration of 52% (w/w). [See the Materials and Methods section for definition of the symbol $pH^* (T^\circ C)$.] Though activity, measured at $pH 8.2$ after suitable dilution, decreased by 13% on addition of the solvent, this may have been due to the presence of small amounts of impurities, and further decreases in activity were very small, amounting to only 5% loss after 2 h at $19^\circ C$. Holding the sample at $-70^\circ C$ for 2 h did not result in any loss of activity, despite the high value of $pH^* (-70^\circ C)$ of 12.5 .

We then carried out assays of xanthine oxidase activity in the presence of dimethyl sulphoxide. Results are presented in Figs. 1 and 2. The form of the curve in Fig. 1 shows clearly that effects of the

Fig. 1. Effect of varying concentrations of dimethyl sulphoxide on the activity of xanthine oxidase

Percentage activity, measured as initial $\Delta A_{295}/min$ at $23.5^\circ C$, is plotted against the concentration of dimethyl sulphoxide. The medium contained 0.1 mM xanthine in Ches buffer (40 – 100 mM), $pH^* (20^\circ C)$ 10.3 ± 0.1 . Reaction was initiated by addition of the enzyme. The concentration of dimethyl sulphoxide used in subsequent work was 7.7 M (57% , w/w).

Fig. 2. Double-reciprocal plots of xanthine oxidase activity at various xanthine concentrations, with and without dimethyl sulphoxide

Reciprocal activity in arbitrary units is plotted against the reciprocal of xanthine concentration (mm). Dimethyl sulphoxide, when present, was at a concentration of 57% (w/w). Activity was measured as initial $\Delta A_{295}/\text{min}$ at 23.5°C, in a medium containing 20 mM-Ches buffer, pH or pH^* (20°C) 10.1. ▲, Samples with dimethyl sulphoxide; ●, samples without dimethyl sulphoxide.

solvent on enzyme activity are complex. Although activity decreased to low values at high dimethyl sulphoxide concentrations, these effects were reversible, as was shown above by the stability tests. The double-reciprocal plot (Fig. 2), obtained when the substrate concentration was varied in 57% (w/w) dimethyl sulphoxide, indicates behaviour approximating to that of a non-competitive inhibitor. According to Douzou (1974), such behaviour of solvents is not unexpected. The apparent K_m of about 0.2 mM under these conditions was not changed significantly by the solvent, and is in fair agreement with the literature (Fridovich, 1964; Olson *et al.*, 1974a).

We selected 57% (w/w) dimethyl sulphoxide (i.e. 7.7 M, obtained by mixing 1 vol. of aqueous solution with 1.2 vol. of dimethyl sulphoxide) for our e.p.r. experiments. Solutions in this medium may be supercooled to -85°C or below (Fink, 1976).

E.p.r. experiments on xanthine oxidase

As noted in the introduction, in reactions of xanthine oxidase with excess xanthine at pH 10 followed by the rapid-freezing procedure, appearance of the Very Rapid e.p.r. signal is followed after a brief lag by appearance of the Rapid signal. This lag is of very short duration when the reaction is carried out at 25°C (Edmondson *et al.*, 1973), but

becomes marked at 1°C (Palmer *et al.*, 1964). We sought to generate a Very Rapid signal on its own, slowing down reaction with the substrate by working in 57% (w/w) dimethyl sulphoxide at low temperatures. The procedures used are set out in the Materials and Methods section.

We succeeded in mixing xanthine oxidase with xanthine, in 57% (w/w) dimethyl sulphoxide at -82°C and at pH^* (-82°C) about 13.1, without any detectable reaction having occurred, as judged by absence of e.p.r. signals on recording the spectrum at -150°C. [The value of pH^* (20°C) in this experiment was 9.8.] When the sample was warmed to -60°C [pH^* (-60°C) 12.4] for 10 min, the virtually

Fig. 3. E.p.r. spectra of xanthine oxidase reduced with xanthine in the presence of dimethyl sulphoxide, before and after pH jump

E.p.r. spectra were recorded at -150°C and show the Very Rapid signal. (a) shows the spectrum obtained for pH^* (20°C) 9.8, and (b) that of the same sample after pH jump to pH^* (20°C) 3.8. The experiment was carried out as described in the text. The e.p.r. gain setting in (b) was increased to compensate for the dilution of the sample. 0.225 ml of solution A was mixed, at -82°C, with 0.15 ml of solution B. After incubation for 10 min at -60°C the spectrum of (a) was recorded; 0.32 ml of solution C was then added at -82°C, and after 6 min at this temperature the spectrum of (b) was obtained. Solutions A, B and C all contained 57% (w/w) dimethyl sulphoxide. Other constituents were as follows: A contained xanthine oxidase (0.14 mM-functional active centres) in 35 mM-Caps buffer and 30 mM-Ches buffer, pH^* (20°C) about 10.0; B contained xanthine (7 mM) in dilute NaOH, pH^* (20°C) 10.1; C contained maleic acid (200 mM) adjusted to pH^* (20°C) 3.0 with NaOH. The arrow, here and in Fig. 4, corresponds to $g = 2.0037$.

pure Very Rapid signal shown in Fig. 3(a) appeared. Integrated intensity of the signal corresponded to 3.2% of the functional molybdenum concentration.

We then performed a pH-jump experiment on the sample of Fig. 3(a). We mixed maleic acid buffer with the sample in a bath at -82°C and maintained it at this temperature for 6 min after mixing. No significant change in the e.p.r. spectrum was observed (Fig. 3b), either in form or in intensity of the signal when allowance was made for the dilution. The acid added in this experiment decreased pH^* (20°C) to 3.8, corresponding to pH^* (-82°C) 4.6.

Experiments similar to the one described above were carried out a number of times, with the same result. The incubation period at -82°C , following the pH jump, could be extended to at least 30 min without any change in the signal occurring. For the initial development of the Very Rapid signal at -60°C , 10 min was found to be the optimum reaction time, if contamination with Rapid signal was to be avoided. In some experiments we observed intensities of the signal rather greater than the value of 3.2% reported above. However, in no case was it as intense as the Very Rapid signal obtained in the absence of dimethyl sulphoxide, under optimum conditions in rapid-freezing experiments. This was presumably due to inhibition by the organic solvent, though this in no way affects our conclusions.

In additional experiments, which will not be described in detail, we varied the temperature, the reaction time and the pH value extensively. We found that, after mixing the enzyme with xanthine at pH^* (-82°C) 13.1, the temperature could be raised to -72°C , and held there for as long as 1 h, without any observable reaction taking place. At -62°C , reaction for periods in excess of 10 min gave mixtures of Very Rapid signal with increasing proportions of Rapid signal. Signals from reduced iron-sulphur centres could also be observed at liquid-helium temperatures in all samples where molybdenum signals were seen. The general behaviour of the system on increasing the reaction time appeared similar to that which occurs at 20 – 25°C (Edmondson *et al.*, 1973; Pick, 1971). Observation of the Very Rapid signal without Rapid signal indicates (Olson *et al.*, 1974b) that enzyme molecules have accepted, at this stage of the reaction, no more than two electrons per active centre. This was confirmed by the observation of relatively weak iron-sulphur signals, only, under these conditions (e.g. Fe-S I plus Fe-S II signals, 8–15% developed, at the time of maximum Very Rapid signal).

Finally, with a similar system, we varied the pH^* value. When the reaction was allowed to proceed at lower pH^* values [e.g. pH^* (20°C) 6.0] at -62°C , then Rapid was the predominant molybdenum signal at all reaction times.

Although Edmondson *et al.* (1973) did so, we chose not to carry out upward pH-jump experiments, for the reasons indicated in the introduction. The Rapid signal, as generated by reduction with xanthine, is not a single chemical species (Bray & Vänngård, 1969; Bray *et al.*, 1978; Gutteridge *et al.*, 1978a), whereas the Very Rapid signal is. Thus no simple result from such an experiment could be expected.

E.p.r. experiments on nitrate reductase

In view of the apparent disagreement between our pH-jump results on xanthine oxidase and those of Edmondson *et al.* (1973), we thought it prudent to carry out control experiments on another molybdenum-containing enzyme. Nitrate reductase has a well-defined pK , detectable by e.p.r., of 8.27 (Vincent & Bray, 1978). We repeated, as exactly as possible, the experiments of Fig. 3, but replacing xanthine oxidase with nitrate reductase. The initial spectrum at pH^* (20°C) 9.5 is shown in Fig. 4(a). In order to reproduce accurately the pH change we added xanthine to the sample as well as maleic acid. The final spectrum after incubation for 6 min at -82°C , following pH jump at this temperature, is shown in Fig. 4(b), and differs markedly from that of Fig. 4(a). By comparing the two spectra with standard nitrate reductase spectra (Vincent & Bray, 1978; Williams-Smith *et al.*, 1977), it was possible to obtain estimates of apparent pH in the samples under the conditions of the e.p.r. Such comparisons made it clear that Figs. 4(a) and 4(b) corresponded to virtually pure high- and low-pH forms respectively of nitrate

Fig. 4. *E.p.r. spectra of nitrate reductase before and after pH jump*

The experiment was carried out as in Fig. 3, but using an aqueous solution of nitrate reductase (70 mg/ml; 320 units/mg) in place of the xanthine oxidase. (a) corresponds to pH^* (20°C) 9.5 and (b) to that after pH jump, at -82°C , to pH^* (20°C) 3.8.

reductase, indicating apparent pH values of 10 or more and of less than 6 in the two samples, in agreement with the calculated pH^* values.

In other experiments, we varied the time interval between pH jump and freezing the sample or re-incubated samples that had already been 'jumped'. In all cases, we found the spectral change to be complete at the shortest reaction time (2 min; this time includes that required for thawing and mixing the very viscous samples as well as that for freezing). No further e.p.r. spectral changes took place, within the next 30 min at least.

Conclusions

The techniques described in the present paper are quite simple, and should prove useful for further e.p.r. studies on the mechanisms of action, not only of xanthine oxidase, but of other metalloenzymes also. Our subzero pH-jump experiments provided no evidence whatever favouring pH-dependent equilibrium between the Rapid and Very Rapid e.p.r. signals from xanthine oxidase. The validity of our procedures was confirmed by the strikingly different results obtained on pH-jump of nitrate reductase. These were in agreement with expectations based on titrations at 20°C in the absence of dimethyl sulphoxide by Vincent & Bray (1978). The pK value for nitrate reductase under these conditions is 8.27. Olson *et al.* (1974b) interpreted the data of Edmondson *et al.* (1973) as indicating a pK of about 9 for an equilibrium between the Rapid and Very Rapid species of xanthine oxidase. Our results are not compatible with a pK value in this region, unless the highly improbable assumption is made that at -82°C equilibration of protons with xanthine oxidase is very slow indeed, in contrast with their rapid equilibration with nitrate reductase.

The finding prompts re-examination of the data of Edmondson *et al.* (1973). They worked with a reacting system in which a finite time (5–7 ms) elapsed between the change of pH and stopping the reaction by freezing. This time is quite sufficient either for an intermediate to decay, perhaps to an e.p.r.-undetectable species, or for additional xanthine molecules to react. In contrast, in our experiments, reaction between xanthine oxidase and xanthine was effectively stopped at the temperature of the pH jump, as is demonstrated by our failure to observe signals on initial mixing at this temperature. However, deprotonation could still take place at -82°C, as is shown by the nitrate reductase work. The downward pH jump of 3.6 units of Edmondson *et al.* (1973) seems to have brought about a change from maybe 90% Very Rapid signal to about 10% Very Rapid signal. This is not reconcilable with a simple pK .

That the Rapid and Very Rapid signals are not, after all, in prototropic equilibrium has important

bearings on the roles and structures of the signal-giving species in the enzymic reaction. Uniqueness of the Very Rapid species is re-emphasized. Further information on its structure should be provided by generating the signal by using xanthine labelled in the C-8 position with ^{13}C (Tanner *et al.*, 1978). The Rapid signal is detectable over at least the pH range from 6 to 10 (Bray *et al.*, 1964). Its amplitude was reported to be independent of pH in this range, after reaction with xanthine for 0.4 s. Though quantitative studies on intensity as a function of pH have not been reported for the 'uncomplexed' form of the Rapid signal, we suggest that the pK for loss of its exchangeable proton must be relatively high. Presumably, the hypothetical deprotonated form of the Rapid signal has not so far been detected, either because of the high value of its pK , or alternatively because in the deprotonated form internal oxidation-reduction reactions between molybdenum and other constituents of the enzyme do not favour molybdenum(V), so rendering the species undetectable by e.p.r. Estimation of the value of this pK should be possible from measurement of the rate of exchange for protons of the Rapid signal (Gutteridge *et al.*, 1978b).

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References

- Bray, R. C. (1961) *Biochem. J.* **81**, 189–195
- Bray, R. C. (1975) *Enzymes 3rd Ed.* **12**, 299–419
- Bray, R. C. & Meriwether, L. S. (1966) *Nature (London)* **212**, 467–469
- Bray, R. C. & Vänngård, R. (1969) *Biochem. J.* **114**, 725–734
- Bray, R. C., Palmer, G. & Beinert, H. (1964) *J. Biol. Chem.* **239**, 2667–2676
- Bray, R. C., Barber, M. J. & Lowe, D. J. (1978) *Biochem. J.* **171**, 653–658
- Cohen, H. J., Fridovich, I. & Rajagopalan, K. V. (1971) *J. Biol. Chem.* **246**, 374–382
- Douzou, P. (1974) *Methods Biochem. Anal.* **22**, 401–512
- Douzou, P. (1977a) *Cryobiochemistry: An Introduction*, Academic Press, New York
- Douzou, P. (1977b) *Adv. Enzymol. Relat. Areas Mol. Biol.* **45**, 157–272
- Douzou, P. & Leterrier, F. (1970) *Biochim. Biophys. Acta* **220**, 338–340
- Douzou, P., Sireix, R. & Travers, F. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **66**, 787–792
- Edmondson, D., Ballou, D., Van Heuvelen, A., Palmer, G. & Massey, V. (1973) *J. Biol. Chem.* **248**, 6135–6144
- Fink, A. L. (1976) *J. Theor. Biol.* **61**, 419–445
- Fridovich, I. (1964) *J. Biol. Chem.* **239**, 3519–3521
- Gutteridge, S., Tanner, S. J. & Bray, R. C. (1978a) *Biochem. J.* **175**, 869–878

- Gutteridge, S., Tanner, S. J. & Bray, R. C. (1978b) *Biochem. J.* **175**, 887–897
- Hart, L. I., McGartoll, M. A., Chapman, H. R. & Bray, R. C. (1970) *Biochem. J.* **116**, 851–864
- Hui Bon Hoa, G. & Douzou, P. (1973) *J. Biol. Chem.* **248**, 4649–4654
- Lowe, D. J., Barber, M. J., Pawlik, R. T. & Bray, R. C. (1976) *Biochem. J.* **155**, 81–85
- Maurel, P., Hui Bon Hoa, G. & Douzou, P. (1975) *J. Biol. Chem.* **250**, 1376–1382
- Olson, J. S., Ballou, D. P., Palmer, G. & Massey, V. (1974a) *J. Biol. Chem.* **249**, 4350–4362
- Olson, J. S., Ballou, D. P., Palmer, G. & Massey, V. (1974b) *J. Biol. Chem.* **249**, 4363–4382
- Palmer, G., Bray, R. C. & Beinert, H. (1964) *J. Biol. Chem.* **239**, 2657–2666
- Pick, F. M. (1971) Ph.D. Thesis, University of London
- Stiefel, E. I., Newton, W. E., Watt, G. D., Hadfield, K. L. & Bulen, W. A. (1977) *Adv. Chem. Ser.* **162**, 353–388
- Tanner, S. J. & Bray, R. C. (1978) *Biochem. J. Soc. Trans.* **6**, in the press
- Tanner, S. J., Bray, R. C. & Bergmann, F. (1978) *Biochem. Soc. Trans.* **6**, in the press
- Vincent, S. J. & Bray, R. C. (1978) *Biochem. J.* **171**, 639–647
- Williams-Smith, D. L., Bray, R. C., Barber, M. J., Tsopanakis, A. D. & Vincent, S. P. (1977) *Biochem. J.* **167**, 593–600