Formamide as a substrate of xanthine oxidase

Fraser F. MORPETH,* Graham N. GEORGE and Robert C. BRAY

School of Chemistry and Molecular Sciences, University of Sussex, Falmer, Brighton BN1 9QJ, U.K.

(Received 28 November 1983/Accepted 8 February 1984)

Formamide is a substrate of xanthine oxidase. At pH 8.2 and 1.14 mM-O₂, $V_{\max(\text{app.})}$ is 3.1 s^{-1} and $K_{\text{m}(\text{app.})}$ is 0.7 M. Mo(V) e.p.r. signals obtained by treating the enzyme with formamide were studied, and these provide new information about the ligation of molybdenum in the enzyme and about the enzymic mechanism. The substrate is the first compound that is not a nitrogen-containing heterocycle to give a Very Rapid signal. This supports the hypothesis that the Very Rapid signal, though it is not detectable with all substrates, represents an essential intermediate in turnover. Formamide also gives the Inhibited signal and is the first non-aldehyde substrate to do so. The Rapid type 1 signal obtained in the presence of formamide was examined in H₂O enriched with ²H or with ¹⁷O. The single oxygen atom detectable in the signal is shown to be strongly and anisotropically coupled. This indicates that this atom remains as an oxo ligand of molybdenum in this signal-giving species. Other structural features of this species are discussed.

The molybdenum-, iron-sulphur- and flavin-containing enzyme xanthine oxidase (Bray, 1975) is remarkable for its very wide substrate specificity. It is known to catalyse the hydroxylation of a large number of nitrogen-containing heterocyclic compounds. These include compounds of no less than twelve different heterocyclic ring systems, in some cases with a variety of substituents in different positions (Krenitsky *et al.*, 1972; Rosemeyer & Seela, 1983). In addition, the enzyme catalyses the oxidation of many aldehydes (Booth, 1938; Morpeth, 1983). These reducing substrates are transformed to products at the molybdenum centre of the enzyme, and e.p.r. spectroscopy of Mo(V) in xanthine oxidase has provided much information on the structure of this centre and on the mechanism of the enzymic reaction (see, e.g., Bray, 1980). The various Mo(V) e.p.r. signals that are observed on reducing the enzyme with substrates are to some extent characteristic on the substrate that is employed. Whereas the Rapid signal is seen with all substrates and corresponds to the normal reduced form of the enzyme, on the other hand the Inhibited and Very Rapid signals are less widely observed. The former signal corresponds to the product of an inhibitory side reaction occurring during substrate turnover, and the Very Rapid signal is believed to represent a transient enzyme-bound intermediate of turnover.

* Present address: Department of Food Technology, University of Reading, Reading, Berks. RG1 5AQ, U.K.

We describe kinetic and e.p.r. studies with a simple new substrate of the enzyme, formamide. A preliminary report of this work has appeared (Morpeth *et al.*, 1983).

Materials and methods

Materials

Xanthine oxidase was prepared and concentrations of the functional enzyme determined as described in previous publications (Hart *et al.*, 1970; Bray, 1975, 1982). All experiments were carried out in 50 mM-Bicine [NN-bis-(2-hydroxyethyl)glycine]/NaOH buffer, pH 8.2. Formamide was obtained from Sigma Chemical Co. (Poole, Dorset, U.K.) and was used without further purification.

Steady-state kinetics and optical measurements

O₂ consumption in steady-state kinetic experiments was followed by using an oxygen electrode (Yellow Springs Instrument Co. oxygen probe, from Anachem, Luton, Beds., U.K.). Assays were initiated by injecting xanthine oxidase, to a final concentration of 0.4–0.8 μM , into the formamide solution. Experiments with 2,6-dichlorophenol-indophenol as oxidizing substrate were performed aerobically, by measuring A_{600} . Measurements of bleaching of the visible absorption of xanthine oxidase in the presence of formamide were carried

out in an anaerobic spectrophotometer cell by using a Cary 210 spectrophotometer.

E.p.r. measurements

Samples of xanthine oxidase (usually about 0.3 mM) were placed in e.p.r. tubes, where appropriate under anaerobic conditions. The substrate was added and the samples were frozen after appropriate times. For the time-course experiments the tubes were rapidly frozen by plunging them into isopentane at about -100°C . Experiments in ^{17}O -enriched water were carried out as described by Bray & Gutteridge (1982), and those in $^2\text{H}_2\text{O}$ as described by Gutteridge *et al.* (1978b).

E.p.r. spectra were recorded on a Varian E9 spectrometer equipped with a dual-sample cavity and linked to a DEC PDP 11/10 computer and a visual-display system (Bray *et al.*, 1978). Recording conditions for e.p.r. spectra were generally 9.3 GHz, 120 K, microwave power 10 mW and modulation amplitude 0.16 mT. For measurements at 35 GHz, the temperature and the modulation amplitude were the same and the attenuation was 10 decibels. Integrations employed Cu^{2+} -EDTA as standard, with correction for transition probability (Aasa & Vännngård, 1975). For further manipulations and for simulations, e.p.r. spectra were transferred to the University of Sussex DEC VAX 11/780 computer, via a link. Iterative or manually controlled simulation routines (George, 1983, and unpublished work) allowed for non-coincident axes of *g* and *A* and included second-order terms.

Results

Steady-state turnover of formamide by xanthine oxidase

O_2 was found to be consumed quite rapidly by xanthine oxidase in the presence of relatively high concentrations of formamide. The reaction taking place must be oxidation of formamide to carbamic acid, which breaks down to give CO_2 and NH_3 . Preliminary kinetic experiments showed that formamide concentrations greater than 20% (v/v) caused the enzyme to lose activity with time, resulting in non-linear assay plots. Also, at formamide concentrations less than 1% (v/v), the sensitivity of our oxygen-electrode system was not adequate to make accurate initial-rate measurements. Fig. 1 shows a double-reciprocal plot over the limited substrate concentration range available to us. The parameters calculated from this graph were $K_{\text{m(app.)}}$ 0.7 M and $V_{\text{max(app.)}}$ 3.1 s^{-1} (calculated on the basis of functional active centres), for pH 8.2 and 25°C , with 1.14 mM- O_2 . When 2,6-dichlorophenol-indophenol was used as oxidizing substrate, significant inhibition of enzyme-catalysed oxidation of formamide occurred at indophenol

Fig. 1. Initial-rate measurements of xanthine oxidase-catalysed oxidation of formamide by O_2 . e/v_0 , the reciprocal of the initial specific rate (where e is the molar concentration of functional xanthine oxidase active centres and v_0 is the initial rate of O_2 consumption), is plotted against the reciprocal of the formamide concentration. The reaction was carried out at 25°C and pH 8.2 in 50 mM-Bicine/NaOH buffer, at a constant concentration of 1.14 mM- O_2 .

concentrations greater than about $12.5 \mu\text{M}$. Similar behaviour is observed with xanthine as reducing substrate (Bray, 1956). Despite this complication, approximate values, for this concentration of indophenol, of $K_{\text{m(app.)}}$ 2 M and $V_{\text{max(app.)}}$ 2.8 s^{-1} , were obtained (results not shown).

Optical measurements of reduction of the enzyme by formamide

When xanthine oxidase (approx. 70% functional, total active-centre concentration $16 \mu\text{M}$) and formamide (0.2 M) were mixed anaerobically, no rapid bleaching of the visible absorption of the enzyme was observed in the first few minutes of the reaction, and only slow decreases of absorption took place thereafter. At the end of the reaction, after 24 h, the typical spectrum of fully reduced xanthine oxidase was observed, A_{450} having decreased by 70% (cf. Massey *et al.*, 1969). We found no evidence at intermediate times for any spectral transients such as those seen with lumazine (Davis *et al.*, 1982).

E.p.r. signals observed and the time course of their development

Under appropriate conditions, as described in the captions to the Figures, we observed Mo(V)

Fig. 2. *Very Rapid, Rapid and Inhibited Mo(V) e.p.r. signals, at 9.3 GHz, obtained with formamide* (a) shows the Very Rapid signal, (b) the Rapid signal and (c) the Inhibited signal. (a') and (c') are computer simulations (obtained as described in the Materials and methods section by using the parameters listed in Table 1) of (a) and (c) respectively. Conditions for generating the signals were as follows. In (a) the enzyme was reduced with formamide (0.5M) in an e.p.r. tube, the solution was gently bubbled with O₂ gas for approx. 2 min, and then the solution was quickly frozen. In (b), the enzyme was mixed under anaerobic conditions with formamide (0.5M), and then the sample was frozen after approx. 2 min. In (c) the enzyme was treated with formamide (0.5M) in an open vessel at 4°C for 3 days, and then substrate that had not reacted was removed by gel filtration. The arrow corresponds to $g = 2.0037$.

e.p.r. signals of the three types that are obtainable from active xanthine oxidase in the presence of other substrates, namely Very Rapid, Rapid and Inhibited [Figs. 2(a), 2(b) and (c) respectively; parameters of the signals are considered below].

We carried out a limited investigation (not illustrated) of the time course of development of the signals during anaerobic reduction of the enzyme by formamide at pH 8.2. Full development of the Rapid signal was rather slow (t_1 about 20 min),

maximum conversion into the signal-giving species corresponding to about 30% of the functional enzyme. FADH[•] free-radical signals reached maximum intensity after about 10 min, then declined. Traces of the Very Rapid signal were also seen as a transient during the first 10 min of reaction, maximum intensity being attained after 1–2 min.

Parameters of the e.p.r. signals

Computer simulations of the Very Rapid and Inhibited signals, obtained by using the parameters listed in Table 1, are illustrated in Figs. 2(a') and 2(c'). The Rapid signal was studied in more detail by using ²H₂O or ¹⁷O-enriched water and with the help of e.p.r. spectra recorded at 35 GHz as well as at 9 GHz. Experimental and simulated e.p.r. spectra are shown in Figs. 3 and 4.

Among the limited number of substrates (Bray *et al.*, 1967; George, 1983) that give rise to the Very Rapid signal, formamide is more reminiscent of 1-methylxanthine (Gutteridge *et al.*, 1978a) than of xanthine (Bray & Vänngård, 1969), since it gives the Rapid signal in its type 1 form, with no evidence for the presence of any of the type 2 form. The pureness, for formamide, of the Rapid type 1 form is shown by the quality of the simulations, at the two frequencies and in ²H₂O as well as in ¹H₂O [Figs. 3(a), 3(a'), 3(b), 3(b'), 4(a), 4(a'), 4(b) and

4(b')]. In the Rapid type 1 signal a strongly coupled and a weakly coupled proton are linked to molybdenum. For the latter proton, splittings were evaluated with particular care in the *g*₂ and *g*₃ regions, where resolved splittings are not seen, with the help of second-derivative spectra (results not shown; see George, 1983).

Bray & Gutteridge (1982) demonstrated by using ¹⁷O at two different enrichments, with the help of computer-generated e.p.r. difference spectra, that several Mo(V) signal-giving species from xanthine oxidase exhibit coupling of more than one oxygen atom to the molybdenum. However, in the Rapid type 1 signal (generated with purine) only a single coupled ¹⁷O was involved. No attempt at quantitative interpretation of ¹⁷O coupling in this signal was made by these workers, though a preliminary and rather unsatisfactory simulation was published earlier by Gutteridge *et al.* (1979).

The formamide Rapid signal seemed particularly suitable for investigation of the coupling to ¹⁷O. We confirmed that only a single coupled oxygen atom was detectable, and Figs. 3(c) and 4(c) show experimental difference spectra corresponding to 100% enrichment with the isotope, at 9 and 35 GHz respectively. The corresponding simulations are shown in Figs. 3(c') and 4(c'). Though the fit of the simulated spectra is not completely

Table 1. Parameters of Mo(V) e.p.r. signals obtained by treating xanthine oxidase with formamide

Signals were generated and recorded as described in the Materials and methods section. Values listed are the ones used for the simulations illustrated in Figs. 2, 3 and 4. *g*-values are believed to be accurate to ± 0.0003 . An iterative fitting routine was used to obtain all values, except those for ¹⁷O. Half-linewidths and hyperfine splittings are in mT. Angles X-O-X', Y-O-Y' and Z-O-Z' are angles of non-coincidence (in degrees), where X, Y and Z refer to the axes (3, 2 and 1) in which *g* is diagonal, and X', Y' and Z' refer to the axes in which A(¹⁷O) is diagonal. Data from the literature are listed for comparison.

Substrate and signal	<i>g</i> -values (linewidths)				Hyperfine coupling (angles)				Reference
	1	2	3	Av.	Nucleus (Z-O-Z') (Y-O-Y') (X-O-X')	1	2	3	
Formamide Very Rapid	2.0229 (0.20)	1.9551 (0.16)	1.9478 (0.21)	1.9753 (0.19)					Present work
Xanthine Very Rapid	2.0252 (0.19)	1.9550 (0.16)	1.9494 (0.19)	1.9765 (0.18)					Tanner <i>et al.</i> (1978)
Formamide Inhibited	1.9832 (0.31)	1.9739 (0.21)	1.9600 (0.20)	1.9724 (0.24)	¹ H	(Not detected)			Present work
Formaldehyde Inhibited	1.9911 (0.20)	1.9772 (0.14)	1.9513 (0.17)	1.9732 (0.17)	¹ H*	0.50	0.34	0.54	0.46 Tanner <i>et al.</i> (1978)
Formamide Rapid type 1	1.9904 (0.18)†	1.9712 (0.18)†	1.9663 (0.18)†	1.9760 (0.18)	¹ H	1.22	1.26	1.32	1.27 Present work
					¹ H‡	0.46	0.17	0.16	
					¹⁷ O	0.15 (35)	0.30 (0)	1.60 (35)	
1-Methylxanthine Rapid type 1	1.9886 (0.18)	1.9691 (0.15)	1.9646 (0.15)	1.9741 (0.16)	¹ H	1.30	1.39	1.40	1.36 Malthouse <i>et al.</i> (1981b); Gutteridge <i>et al.</i> (1978a)
					¹ H	0.40	0.30	0.20	

* Non-exchangeable proton.

† Values were increased to 0.30, 0.28 and 0.28 respectively for simulations at 35 GHz.

‡ Evaluated with the help of second-derivative spectra.

Fig. 3. Effect of using water enriched with ^2H or with ^{17}O on the e.p.r. spectrum of the formamide Rapid type 1 signal at 9.3GHz

(a) shows the spectrum in ordinary water, (b) that in $^2\text{H}_2\text{O}$ and (c) that in H_2^{17}O . For (c), water of 54% enrichment with ^{17}O was employed and the spectrum shown is a difference spectrum obtained by subtracting out the appropriate amount (Bray & Gutteridge, 1982) of spectrum (a). (a'), (b') and (c') are computer simulations of (a), (b) and (c) respectively, and were obtained by using the parameters listed in Table 1. The stick diagram shows the transitions at the principal g-values, which are split by coupling to the two exchangeable protons. The arrow corresponds to $g = 2.0037$. The signals were generated as for Fig. 2(b).

satisfactory, nevertheless the single set of parameters (Table 1) used at the two frequencies probably comes close to the true values. In par-

ticular, the large anisotropy of $A(^{17}\text{O})$ can be seen directly in Fig. 4(c), since hyperfine lines in the g_1 region are not resolved, leaving the low-field

Fig. 4. Effect of using water enriched with ^2H or with ^{17}O on the e.p.r. spectrum of the formamide Rapid type 1 signal at 35.0 GHz

The spectra of (a), (b) and (c) correspond to samples similar to those used in Figs. 3(a), 3(b) and 3(c), and the corresponding simulations (a'), (b') and (c') were obtained by using the parameters listed in Table 1.

doublet of the spectrum virtually unaffected by the isotope, whereas well-resolved ^{17}O features can be seen in the g_2 and g_3 region.

Discussion

Formamide is the second simplest substrate, after formaldehyde, for xanthine oxidase. Although the value of $K_{m(\text{app.})}$ is high, on the other hand $V_{\text{max.}(\text{app.})}$ is also quite high, being of the order of 15% of that for xanthine (Massey *et al.*, 1969; Bray, 1975) or for formaldehyde (Morpeth, 1983). The fact that $V_{\text{max.}(\text{app.})}$ is about the same with two different oxidizing substrates is consistent with the

re-oxidative part of the catalytic cycle not being rate-limiting in turnover.

From the chemical point of view, the simplicity of the formamide molecule makes studies with it particularly interesting. On the one hand its structure is analogous to that of aldehydes, and on the other it has, in common with the heterocyclic substrates of xanthine oxidase, a nitrogen atom adjacent to the carbon at which the hydroxylation reaction takes place. Optical studies indicated no special features during the reaction. On the other hand, e.p.r. studies with formamide provided information bearing on a number of points. Formamide is noteworthy as being only the second

compound to give rise to both Very Rapid and Inhibited signals in addition to Rapid signals. The other compound that does this (2-amino-4-hydroxy-6-formylpteridine; Malthouse *et al.*, 1981a) is a heterocyclic aldehyde, and data on it are complicated by the fact that the enzyme might in principle act either by oxidizing the formyl group or by hydroxylating the 7-position of the pteridine ring.

We first consider the data relating to the Very Rapid signal. During anaerobic reaction of the enzyme by formamide, development of all the e.p.r. signals was quite slow. However, the general pattern of the reduction process seemed similar to that with substrates such as xanthine or 1-methylxanthine (Edmondson *et al.*, 1972), though with a time-scale slower by a factor of the order of 10^4 . Such a factor would be accounted for by the difference of the steady-state parameters, $K_{m(\text{app.})}$ and $V_{\text{max.}(\text{app.})}$. For these purine substrates, kinetic competence of the intermediate represented by the Very Rapid signal is not in doubt (Bray, 1980), and the signal from formamide thus seems likely to be kinetically competent also. Formamide, an aldehyde-like compound, is the first substrate of the enzyme other than a nitrogen-containing heterocycle to give rise to a Very Rapid signal. Observation of the signal thus substantially strengthens the view, first hinted at by Bray *et al.* (1967) and recently supported by George (1983), that the Very Rapid signal represents an essential enzyme-bound intermediate in turnover of all substrates that is, however, not detectable with some substrates, either for kinetic reasons or because an insignificant fraction of the species, which exist predominantly as Mo(IV), is present in the Mo(V) state.

Formamide is the first non-aldehyde substrate to give the Inhibited signal. However, it does contain a formyl group, and formation of the signal is consistent with the structure proposed for the signal-giving species (Bray & Gutteridge, 1982; Morpeth & Bray, 1984). The former workers proposed that the carbonyl group reacts with molybdenum to form a cyclic structure. The formamide Inhibited signal lacks the hyperfine splitting by a carbon-bound proton characteristic (Pick *et al.*, 1971) of

the formaldehyde Inhibited signal. On the other hand, despite much reduced g -value anisotropy, the value of $g_{\text{av.}}$ for the formamide Inhibited signal is close to that for the formaldehyde Inhibited species. Morpeth & Bray (1984) showed that for aldehydes with a bulky or electron-withdrawing substituent higher g -values are observed.

In general, for xanthine oxidase, the relationship between Rapid type 1 and type 2 signal-giving species is not fully understood. Thus it is not certain why formamide yields the former and not the latter. However, our studies with the formamide Rapid type 1 signal serve to establish an important point. They show for the first time that the coupling of molybdenum to ^{17}O in this species is highly anisotropic. The magnitude of this coupling is in the range expected for ligation of oxygen to molybdenum. The anisotropy indicates a large p -orbital contribution in the bonding of this ligand to the metal atom, which would be expected for a terminally bound oxygen atom. The anisotropy thus provides the first real evidence (cf. Bray & Gutteridge, 1982) that the coupled oxygen atom is a terminal oxygen ligand of molybdenum rather than being present as, e.g., a hydroxyl ligand.

Possible structures for the Rapid type 1 signal-giving species are illustrated in Scheme 1(a-c). The cyanide-labile sulphur atom is in an equatorial ligand position, bearing the strongly coupled proton (Gutteridge *et al.*, 1978b; Malthouse *et al.*, 1981b; George & Bray, 1983a,b). The structure in Scheme 1(a), with the oxygen atom in an equatorial position and the weakly coupled proton hydrogen-bonded to it, corresponds to the one suggested by Bray & Gutteridge (1982). However, we have added, in an axial position, the ligand X, which is supported to exert a strong ligand field at the molybdenum atom. This is required to cause the ground-state $4d$ orbital to be in the equatorial plane perpendicular to the Mo-X bond. The possible nature of X is quite uncertain. It is conceivable that it is a second terminally bound oxygen ligand (Scheme 1b). However, the results with ^{17}O , indicating (cf. Bray & Gutteridge, 1982) only a single e.p.r.-detectable oxygen ligand, provide no support for such a structure. Hence, we favour a structure of the form in Scheme 1(c). Here, the

Scheme 1. Alternative structures for the Rapid type 1 e.p.r. signal-giving species
The favoured structure is the one shown in (c) (see the text).

coupled oxygen atom is shown in an axial position, even though the ^{17}O coupling is larger than that expected from studies (Hanson *et al.*, 1981) of a model compound. We suggest that this could be due to a somewhat distorted ligand geometry in the enzyme, so that the assumption of a simple octahedral ligand field no longer applies. Under circumstances of low symmetry, a larger coupling to an axial (oxygen) ligand might be expected, as participation of excited state *d*-levels could result in a ground state no longer completely in the equatorial plane.

It is not certain whether the substrate (or other modifier molecule) bound at the molybdenum site in Rapid-signal-giving species generated by substrates such as formamide is actually a ligand of the metal atom. In earlier work (Gutteridge *et al.*, 1978a; Bray *et al.*, 1979) it was assumed that this was the case. However, parameters within the Rapid type 1 family of signals are perhaps too much alike (compare, e.g. signals from formamide and 1-methylxanthine in Table 1) for the substrate to be a ligand, unless it were present in an axial type of position. Perhaps in fact it is not unlikely that substrates are ligated in such a position, *trans* to the oxygen atom of Scheme 1(c). With this structure, owing to the *trans* effect, the ligation would be labile, as is expected in a Michaelis type of complex.

The work was supported by grants from the Medical Research Council and the Science and Engineering Research Council.

References

- Aasa, R. & Vänngård, T. (1975) *J. Magn. Reson.* **19**, 308–315
- Booth, V. H. (1938) *Biochem. J.* **32**, 494–502
- Bray, R. C. (1956) Ph.D. Thesis, University of London
- Bray, R. C. (1975) *Enzymes 3rd Ed.* **12**, 299–419
- Bray, R. C. (1980) *Adv. Enzymol. Relat. Areas Mol. Biol.* **51**, 107–165
- Bray, R. C. (1982) in *Flavins and Flavoproteins* (Massey, V. & Williams, C. H., eds.), pp. 775–785, Elsevier/North-Holland, New York
- Bray, R. C. & Gutteridge, S. (1982) *Biochemistry* **21**, 5992–5999
- Bray, R. C. & Vänngård, T. (1969) *Biochem. J.* **114**, 725–734
- Bray, R. C., Knowles, P. F. & Meriwether, L. S. (1967) in *Magnetic Resonance in Biological Systems* (Ehrenberg, A., Malmström, B. G. & Vänngård, T., eds.), pp. 249–264, Pergamon Press, Oxford
- Bray, R. C., Barber, M. J. & Lowe, D. J. (1978) *Biochem. J.* **171**, 653–658
- Bray, R. C., Gutteridge, S., Stotter, D. A. & Tanner, S. J. (1979) *Biochem. J.* **177**, 357–360
- Davis, M. D., Olson, J. S. & Palmer, G. (1982) *J. Biol. Chem.* **257**, 14730–14737
- Edmondson, D., Ballou, D., Van Heuvelen, A., Palmer, G. & Massey, V. (1972) *J. Biol. Chem.* **248**, 6135–6144
- George, G. N. (1983) D.Phil. Thesis, University of Sussex
- George, G. N. & Bray, R. C. (1983a) *Biochemistry* **22**, 1013–1021
- George, G. N. & Bray, R. C. (1983b) *Biochemistry* **22**, 5443–5452
- 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
- Gutteridge, S., Malthouse, J. P. G. & Bray, R. C. (1979) *J. Inorg. Biochem.* **11**, 355–360
- Hanson, G. R., Brunette, A. A., McDonnell, A. C., Murray, K. S. & Wedd, A. G. (1981) *J. Am. Chem. Soc.* **103**, 1953–1959
- Hart, L. I., McGartoll, M. A., Chapman, H. R. & Bray, R. C. (1970) *Biochem. J.* **116**, 851–864
- Krenitsky, T. A., Neil, S. M., Elion, G. B. & Hitchings, G. H. (1972) *Arch. Biochem. Biophys.* **150**, 585–599
- Malthouse, J. P. G., Williams, J. W. & Bray, R. C. (1981a) *Biochem. J.* **197**, 421–425
- Malthouse, J. P. G., George, G. N., Lowe, D. J. & Bray, R. C. (1981b) *Biochem. J.* **199**, 629–637
- Massey, V. M., Brumby, P. E., Komai, H. & Palmer, G. (1969) *J. Biol. Chem.* **244**, 1682–1691
- Morpeth, F. F. (1983) *Biochim. Biophys. Acta* **744**, 328–334
- Morpeth, F. F. & Bray, R. C. (1984) *Biochemistry* in the press
- Morpeth, F. F., George, G. N. & Bray, R. C. (1983) in *Some Recent Developments in the Chemistry of Chromium, Molybdenum and Tungsten* (Dilworth, J. R. & Lippert, M. F., eds.), p. P9, Royal Society of Chemistry, Dalton Division, London
- Pick, F. M., McGartoll, M. A. & Bray, R. C. (1971) *Eur. J. Biochem.* **18**, 65–72
- Rosemeyer, H. & Seela, F. (1983) *Eur. J. Biochem.* **134**, 513–515
- Tanner, S. J., Bray, R. C. & Bergmann, F. (1978) *Biochem. Soc. Trans.* **6**, 1327–1330