Stopped-Flow Spectrophotometric Studies on the Reaction of Turkey Liver Xanthine Dehydrogenase with Reducing Substrates

By ÍDE NÍ FHAOLÁIN,* MICHAEL J. HYNES† and MICHAEL P. COUGHLAN*
Departments of *Biochemistry and †Chemistry, University College, Galway, Ireland

(Received 30 June 1977)

The kinetics of reduction of turkey liver xanthine dehydrogenase by substrates were investigated by stopped-flow spectrophotometry. The results may be explained in terms of the known redox potentials of the various centres in the enzyme [Barber, Bray, Cammack & Coughlan (1977) *Biochem. J.* **163**, 279–289]. They are, moreover, consistent with the scheme [Olson, Ballou, Palmer & Massey (1974) *J. Biol. Chem.* **249**, 4363–4382] in which reduction occurs in three consecutive steps, one molecule of substrate reacting with the active site at each step. First-order rate constants believed to correspond respectively to the combined first and second steps and to the third step in the reduction by excess of xanthine and of NADH were determined. The rates of reaction with these substrates in the combined first and second steps are independent of the degree of enzyme functionality.

The reduction of xanthine oxidase by substrates has been extensively studied by spectrophotometric, rapid-reaction and e.p.r. techniques [see reviews by Massey (1973) and Bray (1975)]. The relative (Olson *et al.*, 1974*b*) and absolute (Cammack *et al.*, 1976) oxidation–reduction potentials of the molybdenum, flavin and iron–sulphur prosthetic groups have been determined, as has the distribution of electrons among these groups on reduction of the enzyme. Such studies have increased enormously our understanding of the mechanism of action of this enzyme (see e.g. Olson *et al.*, 1974*a,b*). By contrast with xanthine oxidase, the reaction of xanthine dehydrogenases with reducing substrates has been investigated in detail only by e.p.r. techniques (Dalton *et al.*, 1976; Barber *et al.*, 1976). In the present paper we report the results of stopped-flow spectrophotometric studies on turkey liver xanthine dehydrogenase. Interpretation of these results is aided by the determination of the redox potentials of the various prosthetic groups in this enzyme (Barber *et al.*, 1977).

Experimental

Materials and reagents

Fresh-frozen turkey livers were obtained from Castlemahon Co-Op, Newcastle West, Co. Limerick, Ireland, and stored at -20°C until required. Enzyme was isolated from this source by a slight modification of the method of Cleere (1976). Thus the amounts of $(\text{NH}_4)_2\text{SO}_4$ used in the first and second fractionation steps were decreased by 3 g/100 ml in each case. Although less active samples were used in the present

experiments, enzyme so prepared was usually 70–80% functional (cf. below) and was concentrated by ultrafiltration and stored as frozen beads at liquid- N_2 temperature until required. Samples of different degrees of functionality were prepared by mixing native enzyme and cyanide-inactivated enzyme in the appropriate proportions (Cleere *et al.*, 1974).

Xanthine was obtained from Sigma (London) Chemical Co., London S.W.6, U.K.; NAD^+ and NADH were from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; sodium dithionite was from BDH, Poole, Dorset, U.K.

Enzyme assay

Reactions were carried out at 30°C in 0.05 M-potassium phosphate buffer, pH 7.8, containing 0.1 mM-EDTA, in a final volume of 1.0 ml. The oxidation of xanthine (0.15 mM) by NAD^+ (0.5 mM) was followed at 340 nm, by using a Unicam SP. 1800 spectrophotometer equipped with thermostatically controlled cuvette holder and continuous chart recorder. The activity was expressed as the activity/flavin ratio (see Avis *et al.*, 1956), obtained by dividing the change in A_{340}/min by the A_{450} of the enzyme used in the assay. Fully functional enzyme has a value of 325 under these conditions (Cleere *et al.*, 1974).

Protein and substrate determination

Enzyme active-centre concentration (two active centres per molecule) was determined by the previously determined A_{450} value of 36 550 litre $\cdot$ mol $^{-1} \cdot \text{cm}^{-1}$ (Cleere & Coughlan, 1975). Functional active-

centre concentration was measured from the activity/flavin ratio (see above). Xanthine and NADH concentrations in the standard buffer were determined at 278 nm ($\epsilon=8768 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) and at 340 nm ($\epsilon=6300 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) respectively.

Rapid-reaction studies

Fast phases of reduction were monitored by using a stopped-flow spectrophotometer (Applied Photo-physics Ltd., London W1X 3HA, U.K.) The drive syringes and the mixing chamber were maintained at $25 \pm 0.1^\circ\text{C}$. Kinetic data were recorded with a Tektronix model 5100 storage oscilloscope. The stored traces were photographed by using a Polaroid camera. First-order rate constants were obtained from plots of $-\ln(A-A_\infty)$ versus time, where A_∞ is the absorbance at infinite time. For consecutive reactions it was necessary to treat the value of A_∞ for the fast steps as a variable. The value was adjusted to obtain linear pseudo-first-order plots. Reaction conditions are described in the text.

Results

The reaction of xanthine oxidase with reducing substrates occurs in a number of distinct phases (Gutfreund & Sturtevant, 1959; Massey, 1973; Bray, 1975). Indeed, using e.p.r. techniques alone, Swann & Bray (1972) were able to distinguish no less than four phases in the reduction of this enzyme. Olson *et al.* (1974b) have also commented in detail on the various steps in reduction. The reaction of turkey liver xanthine dehydrogenase with substrates is apparently biphasic, as judged by conventional spectrophotometry, a fast phase being followed by a slow phase (Í. Ní Fhaoláin & M. P. Coughlan, unpublished work). The apparent biphasic nature of such reduction may be something of an oversimplification in that 'sub-phases' may have been masked. Thus in order to examine the fast phase(s) of substrate reduction of xanthine dehydrogenase in more detail, we turned to stopped-flow techniques.

Because of the low reactivity of the turkey liver

Fig. 1. Phases II and III in the reduction of turkey liver xanthine dehydrogenase by substrate. First-order plots of phase II (a) and of phase III (b) of the reduction of enzyme by xanthine (Δ) and by NADH ($\square$) at 25°C in 0.05 M -potassium phosphate, pH 7.8, containing 0.1 mM -EDTA. The concentrations of reactants after mixing in the stopped-flow apparatus were: enzyme, $7.5 \mu\text{M}$ with respect to total flavin; xanthine, $375.0 \mu\text{M}$ (a) and $75.0 \mu\text{M}$ (b); NADH $22.5 \mu\text{M}$ (a) and $75.0 \mu\text{M}$ (b). The time scales for reduction by xanthine and by NADH are shown at the top and bottom respectively of each panel. In the above experiments two consecutive reactions were clearly observable. However, to optimize the accuracy of the experimental data the x and y controls of the oscilloscope were adjusted so as to exclude phase II from the screen when phase III was being measured, and vice versa. Note that the negative log of the absorbance change at 450 nm is plotted versus time. Hence the slope increases as the absorbance decreases. The various phases in the reduction of enzyme by substrate are defined in the Discussion section.

enzyme with oxygen (less than 2% of the rate with acceptors such as NAD^+ ; Cleere & Coughlan, 1975; Coughlan & Cleere, 1976), we thought that it might not be necessary to carry out the stopped-flow experiments under strict anaerobic conditions. This assumption was proved to be correct. Thus the first-order rate constant obtained for the fast phase of reduction by excess of xanthine (Figs. 1a, 2 and Table 1) is identical with that obtained in our previous study [cf. Barber *et al.* (1976), and below] in which strict anaerobic conditions were maintained. Moreover the first-order rate constant obtained here is identical with the turnover rate for the enzyme (cf. below). Thus it is clear that the presence of oxygen in the various solutions used did not significantly interfere with the determination of rate constants for the fast phases of reduction of the turkey enzyme.

Kinetics of reaction with reducing substrates

We were able to distinguish two 'fast' phases in the reduction of xanthine dehydrogenase by xanthine or by NADH using stopped-flow techniques (Figs. 1a and 1b). Each phase could be described by a single exponential (note the different time scales for the two phases and for the two substrates). For reasons discussed below we shall refer to the faster of these phases as 'phase II' and to the slower as 'phase III'. In the experiments shown (Figs. 1a and 1b) the first-order rate constants for reduction by xanthine were: phase II, 21.6s^{-1} ; phase III, 0.066s^{-1} . In our previous study (Barber *et al.*, 1976) using stopped-flow techniques at 30°C , pH 8.2 and strict anaerobic conditions, we found the first-order rate constant for the phase corresponding to phase II above to be $33 \pm 1\text{s}^{-1}$. The value of 21.6s^{-1} obtained here at 25°C , pH 7.8 corrects to 33.05s^{-1} when allowance is made for the differences in temperature (factor 1.5; Cleere & Coughlan, 1975) and in pH (factor 1.02; Barber *et al.*, 1976). Clearly, the presence of oxygen does not affect the determination of rate constants for the fast phases of reaction of this enzyme (cf. above).

The catalytic-centre activity of the enzyme-catalysed oxidation of xanthine at 30°C , pH 8.2, is 32.5s^{-1} (Cleere *et al.*, 1974; Cleere & Coughlan, 1975; Barber *et al.*, 1976). Thus phase II is catalytically significant and shows, as with the milk enzyme (Massey *et al.*, 1969; Edmondson *et al.*, 1973; Olson *et al.*, 1974a,b), that reduction by xanthine (or more accurately, release of the products: Olson *et al.*, 1974b) is rate-limiting in turnover. The value obtained in Fig. 1(b) for phase III (0.066s^{-1}) is somewhat lower than the average value for this phase (cf. Table 1), but shows, as expected, that it is not catalytically significant (see the Discussion section).

Stopped-flow traces in our earlier study (Barber *et al.*, 1976) showed that bleaching of the A_{450} of the turkey enzyme by NADH was faster than that by

xanthine but did not measure the rate. Fig. 1 confirms this, the first-order rate constants for phases II and III of reduction by NADH being 111.0s^{-1} and 0.835s^{-1} respectively (note the different time scales for the two phases). These values may be compared with the turnover rate for the enzyme-catalysed oxidation of NADH by dichlorophenol-indophenol of 30s^{-1} (Ní Fhaoláin & Coughlan, 1976). Clearly, phase II is catalytically significant, whereas phase III is not. Moreover reduction of the enzyme by NADH is obviously not rate-limiting in turnover (compare 111s^{-1} with 30s^{-1}). This suggests that with respect to NADH oxidoreductase activity, release of the products or reoxidation of enzyme is the rate-limiting factor.

The effects of varying the ratio of substrate to enzyme on phase II of the reduction of the turkey enzyme by xanthine or by NADH are shown in Fig. 2. The spectral changes occurring on reduction by either substrate could be described by first-order plots regardless of the ratio of substrate to enzyme. However, under pseudo-first-order conditions with substrate in excess the average rate constants obtained for phase II of reduction by xanthine is 21.0s^{-1} , whereas that for NADH is 113s^{-1} .

Table 1 summarizes the average values obtained for the various phases in the reduction of enzyme by substrate. The fact that the absorption changes especially at 550 and 610nm are relatively small, together with the large amounts of enzyme required in

Fig. 2. First-order rate constants for phase II as a function of the ratio of substrate to enzyme

First-order rate constants for phase II of reduction of enzyme ($7.5\mu\text{M}$ with respect to total flavin) by xanthine (Δ) and by NADH ($\square$) were obtained at the various ratios of substrate to total flavin shown. Other conditions were as described in the Experimental section.

Table 1. *First-order rate constants for various stages of reduction of the turkey enzyme by substrates*

First-order rate constants for the fast phases of change in absorbance at the wavelengths indicated were determined by stopped-flow spectrophotometry as described in the Experimental section. The results shown (except for the single observation at 610nm) are the mean values $\pm$ S.E.M. The numbers in parentheses represent the number of values used in calculating the mean.

Phase	Wavelength (nm)	Reducing substrate	k (s ⁻¹)	
			Xanthine	NADH
II	450		21.38 $\pm$ 0.48 (12)	114.4 $\pm$ 1.6 (11)
III	450		0.091 $\pm$ 0.011 (12)	0.77 $\pm$ 0.04 (3)
II	550		—	60.3 $\pm$ 2.2 (27)
III	550		0.07 $\pm$ 0.01 (7)	—
II	610		10.8	—

these studies, prevented us obtaining all of the values that we would have wished to determine. We would have expected, by analogy with xanthine oxidase (Olson *et al.*, 1974b), that the values for the rate constants in phase II of the reduction of xanthine dehydrogenase by excess of NADH should be the same whether the reaction was monitored at 450nm or at 550nm. However, that obtained at 450nm was consistently greater than that at 550nm (Table 1). We do not as yet have a plausible explanation for this observation. The values obtained (Table 1) for the first-order rate constants for phase III of bleaching of A_{450} are close to those predicted from earlier e.p.r. work (Barber *et al.*, 1977; see the Discussion section).

Effects of non-functional enzyme

Morell (1952) showed that the extent of reduction of xanthine oxidase by xanthine occurring in the rapid phase was dependent on the specific activity of the purified enzyme samples used. The reasons for this are discussed below. In passing we may note that Morrell's (1952) findings have been confirmed by Gutfreund & Sturtevant (1959), McGartoll *et al.* (1970) and by Edmondson *et al.* (1972) all of whom used milk xanthine oxidase. Similar observations have been made with rabbit liver aldehyde oxidase (Branzoli & Massey, 1974), chicken liver xanthine dehydrogenase (Nishino *et al.*, 1975) and turkey liver xanthine dehydrogenase (Í. Ní Fhaoláin & M. P. Coughlan, unpublished work).

By contrast with the extent of reduction of turkey liver xanthine dehydrogenase by excess of xanthine, that by NADH is independent of the content of non-functional active sites in the enzyme samples used (Í. Ní Fhaoláin & M. P. Coughlan, unpublished work). The reasons for this expected finding (cf. Edmondson *et al.*, 1973; Ní Fhaoláin & Coughlan, 1976) are discussed below.

Fig. 3 shows that the values of the first-order rate constants for phase II of reduction of the turkey enzyme by excess of substrate are independent of the degree of functionality of the samples used. Thus,

Fig. 3. *First-order rate constants for phase II of reduction of the turkey enzyme by substrate correlated with the content of functional active sites*

Enzyme samples (7.5 μ M with respect to total flavin) of different degrees of functionality (cf. the Experimental section) were treated with 75 μ M-xanthine (Δ) or 75 μ M-NADH ($\square$) at 25°C in 0.05 M-potassium phosphate, pH 7.8, containing 0.1 mM-EDTA. First-order rate constants for phase II were obtained as described in the Experimental section.

with xanthine, it is only the extent of the reaction occurring in the fast phase (cf. above), not the rate of the reaction (Fig. 3), that is dependent on the content of functional active sites. Branzoli & Massey (1974) have made similar observations with aldehyde oxidase. With NADH, neither the extent of reduction of xanthine dehydrogenase (cf. above) nor the rate of reaction (Fig. 3) depend on the degree of enzyme functionality.

Discussion

Molecules of xanthine oxidase, and of the related enzymes xanthine dehydrogenase and aldehyde oxidase, are composed of two identical subunits (Massey, 1973; Bray, 1975; Waud *et al.*, 1975). Each

subunit contains one molybdenum atom, one FAD molecule and four iron-sulphur centres. Enzymes prepared by conventional means contain up to 50% or more of non-functional molecules. These appear to differ from functional molecules only by the presence in the latter and absence from the former of a 'persulphide' group at the molybdenum centre (Edmondson *et al.*, 1972; Cleere *et al.*, 1974). Substrates such as xanthine that bind to the molybdenum site cannot reduce the enzyme if the persulphide group is not intact. NADH, by contrast with xanthine, interacts with flavin rather than the molybdenum centre and so readily reduces both functional and non-functional (desulpho) enzyme molecules (Edmondson *et al.*, 1972; Ní Fhaoláin & Coughlan, 1976).

Xanthine oxidase and xanthine dehydrogenase accept a maximum of six electrons per subunit on full reduction (e.g. Olson *et al.*, 1974b; Barber *et al.*, 1977). These are provided by three molecules of substrate (per subunit), each donating a pair of electrons in consecutive irreversible steps (Olson *et al.*, 1974b). The six electrons per active site are distributed as follows: two to molybdenum, two to flavin and one to each pair of iron-sulphur groups. Determination of the oxidation-reduction equilibria for the systems $\text{Mo(VI)} \rightleftharpoons \text{Mo(V)} \rightleftharpoons \text{Mo(IV)}$ and $\text{FAD} \rightleftharpoons \text{FADH}^{\cdot} \rightleftharpoons \text{FADH}_2$ and one-electron reduction of each iron-sulphur centre, permitted calculation of the distribution of electrons among the prosthetic groups at various stages of reduction (i.e. zero to six electrons per active site; Olson *et al.*, 1974b; Cammack *et al.*, 1976; Barber *et al.*, 1977). Moreover, knowledge of the equilibrium distribution of electrons allowed calculation of the time course of reduction (Olson *et al.*, 1974b).

Reaction with xanthine

Each molecule of xanthine donates a pair of electrons to the enzyme. Thus the steps involved in reduction of fully functional xanthine dehydrogenase may be written as follows (where XD represents a half-molecule of enzyme and the subscript denotes the number of electrons accepted):

We will call these stages of reduction phases I, II and III.* Previous investigations have shown that phases I and II are catalytically significant, whereas the rate of conversion of the four-electron into the six-electron reduced species is not (Massey *et al.*, 1969; Olson *et al.*, 1974b; Cammack *et al.*, 1976; Barber *et al.*, 1977).

* The numbering system is not the same as that used by Swann & Bray (1972).

Olson *et al.* (1974b) have shown that the rate of each step in reduction by excess of xanthine depends on the fraction of molybdenum present as Mo(VI). For xanthine dehydrogenase, Barber *et al.* (1977) have shown that in form XD_{4e} only 0.7% of the molybdenum remains as Mo(VI). Accordingly from the ratio 100/0.7 one would expect the first-order rate constant in the reduction of the turkey enzyme by excess of xanthine of 21.38 s⁻¹ (cf. Table 1) is, as we have shown above, commensurate with the catalytic-centre activity. Accordingly we assigned it to phase II (which is catalytically significant as discussed above), although it may in fact be the combined first-order rate constant for phases I and II. Assuming that this is in fact the case, the ratio between the rate constant for phase I to be 143 times greater than that for phase III. The first-order rate constant 21.38 s⁻¹ above and that assigned to phase III, i.e. 0.091 s⁻¹ (Table 1), is 235, which is in reasonable agreement with the predicted ratio of 143. Barber *et al.* (1977) predicted, by analogy with the milk enzyme, that the half-time for the conversion of form XD_{4e} into XD_{6e} should be 5 s. From the value of the first-order rate constant (0.091 s⁻¹) that we assigned to phase III we can calculate a half-time of 7 s. Clearly therefore we have assigned rate constants to the appropriate phases, although the presence of oxygen in the stopped-flow experiments may have led to some underestimation of the rate constants for phase III.

Reaction with NADH

Massey *et al.* (1969) found that the reduction of xanthine oxidase by excess of NADH was essentially biphasic. A fast phase (presumably corresponding to phases I and II as defined here) in which 70% of the total reduction took place was followed by a slow phase (phase III as defined here) extending over a period of hours which contributed the remaining 30% of the absorbance change. Olson *et al.* (1974b) explained the slowness of the latter phase on the basis of the calculated distribution of electrons at various stages of reduction. Thus three molecules of NADH each donate a pair of electrons in consecutive steps to each subunit. However, in form XO_{4e} (where XO represents a half-molecule of xanthine oxidase) only 2% of the flavin remains fully oxidized and therefore available for reaction with NADH. Thus the rate of conversion of form XO_{4e} into XO_{6e} should be one-fiftieth that of the first step.

The rate of phase II (or possibly phases I and II) in the reduction of xanthine dehydrogenase by excess of NADH is over 100-fold higher than that of phase III (cf. Table 1). To explain this observation, account must be taken of the electron distribution in form XD_{4e} as well as that in the non-functional form, XD(ds)_{4e} [where XD(ds) represents a half-molecule of the desulpho form of xanthine dehydro-

genase], since we are dealing with a mixed population of molecules. In form XD_{4e}, by contrast with form XO_{4e}, most of the flavin remains in the fully oxidized form (Barber *et al.*, 1977). These workers considered the difficulty in accepting a pair of electrons from the third molecule of NADH to be a thermodynamic rather than a kinetic problem. The redox potentials for the systems XD₀/XD_{2e}, XD_{2e}/XD_{4e} and XD_{4e}/XD_{6e} were calculated to be -277 mV, -314 mV and -366 mV respectively (Barber *et al.*, 1977). These values may be compared with that of -350 mV for the NAD⁺/NADH pair under similar conditions. From these values we can calculate the equilibrium constants for phases I, II and III of reduction by excess of NADH to be 289, 16.6 and 0.29 respectively. Clearly, for phases I and II the equilibria are favourable, whereas that for phase III is not. The possible effects of NADH (or of NAD⁺) binding on the redox potentials were not allowed for in the work of Barber *et al.* (1977) and may, in fact, modify the above calculations somewhat. However, the results are consistent with the prediction of Barber *et al.* (1977) that even in the presence of a substantial excess of NADH the rate of phase III is considerably less than that of the earlier phases (Table 1).

The redox potential of molybdenum in the desulpho form of xanthine oxidase is much more negative than that in the functional enzyme (Cammack *et al.*, 1976). If the same situation obtains in xanthine dehydrogenase, the probability of electrons being located on the molybdenum of form XD(ds)_{4e} will be low. This will have the effect of decreasing the amount of oxidized flavin remaining in form XD(ds)_{4e} relative to that in form XD_{4e} (R. C. Bray, personal communication). Consequently the rate of phase III in the reduction of the desulpho form of xanthine dehydrogenase by excess of NADH should also be much less than that for the earlier phases.

References

- Avis, P. G., Bergel, F., Bray, R. C., James, D. W. F. & Shooter, K. V. (1956) *J. Chem. Soc.* 1212-1226
- Barber, M. J., Bray, R. C., Lowe, D. J. & Coughlan, M. P. (1976) *Biochem. J.* **153**, 297-307
- Barber, M. J., Bray, R. C., Cammack, R. & Coughlan, M. P. (1977) *Biochem. J.* **163**, 279-289
- Branzoli, U. & Massey, V. (1974) *J. Biol. Chem.* **249**, 4346-4349
- Bray, R. C. (1975) *Enzymes 3rd Ed.* **12**, 299-419
- Cammack, R., Barber, M. J. & Bray, R. C. (1976) *Biochem. J.* **157**, 469-478
- Cleere, W. F. (1976) Ph.D. Thesis, University College, Galway
- Cleere, W. F. & Coughlan, M. P. (1975) *Comp. Biochem. Physiol. B* **50**, 311-322
- Cleere, W. F., O'Regan, C. & Coughlan, M. P. (1974) *Biochem. J.* **143**, 465-468
- Coughlan, M. P. & Cleere, W. F. (1976) in *Flavins and Flavoproteins* (Singer, T. P., ed.), pp. 576-581, Elsevier, Amsterdam
- Dalton, H., Lowe, D. J., Pawlik, R. T. & Bray, R. C. (1976) *Biochem. J.* **153**, 287-295
- Edmondson, D., Massey, V., Palmer, G., Beacham, L. M. III & Elion, G. B. (1972) *J. Biol. Chem.* **247**, 1597-1604
- Edmondson, D., Ballou, D., Van Heuvelen, A., Palmer, G. & Massey, V. (1973) *J. Biol. Chem.* **248**, 6135-6144
- Gutfreund, H. & Sturtevant, J. M. (1959) *Biochem. J.* **73**, 1-6
- Massey, V. (1973) in *Iron-Sulphur Proteins* (Lovenberg, W., ed.), pp. 301-360, Academic Press, New York
- Massey, V., Brumby, P. E., Komai, H. & Palmer, G. (1969) *J. Biol. Chem.* **244**, 1682-1691
- McGartoll, M. A., Pick, F. M., Swann, J. C. & Bray, R. C. (1970) *Biochim. Biophys. Acta* **212**, 523-526
- Morell, D. B. (1952) *Biochem. J.* **51**, 657-666
- Ní Fhaoláin, Í. & Coughlan, M. P. (1976) *Biochem. J.* **157**, 283-285
- Nishino, T., Itoh, R. & Tsushima, K. (1975) *Biochim. Biophys. Acta* **403**, 17-22
- 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
- Swann, J. C. & Bray, R. C. (1972) *Eur. J. Biochem.* **26**, 407-415
- Waud, W. R., Brady, F. O., Wiley, R. D. & Rajagopalan, K. V. (1975) *Arch. Biochem. Biophys.* **169**, 695-701