Biochem. J. (1982) **203**, 505–510
Printed in Great Britain

The steady state kinetics of the NADH-dependent nitrite reductase from *Escherichia coli* K12

The reduction of single-electron acceptors

Ronald H. JACKSON,* Jeffrey A. COLE and Athel CORNISH-BOWDEN†
Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

(*Received 7 January 1982/Accepted 8 February 1982*)

The kinetic characteristics of the diaphorase activities associated with the NADH-dependent nitrite reductase (EC 1.6.6.4) from *Escherichia coli* have been determined. The values of the apparent maximum velocity are similar for the reduction of $Fe(CN)_6^{3-}$ and mammalian cytochrome *c* by NADH. These reactions may therefore have the same rate-limiting step. NAD^+ activates NADH-dependent reduction of cytochrome *c*, and the apparent maximum velocity for this substrate increases more sharply with the concentration of NAD^+ than for hydroxylamine. The simplest explanation is that NAD^+ activation of hydroxylamine reduction derives solely from activation of steps involved in the reduction of cytochrome *c*, a flavin-mediated reaction, but these steps are only partly rate-limiting for the reduction of hydroxylamine. At 0.5 mM-NAD^+, the apparent maximum velocity was 2.3 times higher for 0.1 mM-cytochrome *c* as substrate than for 100 mM-hydroxylamine, suggesting that the rate-limiting step during hydroxylamine reduction is a step that is not involved in cytochrome *c* reduction. A scheme is proposed that can account for the pattern of variation with [NAD^+] of the Michaelis–Menten parameters for hydroxylamine and for NADH with hydroxylamine or cytochrome *c* as oxidized substrate.

The NADH-dependent nitrite reductase from *Escherichia coli* K12 (NADH:nitrite oxidoreductase, EC 1.6.6.4) catalyses both the six-electron reduction of nitrite and the two-electron reduction of hydroxylamine to ammonia (Coleman *et al.*, 1978). Prosthetic groups include sirohaem, FAD and one or more iron–sulphur centres (Jackson *et al.*, 1981*a*). The enzyme is activated by the oxidized product NAD^+, but inhibited by high concentrations of the reduced substrate, NADH. These kinetic characteristics are consistent with a model that postulates that there is a single pyridine nucleotide binding site: according to this model, NAD^+ activates by antagonizing over-reduction of the enzyme by NADH (Scheme 1 in Jackson *et al.*, 1981*b*).

Nitrite reductase also catalyses the reduction of the single-electron acceptors horse-heart cytochrome *c*, dichlorophenolindophenol and $K_3Fe(CN)_6$ by NADH. These activities are independent of the haem group (Jackson *et al.*, 1981*a*). Similar 'diaphorase' activities are detected with other flavoproteins that are specific for reduced pyridine nucleotides, and these activities are also mediated by the flavin prosthetic group (Massey *et al.*, 1969). According to the scheme postulated by Jackson *et al.* (1981*b*), the rate-limiting step for nitrite reduction occurs after the formation of the two-electron-reduced enzyme species, EH_2: if this is correct, the diaphorase activity of nitrite reductase should be more sensitive than the rates of reduction of nitrite or hydroxylamine to changes in the concentrations of NAD^+ and NADH. A kinetic analysis of the diaphorase activity has therefore been undertaken to test this prediction and to provide further insight into the sequence of electron transfer reactions during the reduction of nitrite or hydroxylamine.

* Present address: Molecular Biology Division, Veterans' Administration Hospital, University of California, San Francisco, CA 94121, U.S.A.
† To whom correspondence should be addressed.

Materials and methods

Enzyme and substrate

Nitrite reductase was purified from *E. coli* strain 0R75Ch15 as described by Jackson *et al.* (1981*a*),

0306-3275/82/050505-06$01.50/1

and appeared to be more than 90% pure as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Horse-heart cytochrome *c* (grade 3) was obtained from Sigma.

Measurement of NADH-dependent 'diaphorase' activities

Open sample and reference cuvettes (1.5ml) contained various concentrations of NAD^+, NADH and cytochrome *c* or $K_3Fe(CN)_6$ in 1ml of buffer [50mM-Tris/HCl (pH8.0)/5mM-EDTA] at 30°C. The reaction was initiated by adding the enzyme to the sample cuvette. 'Diaphorase' activities were calculated from the rate of change of absorbance at 340nm for reduction of $K_3Fe(CN)_6$ or at 550nm for reduction of cytochrome *c*. Rates were corrected for the non-enzymic reduction of $Fe(CN)_6^{3-}$ by NADH. Initial rates were calculated from orthogonal polynomials fitted to the progress curves (Booman & Niemann, 1956). The molar absorbance coefficient for reduced minus oxidized cytochrome *c* was taken to be $\varepsilon_{550} = 2.1 \times 10^4 cm^{-1} \cdot M^{-1}$ (Masters *et al.*, 1965).

For each determination of Michaelis–Menten parameters, eight concentrations of the varied substrate were used, three of the determinations being in duplicate. Values of the parameters were calculated as in Jackson *et al.* (1981*b*). The ranges of substrate concentrations used were 3–100μM for cytochrome *c*, 0.1–2.0mM for $Fe(CN)_6^{3-}$, 5–200μM for NADH when NH_2OH was the oxidant and from 5μM to a concentration above which deviation from Michaelis–Menten kinetics was readily apparent with cytochrome *c* as oxidant.

Each point in the Figures represents a single determination, the same volume of the same preparation being used for all determinations.

Results and discussion

The peak of NADH-dependent cytochrome *c* reductase eluted from the DEAE-Sephadex column in the last step of the purification procedure was completely coincident with the peak of nitrite reductase activity (Fig. 1); the ratio of the two activities across the peak was 5.56 ± 0.16. By the criterion of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, the enzyme eluted from this column was 95% pure and no single contaminant accounted for more than 1% of the total protein. It follows that the cytochrome *c* reductase activity is almost certainly due to nitrite reductase rather than to a contaminant, because such a contaminant, if it existed, would need to have a catalytic constant of at least $1.4 \times 10^5 s^{-1}$ to account for the observed behaviour.

To establish the stoichiometry of the reduction of cytochrome *c* by NADH, the amount of NADH

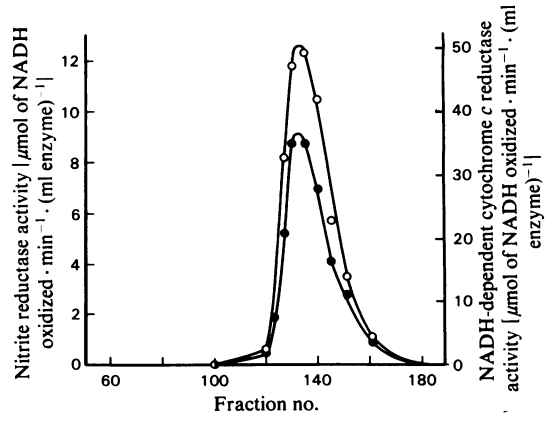


Fig. 1. *Coincidence of nitrite reductase and diaphorase activities*

Elution profiles from the DEAE-Sephadex column in the last step of the purification procedure are shown for NADH-dependent cytochrome *c* reductase activity (○) and for nitrite reductase activity in the presence of 0.1mM-NADH, 0.2mM-NAD^+ and 2mM-NO_2^- (●).

oxidized during the reduction of a fixed amount of cytochrome *c* was measured by the change in absorbance at 340nm and compared with the change in absorbance at 550nm. Results were corrected for the amount of NADH oxidized in the absence of cytochrome *c*. For every NADH molecule oxidized, 1.96 ± 0.08 (five determinations, S.E.M.) molecules of cytochrome *c* were reduced.

The values of $K_m^{app.}$ and $V^{app.}$ for reduction of cytochrome *c* and $Fe(CN)_6^{3-}$ were determined with 0.2mM-NADH and 0.7mM-NAD^+ (Table 1). The similar values of $V^{app.}$ for the reduction of cytochrome *c* and $Fe(CN)_6^{3-}$ suggest that the rate-limiting step is the same for the two reactions. Furthermore, similar rates of oxidation of 0.2mM-NADH were obtained with either 1mM-$Fe(CN)_6^{3-}$ or 0.1mM-cytochrome *c*, when the NAD^+ concentration was 0.7, 1.0 or 5.0mM.

The addition of 1mM-NO_2^- to assay mixtures did not significantly lower the rate of NADH-dependent reduction of cytochrome *c*, as measured by the change in absorbance at 550nm (Table 2). Further, the rate of NADH oxidation with 1mM-NO_2^- and 1mM-$Fe(CN)_6^{3-}$ is the same as, or a little more than, the rate with 1mM-$Fe(CN)_6^{3-}$ alone. If NO_2^- competed for the same site as cytochrome *c* and $Fe(CN)_6^{3-}$, it would be expected to have an apparent inhibition constant equal to its K_m (Cornish-Bowden, 1979), and hence to decrease the rate of NADH-dependent reduction of cytochrome *c* by 77% and that of $Fe(CN)_6^{3-}$ by 90%. That this was not observed confirms the conclusion from the effects of

Table 1. *Kinetic constants for diaphorase reactions*

All kinetic constants were determined in the presence of 0.7mM-NAD^+. Reactions were initiated with 20μl samples of enzyme (5nkat of nitrite reductase/ml when hydroxylamine was the oxidized substrate; 1nkat/ml for cytochrome *c* reduction). Values of $V^{app.}$ have been corrected for this difference in enzyme concentration. Abbreviation: n.d., not determined with this enzyme preparation.

Variable substrate	Constant substrate	$K_m^{app.}$ (μM)	$V^{app.}$ [μmol of NADH oxidized · min^{-1} · (ml enzyme)$^{-1}$]
Cytochrome *c*	0.2mM-NADH	3.9	27.9
$K_3Fe(CN)_6$	0.2mM-NADH	100	29.8
NADH	0.1mM-cytochrome *c*	48.8	34.4
NO_2^-	0.2mM-NADH	11.0	n.d.
NH_2OH	0.2mM-NADH	2910	15.0

Table 2. *Competition between diaphorase acceptors and nitrite during NADH-dependent reduction*

All reaction mixtures contained 0.7mM-NAD^+.

Experiment	Substrates added	Rate ± standard deviation [μmol of NADH oxidized · min^{-1} · (ml of enzyme)$^{-1}$]
Effect of NO_2^- on cytochrome *c* reduction measured at 550nm	200μM-NADH, 0.1mM-cytochrome *c*	36.3 ± 1.3
	200μM-NADH, 0.1mM-cytochrome *c*, 1mM-NO_2^-	35.9 ± 0.7
Competition between $Fe(CN)_6^{3-}$ and NO_2^- for NADH oxidation, measured at 340nm	200μM-NADH, 1mM-NO_2^-	7.5 ± 0.2
	200μM-NADH, 1mM-$Fe(CN)_6^{3-}$	42.1 ± 1.5
	200μM-NADH, 1mM-$Fe(CN)_6^{3-}$, 1mM-NO_2^-	42.7 ± 1.5
Competition between cytochrome *c* and NO_2^- for NADH oxidation, measured at 340nm	100μM-NADH, 1mM-NO_2^-	4.5
	100μM-NADH, 0.1mM-cytochrome *c*	33.1 ± 1.1
	100μM-NADH, 0.1mM-cytochrome *c*, 1mM-NO_2^-	33.7 ± 2.1

CN^- (Jackson *et al.*, 1981*a*) that NO_2^- and cytochrome *c* or $Fe(CN)_6^{3-}$ accept electrons at separate sites. It also suggests that the inhibition constant for NO_2^- is very high and that a slow step occurs during nitrite reduction after the formation of the reduced enzyme species that donates electrons to cytochrome *c* or $Fe(CN)_6^{3-}$.

Effect of NAD^+ on the rate of cytochrome c reduction by NADH

The rate of cytochrome *c* reduction was determined by measuring the rate of increase in absorbance at 550nm with 200μM-NADH and 0.1mM-cytochrome *c* but no added NAD^+. The rate was not constant but increased with time, increasing by 40% in the first 30s after mixing. A similar acceleration was observed when the rate of oxidation of NADH by NO_2^- or NH_2OH was monitored (Coleman *et al.*, 1978). This activation of cytochrome *c* reduction by NAD^+ is, however, the first direct demonstration that the formation of reduced product accelerates concomitantly with oxidation of NADH. As was observed for the reduction of NO_2^- and hydroxylamine (Jackson *et al.*, 1981*b*), NADH is a substrate inhibitor of cytochrome *c* reduction. A plot of the rate of reaction against [NADH] at 0.05mM-NAD^+ and 0.1mM-cytochrome *c* deviated from the pattern predicted by the Michaelis–Menten equation, the rate decreasing as [NADH] increased from 200μM to 800μM.

The effect of NAD^+ on the Michaelis–Menten parameters for NADH was investigated with 0.1mM-cytochrome *c* as constant substrate. Over the range 0.02–0.5mM, $V^{app.}$ increased sharply before reaching a plateau or slowly declining at higher [NAD^+] (Fig. 2*a*). $K_m^{app.}$ for NADH increased non-linearly with [NAD^+] (Fig. 2*b*), as did $K_m^{app.}/V^{app.}$. Thus, the variation of $K_m^{app.}$ and $K_m^{app.}/V^{app.}$ for NADH with [NAD^+] was qualitatively similar to that when 100mM-hydroxylamine was the oxidized substrate (Jackson *et al.*, 1981*b*).

There were three striking differences in the behaviour of the Michaelis–Menten parameters for NADH when 0.1mM-cytochrome *c* rather than 100mM-hydroxylamine was the oxidized substrate. First, the values of $V^{app.}$ for oxidation of NADH were much higher for oxidation of cytochrome *c* than for oxidation of hydroxylamine (Fig. 2*a*). This may be due to an extra rate-limiting step during hydroxylamine reduction that is not involved in cytochrome *c* reduction. Alternatively, there may be nitrite reductase molecules that lack sirohaem in

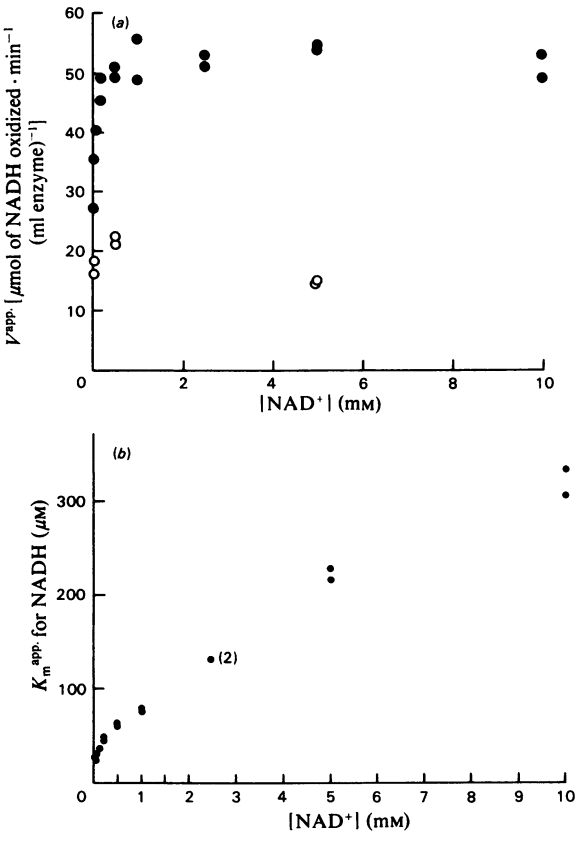


Fig. 2. *Variation with [NAD$^+$] of the apparent Michaelis–Menten parameters for NADH for the NADH-dependent reduction of cytochrome c*

Measurements were made in 50mM-Tris/HCl buffer at pH 8.0 and 30°C in the presence of 5mM-EDTA. Experiments were done with 0.1mM-cytochrome *c* (●) as substrate or with 100mM-hydroxylamine (○) for comparison, and a single enzyme preparation was used for all determinations. (*a*) Variation of $V^{app.}$ with [NAD$^+$]; (*b*) variation of $K_m^{app.}$ with [NAD$^+$].

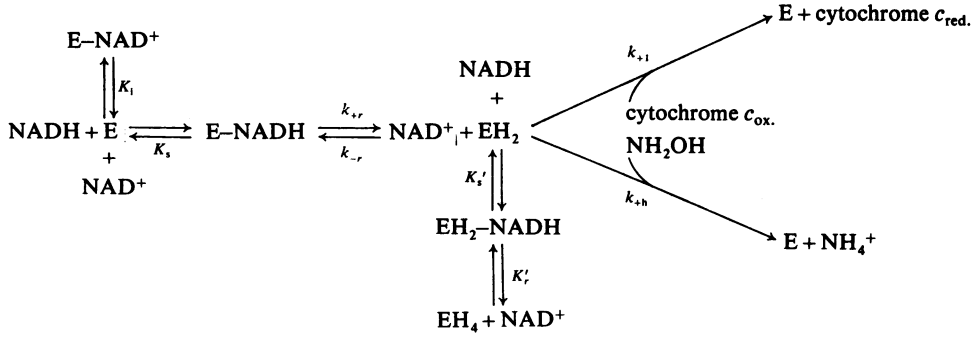
spite of retaining FAD: such molecules might be able to catalyse the NADH-dependent reduction of cytochrome *c* but not that of hydroxylamine. Although such heterogeneity in the enzyme preparation might contribute to the differences in $V^{app.}_{NADH}$ for NADH, the ε_{386} for the preparation of nitrite reductase used in this comparison was 5.6×10^4. Assuming that the molar absorbance at 386nm of sirohaem in *E. coli* nitrite reductase is the same as in spinach nitrite reductase [$7.6 \times 10^4 cm^{-1} \cdot (M\ haem)^{-1}$; Lancaster *et al.*, 1979], this preparation contained 0.69mol of sirohaem per mol of subunit. This suggests that heterogeneity may be insufficient to account for the full difference observed. As the sirohaem content of nitrite reductase was not determined by a rigorous method, however, independent confirmation of the presence of two slow steps during hydroxylamine reduction is required. The molar catalytic activity of the NADPH-dependent sulphite reductase of *E. coli* (which also contains sirohaem) for cytochrome *c* reduction is also substantially higher than for reduction of hydroxylamine, NO_2^- or SO_3^{2-} (Siegel *et al.*, 1974). For this enzyme, the reduction of FAD by NADPH appears to be rate-limiting for the NADPH-dependent reduction of cytochrome *c* (Siegel *et al.*, 1971). Two further steps, electron transfer between flavin and haem, and release of S^{2-} from sirohaem appear sufficiently slow to be rate-limiting for SO_3^{2-} reduction (Siegel & Kamin, 1968; Rueger & Siegel, 1976).

The second difference from the behaviour with hydroxylamine was that activation by NAD$^+$ is more pronounced for the reduction of cytochrome *c*. Thus, $V^{app.}$ for cytochrome *c* reduction increases by 80% on increasing the NAD$^+$ concentration from 0.025mM to 0.5mM, whereas for hydroxylamine reduction it increases by only 41% over the same range. The simplest explanation is that NAD$^+$ activation of hydroxylamine reduction derives solely from activation of steps involved in the reduction of cytochrome *c*, a flavin-mediated reaction that is not dependent on the haem and is only partially rate-limiting in the reduction of hydroxylamine.

The third difference in the behaviour with cytochrome *c* is that the sharp decline in $V^{app.}$ for NADH between 0.5mM-NAD$^+$ and 5mM-NAD$^+$ seen with hydroxylamine as oxidized substrate does not occur with 0.1mM-cytochrome *c*. Although this may be because cytochrome *c* reduction follows a different type of mechanism from hydroxylamine reduction, the common features of the two activities suggest that the mechanisms are similar: both activities are activated by NAD$^+$, inhibited by the substrate NADH and show qualitatively similar variation of $K_m^{app.}$ and $K_m^{app.}/V^{app.}$ for NADH with NAD$^+$.

Model for the mechanism of the NADH-dependent reduction of cytochrome c and hydroxylamine

A model similar to Scheme 1 of Jackson *et al.* (1981*b*) can be applied to the NADH-dependent reduction of cytochrome *c* (Scheme A). Again, NADH can 'over-reduce' the enzyme to an inactive form EH_4. NAD$^+$ inhibits as a product inhibitor by reoxidizing EH_2 to E and by competing with NADH for binding to E. NAD$^+$ activates by reoxidizing EH_4 to EH_2. Scheme A differs from Scheme 1, however, in not assuming that the steps forming EH_2 are at equilibrium. Thus it does not assume that the formation of EH_2 is much faster than the rate of



Scheme A. *Model for the reduction of cytochrome c and hydroxylamine by NADH*

This model differs from Scheme 1 of Jackson *et al.* (1981*b*) by the inclusion of the reaction of EH_2 with oxidized cytochrome *c* and by not treating the reaction between E–NADH and $NAD^+ + EH_2$ as an equilibrium. The equation derived from this model with the equilibrium and rate constants shown is given in the text. Some of the reactions are treated as equilibria to avoid making the analysis more complicated than the experimental data justify. If this were not done the range of behaviour permitted by the model would be increased, but no behaviour allowed by the model as shown would be excluded.

reoxidation of EH_2 by cytochrome *c*, rate constant k_{+1}. Indeed, it is rare for electron transfer to the oxidant to be rate-limiting in multi-centre redox enzymes (Palmer & Olson, 1980), and so it is more likely that k_{+1} is much greater than k_{+r}, the rate constant for the reduction of nitrite reductase by NADH to give EH_2 with release of the oxidized product NAD^+.

The equation derived from this model for the reduction of cytochrome *c* by NADH is as follows:

$$v = \frac{k_{+r}e_0}{1 + \frac{K_s}{[NADH]} + \frac{[NAD^+]K_sk_{-r}}{[NADH]k_{+1}} + \frac{[NAD^+]k_{-r}}{k_{+1}} + \frac{k_{+r}}{k_{+1}} + \frac{K_s[NAD^+]}{K_i[NADH]} + \frac{[NAD^+]^2K_sk_{-r}}{[NADH]k_{+1}K_i} + \frac{[NADH]k_{+r}}{K_s'k_{+1}} + \frac{[NADH]k_{+r}K_r'}{[NAD^+]k_{+1}K_s'}}$$

It is consistent with the observed non-linear increase in $K_m^{app.}$ for NADH with increasing $[NAD^+]$ and with the apparent deviation from linearity in the increase with $[NAD^+]$ of $K_m^{app.}/V^{app.}$ for NADH. The plateau or only marginal decline in $V^{app.}$ for NADH at high $[NAD^+]$ is consistent with the model if $k_{+1} \gg k_{-r}[NAD^+]$ for $[NAD^+]$ up to 10mM.

The equation derived from Scheme A for the dependence of the rate of NADH oxidation on [NADH] and $[NAD^+]$ at 100mM-hydroxylamine is the same as the above equation with k_{+1} replaced by k_{+h} throughout. It is of the same form as the equation derived from Scheme 1 of Jackson *et al.* (1981*b*); it therefore predicts the same pattern of variation in the apparent Michaelis–Menten parameters for NADH with $[NAD^+]$ and is compatible with the observed inhibition characteristics of NAD^+ with respect to NADH (Jackson *et al.*, 1981*b*). The rate constant k_{+h} is the rate constant for the slowest step of the following four: (i) the intramolecular transfer of electrons to sirohaem in the two-electron reduced form EH_2; (ii) the binding of hydroxylamine to sirohaem; (iii) the reduction of hydroxylamine; (iv) release of NH_4^+. Scheme A can explain the sharp decline in $V^{app.}$ for NADH when hydroxylamine is the oxidized substrate (Jackson *et al.*, 1981*b*; Fig. 2*a*) if the rate constant k_{+h} is not much greater than $k_{-r}[NAD^+]$. An increase in $[NAD^+]$ will lower $V^{app.}$ for NADH by driving the reaction $E\text{-}NADH \rightleftharpoons EH_2 + NAD^+$ to the left and therefore lowering the concentration of EH_2 in the steady state. As noted above, if Scheme A applies, $k_{+1} \gg k_{-r}[NAD^+]$ for $[NAD^+]$ up to 10mM. This model therefore suggests that k_{+h} is substantially lower than k_{+1}. The data are compatible with $E\text{–}NADH \rightleftharpoons EH_2 + NAD^+$ being rate-limiting for cytochrome *c* and hydroxylamine reduction. If, additionally, the rate of re-oxidation of EH_2 by hydroxylamine was slow enough to limit the overall rate of hydroxylamine reduction, then the model would still be consistent with the data.

R. H. J. was supported by a Research Studentship from the Science Research Council.

References

Booman, K. A. & Niemann, C. (1956) *J. Am. Chem. Soc.* **78**, 3642–3646

Coleman, K. J., Cornish-Bowden, A. & Cole, J. A. (1978) *Biochem. J.* **175**, 495–499

Cornish-Bowden, A. (1979) *Fundamentals of Enzyme Kinetics*, pp. 82–85, Butterworths, London and Boston

Jackson, R. H., Cornish-Bowden, A. & Cole, J. A. (1981*a*) *Biochem. J.* **193**, 861–867

Jackson, R. H., Cole, J. A. & Cornish-Bowden, A. (1981*b*) *Biochem. J.* **199**, 171–178

Lancaster, J. R., Vega, J. M., Kamin, H., Orme-Johnson, N. R., Orme-Johnson, W. H., Krueger, R. J. & Siegel, L. M. (1979) *J. Biol. Chem.* **254**, 1268–1272

Massey, V., Müller, F., Feldberg, R., Schuman, M., Sullivan, P. A., Howell, L. G., Mayhew, S. G., Matthews, R. G. & Foust, G. P. (1969) *J. Biol. Chem.* **244**, 3999–4006

Masters, B. S. S., Kamin, H., Gibson, Q. H. & Williams, C. H., Jr. (1965) *J. Biol. Chem.* **240**, 921–931

Palmer, G. & Olson, J. S. (1980) in *Molybdenum and Molybdenum-Containing Enzymes* (Coughlan, M. P., ed.), pp. 187–220, Pergamon, Oxford

Rueger, D. C. & Siegel, L. M. (1976) in *Flavins and Flavoproteins: Proceedings of the 5th International Symposium* (Singer, T. P., ed.), pp. 610–620, Elsevier, Amsterdam

Siegel, L. M. & Kamin, H. (1968) in *Flavins and Flavoproteins: Proceedings of the 2nd International Symposium* (Yagi, K., ed.), pp. 15–40, University Park Press, Baltimore

Siegel, L. M., Kamin, H., Rueger, D. C., Presswood, R. P. & Gibson, Q. H. (1971) in *Flavins and Flavoproteins: Proceedings of the 3rd International Symposium* (Kamin, H., ed.), pp. 523–554, Butterworths, London

Siegel, L. M., Davis, P. S. & Kamin, H. (1974) *J. Biol. Chem.* **249**, 1572–1586