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Studies on Methanol Dehydrogenase from *Hyphomicrobium* X

ISOLATION OF AN OXIDIZED FORM OF THE ENZYME

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1. Double-reciprocal plots of initial reaction rates of methanol dehydrogenase [alcohol–(acceptor) oxidoreductase, EC 1.1.99.8] *in vitro* show patterns of parallel lines. The results with various methanol, ammonia and phenazine methosulphate concentrations can be described by an equation valid for a Ping Pong kinetic mechanism with three reactants. 2. The overall maximum velocity was the same for several primary alcohols, $C_{(2)}$-deuterated ethanols and different electron acceptors, but it was significantly lower for $C_{(1)}$-deuterated substrates. 3. Oxidation of the isolated enzyme with electron acceptors required the presence of ammonia and a high pH. The inclusion of cyanide or hydroxylamine during the incubation was essential to prevent enzyme inactivation. The absorbance spectrum of an oxidized form of the enzyme was clearly different from that of the isolated enzyme and the free radical was no longer present. On addition of substrate, the original absorption spectrum and electron-spin-resonance signal reappeared and a concomitant substrate oxidation was found. This reaction could be carried out at pH 7 and ammonia was not required. 4. Based on the activity of the enzyme with one-electron acceptors, the presence of a free radical and the kinetic behaviour, an oxidation of the enzyme via one-electron steps is proposed.

Methanol dehydrogenase [alcohol–(acceptor) oxidoreductase, EC 1.1.99.8] is an enzyme that is found in methylotrophic bacteria grown on methane or methanol. Anthony & Zatman (1967) were the first to describe some properties of the enzyme from *Pseudomonas* M27. *In vitro*, oxidation of primary alcohols or formaldehyde takes place at pH9 with phenazine methosulphate as an electron acceptor. Furthermore, this reaction requires ammonia or methylamine as activator. In the past decade, several methanol dehydrogenases with similar properties have been isolated from other organisms (an enumeration is given by Bamforth & Quayle, 1978), but no substantial further information about the reaction has been reported.

As previously described (Duine *et al.*, 1978), methanol dehydrogenase from *Hyphomicrobium* X contains an organic free radical and is active with one-electron acceptors. This can be interpreted as an indication that the transfer of redox equivalents proceeds via one-electron steps. To investigate the mechanism, we decided to start kinetic experiments and to study the redox state of the enzyme.

When methanol dehydrogenase is incubated with electron acceptor and activator in the absence of substrate, an unexpected large amount of electron acceptor becomes reduced (Anthony & Zatman, 1964*b*; Goldberg, 1976; Bamforth & Quayle, 1978). The same phenomenon was observed for the purified enzyme from *Hyphomicrobium* X, independent of the electron acceptors used (Duine *et al.*, 1978). Furthermore, we found that after the incubation the activity of the enzyme was lost. It will be clear that under such conditions, kinetic investigations are of little value. At the same time, the observed inactivation is probably responsible for the fact that, so far, the properties of an oxidized enzyme form have not been described.

Cyanide and hydroxylamine appeared to be inhibitors with respect to the alcohol substrate. Their presence during the incubation suppressed the reduction of the electron acceptor and, more important, prevented enzyme inactivation. By applying these observations, we were able to perform a preliminary kinetic analysis and to isolate an oxidized form of the enzyme.

Materials and Methods

Materials

[^{2}H]Methanol and all other chemicals were from E. Merck B.V., Amsterdam, The Netherlands,

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except for the other deuterated compounds, which were from C. Roth G.m.b.H., Karlsruhe, Germany, phenazine ethosulphate, which was from Sigma Chemical Company, St. Louis, MO, U.S.A., Bio-Gel P10, which was from Bio-Rad, Richmond, CA, U.S.A., and a μBondapak C18 column, which was from Waters Associates, Etten-Leur, The Netherlands. The preparation of enzyme and Wurster's Blue have been described previously (Duine *et al.*, 1978). Wurster's Blue is the free radical of *NNN'N'*-tetramethyl-*p*-phenylenediamine.

Kinetic measurements

Substrate and electron-acceptor concentrations are indicated in the Figures. To calculate the rates, a molar absorption coefficient of 22×10^3 litre · mol^{-1} · cm^{-1} for 2,6-dichlorophenol-indophenol (Armstrong, 1964) and of 9×10^3 litre · mol^{-1} · cm^{-1} for Wurster's Blue was used. The latter value was calculated from absorbance measurements at 600nm in the test buffer. The rates are expressed as nmol of 2,6-dichlorophenol-indophenol reduced/min at 22.5°C for an enzyme concentration of 0.1 μM [assuming a mol.wt. of 120000 (Duine *et al.*, 1978)]. In the case of Wurster's Blue, the rate observed was divided by 2 to compare it with the results obtained with the two-electron acceptor 2,6-dichlorophenol-indophenol. Test buffer contained 0.1 M-tetrasodium pyrophosphate, 50mM-NH_4Cl and was brought to pH9.0 with conc. HCl. For the test, KCN and 2,6-dichlorophenol-indophenol were added to a concentration of 1mM and 55μM respectively. The test mixture had a total volume of 1ml and the reaction was started by adding enzyme.

The measured values and concentrations were plotted by the method of Lineweaver & Burk (1934). Michaelis constants are given in mM. Linearity between the initial rate of the reaction and enzyme concentrations three times higher and five times lower was checked. The reaction was found to obey the Michaelis–Menten equation.

Methanol oxidation was followed by gas chromatography (Duine *et al.*, 1978). Cinnamyl alcohol oxidation was followed by high-pressure liquid chromatography (Duine & Frank, 1980) on μBondapak C18 with methanol/water (2:5, v/v) as the eluent. Cinnamyl alcohol was detected at 254nm and cinnamaldehyde was detected at 280nm.

Oxidized enzyme

To methanol dehydrogenase in 0.1 M-tetrasodium pyrophosphate, 50mM-NH_4Cl and 10mM-KCN, pH9.0, 2,6-dichlorophenol-indophenol was added to a concentration of 100μM and phenazine methosulphate to 1mM. The mixture was chromatographed immediately on a Bio-Gel P-10 column in 0.1 M-tetrasodium pyrophosphate buffer, pH9.0, at room temperature. In this procedure the use of Bio-Gel is essential because it was found that Sephadex material reduces the oxidized enzyme. Furthermore, it appeared on several occasions that buffers were contaminated by substrate, probably originating from the laboratory atmosphere.

X-band e.s.r. spectra were measured at room temperature in a standard flat cell (Duine *et al.*, 1978; Westerling *et al.*, 1979).

Results

Kinetic behaviour

Parallel lines in the Lineweaver–Burk plots (Figs. 1 and 2) were obtained for the kinetic measurements on varying the concentrations of methanol and phenazine methosulphate in the presence of 1mM-KCN. The same results were obtained for other substrates and electron acceptors. However, the behaviour of the enzyme was only verifiable over a limited range of concentrations, because at a certain phenazine methosulphate concentration, substrate inhibition occurred (Fig. 3). It is noteworthy, however, that the onset of substrate inhibition was somewhat variable. Extension of the experiments to very low concentrations of ammonia, phenazine methosulphate or methanol gave no reliable results

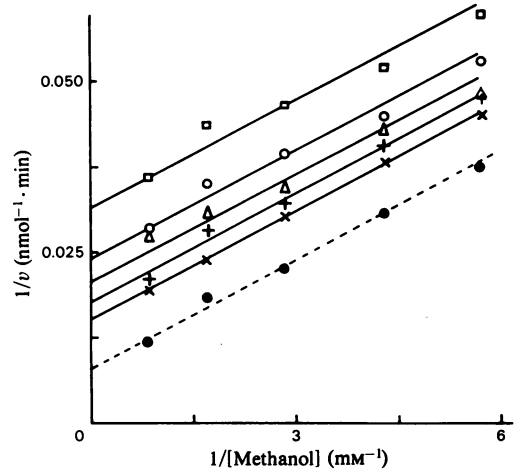

Fig. 1. *Initial-velocity analysis of methanol dehydrogenase with methanol as the variable substrate*
Test buffer contained 0.1 M-tetrasodium pyrophosphate, 50mM-NH_4Cl and was brought to pH9.0 with conc. HCl. For the test, KCN and 2,6-dichlorophenol-indophenol were added to a concentration of 1mM and 55μM respectively. The phenazine methosulphate concentration was held constant at 265μM (□), 398μM (○), 530μM (△), 663μM (+) and 795μM (×). The intercepts on the vertical axis of Fig. 2 were also replotted (●).

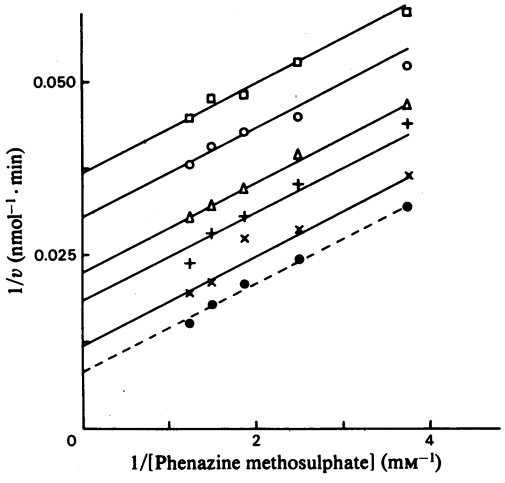

Fig. 2. *Initial-velocity analysis of methanol dehydrogenase with phenazine methosulphate as the variable substrate*

Assay components were as described in the legend to Fig. 1. The methanol concentration was held constant at 174 μM (□), 232 μM (○), 349 μM (△), 581 μM (+) and 1163 μM (×). The intercepts on the vertical axis of Fig. 1 were also replotted (●).

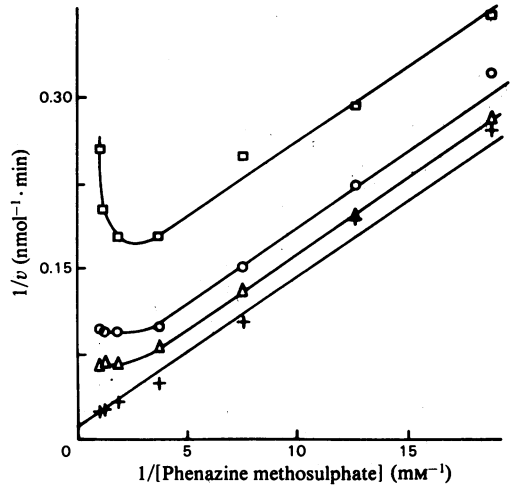

Fig. 3. *Substrate inhibition of methanol dehydrogenase by various concentrations of phenazine methosulphate*

Assay components were as described in the legend to Fig. 1. The methanol concentration was held constant at 23 μM (□), 58 μM (○), 136 μM (△) and 1163 μM (+).

because at rates below 5 nmol of 2,6-dichlorophenol-indophenol reduced/min, its reoxidation, in spite of the presence of cyanide, could no longer be neglected.

The patterns of the plots (Figs. 1 and 2) are indicative of Ping Pong-type kinetics. As was pointed out by Cleland (1970), substrate inhibition can be a valuable tool in deciding whether the observation of parallel lines is due to Ping Pong-type kinetics or to a low value of the constant term in the denominator of the rate equation for a sequential mechanism. Fig. 3 shows that substrate inhibition by phenazine methosulphate can be abolished by higher concentrations of methanol. The competitive substrate inhibition shown here may thus be ascribed to a combination of the electron acceptor with the wrong (i.e. oxidized) enzyme form and is a further confirmation of the Ping Pong-type kinetics.

Kinetic parameters

From the values of the apparent parameters in Tables 1 and 2, it appears that the maximum rate for undeuterated substrates and acceptors is roughly the same, although the values of the Michaelis constants are widely different. At the same time, it appears that the presence of an electron-donating or -withdrawing substituent on the $-CH_2OH$ group has no opposite effect on the value of the Michaelis constant, whereas the volume and the degree of saturation of the substituent do have to some extent.

Additional support for a Ping Pong-type kinetics can be found in the Tables. The Michaelis constant for methanol has the same value for different acceptors (Table 2), whereas the Michaelis constant for phenazine methosulphate is also the same for different substrates (Table 1).

By comparing the maximal rates of [2-$^2H_3$]-ethanol, [1-$^2H_2$]ethanol and ethanol (Table 1), it is clear that the oxidation involves a $C_{(1)}$-bound hydrogen atom.

Activators

A pattern of parallel lines is obtained by varying the ammonia concentration (Fig. 4). The results can be described by a Ping Pong rate equation for three reactants (Cleland, 1963):

$$v = \frac{V[A][D][N]}{K_A[D][N] + K_D[A][N] + K_N[A][D] + [A][D][N]} \quad (1)$$

where A, D and N stand for electron acceptor, substrate and activator respectively, K_A, K_D and K_N are the limiting Michaelis constants for each reactant, V is the limiting maximum rate and v the measured reaction rate. For a fixed ammonia concentration, eqn. (1) can be rearranged to give eqn. (2):

$$v = \frac{V'[A][D]}{K'_A[D] + K'_D[A] + [A][D]} \quad (2)$$

Table 1. *Apparent kinetic parameters for different substrates*

The test mixture contained 1mM-KCN, 50mM-NH_4Cl, 55μM-2,6-dichlorophenol-indophenol and 0.1M-tetrasodium pyrophosphate, pH9.0, with various concentrations of phenazine methosulphate and substrate. The results were calculated as described in Figs. 1 and 2. K'_A, K'_D and V' are the apparent kinetic parameters at a fixed concentration of ammonia and represent the Michaelis constant for electron acceptor, the Michaelis constant for the alcohol and the maximum rate respectively. Eqn. (2) gives the relationship between the symbols.

Substrate	K'_A (mM)	K'_D (mM)	V' (nmol of 2,6-dichlorophenol-indophenol reduced/min)
Methanol	1.0	0.3	115
Ethanol	0.9	0.5	100
Propan-1-ol	0.9	3.0	108
2-Chloroethanol	0.8	2.8	98
Allyl alcohol	0.8	1.5	101
3-Hydroxypropionic acid	1.0	48.8	111
Glycolamide	1.0	49.8	101
2-Phenylethanol	0.8	2.1	82
Cinnamyl alcohol	0.9	0.4	105
[^{2}H]Methanol	0.9	0.3	56
[$^2H_2$]Methanol	0.8	0.3	36
[$^2H_3$]Methanol	0.8	0.3	20
[2-$^2H_3$]Ethanol	0.8	0.8	95
[1-$^2H_2$]Ethanol	0.5	0.6	38

Table 2. *Apparent kinetic parameters for different electron acceptors*

Measurements were made with various methanol and acceptor concentrations. Further details are given in the legend to Table 1.

Electron acceptor	K'_A	K'_D	V'
Phenazine methosulphate	0.8	0.3	93
Phenazine ethosulphate	1.6	0.3	94
Wurster's Blue	0.3	0.4	90

Table 3. *The dependence of the parameters on ammonia concentration*

The measurements were performed with the indicated NH_4Cl concentration and various phenazine methosulphate and methanol concentrations.

[NH_4Cl] (mM)	K'_A	K'_D	V'
5	0.17	0.06	25
8.8	0.33	0.13	45
15	0.49	0.15	56
37.5	0.65	0.18	85

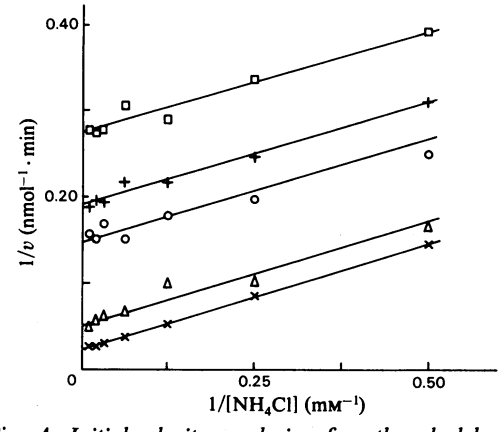

Fig. 4. *Initial-velocity analysis of methanol dehydrogenase with ammonia as variable substrate*

Assay components were as described in the legend to Fig. 1. The methanol and phenazine methosulphate concentrations (μM) were held constant respectively at 23 and 79 (□), 23 and 972 (+), 58 and 132 (○), 1163 and 265 (△) and 1163 and 927 (×).

Here the apparent kinetic parameters represent the limiting parameters multiplied by the factor $[N]/([N]+K_N)$. Eqn. (2) explains why increasing the ammonia concentration results in higher values for the kinetic parameters, as was found in Table 3. By replotting these data, the limiting kinetic parameters for the system methanol/phenazine methosulphate/ammonia were calculated: $V = 150$nmol of 2,6-dichlorophenol-indophenol reduced/min, $K_A = 1.2$mM, $K_D = 0.4$mM and $K_N = 24$mM. It is noteworthy that the value of K_D is not the true limiting parameter, because cyanide was present in the test system.

Anthony & Zatman (1964*b*) screened a range of nitrogen-containing compounds for their applicability as activators. Only ammonia and methyl-

amine were reported to be active. We found that the kinetic behaviour of methylamine was identical with that of ammonia, but a fairly high Michaelis constant of 1.9M was observed. In addition to kinetic measurements, the conversion of methanol over a longer time period was followed by gas chromatography to investigate the role of ammonia. The reaction progress curves indicated that the reaction rates increase with increasing ammonia concentrations, but the total amount of methanol that becomes oxidized is independent of the amount of ammonia. Thus ammonia is not consumed during the reaction and must therefore have a catalytic function.

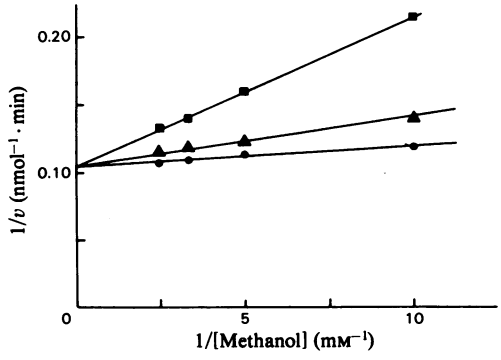

Fig. 5. *Inhibition of methanol dehydrogenase by KCN with methanol as the variable substrate*
Phenazine methosulphate and NH_4Cl concentrations were held constant at 100μM and 50mM respectively. The concentration of KCN was 5mM (■), 1mM (▲) and 0mM (●).

Inhibitors

Methanol oxidation in whole cells of *Pseudomonas* M27 is inhibited by several substances (Anthony & Zatman, 1964*a*). Of these compounds only hydroxylamine and cyanide were found to be inhibitors for the enzyme of *Hyphomicrobium in vitro*. From Fig. 5, it appears that cyanide is a linear competitive inhibitor for methanol with a K_I of 1mM.

Hydroxylamine was an even better competitive inhibitor and from the results shown in Table 4, a K_I of 12μM was calculated. The low value probably reflects the similarity in molecular shape of hydroxylamine and methanol. Concentrations of hydroxylamine greater than 100μM were detrimental to the assay as phenazine methosulphate was decomposed. Hydroxylamine and cyanide were reversible inhibitors because preincubation of the enzyme in their presence had no further influence on the activity.

Table 4. *Inhibition by hydroxylamine*
The text mixture contained the indicated hydroxylamine concentrations and various methanol and phenazine methosulphate concentrations.

[Hydroxylamine] (μM)	K'_A	K'_D	V'
20	0.8	1.1	93
40	1.0	1.8	85
60	0.9	2.5	110

Oxidized enzyme

Preincubation of the enzyme with electron acceptor and ammonia (in the absence of methanol and cyanide) under normal test conditions destroyed the activity (Table 5). The inactivation was strictly dependent on the simultaneous presence of phenazine methosulphate and ammonia. This is also true for the reaction-rate value of the blank in the

Table 5. *Enzyme inactivation by incubation with electron acceptor*
Enzyme was mixed at room temperature with phenazine methosulphate, NH_4Cl and pyrophosphate buffer, pH 9.0, with or without inhibitor. Then water (blank value) or methanol solution was added immediately ($t = 0$min) or after the indicated time of preincubation. The reaction rate (nmol of 2,6-dichlorophenol-indophenol reduced/min) was measured as described in the Materials and Methods section. The final concentrations were: 100μM-phenazine methosulphate, 200μM-methanol, 50mM-NH_4Cl and 55μM-2,6-dichlorophenol-indophenol.

		Reaction rate (nmol of 2,6-dichlorophenol-indophenol reduced/min)		
		v (blank value)	v (with methanol)	
Inhibitor	t (min) ...	0	0	4
None		5.46	8.33	3.15
1mM-KCN		0.21	8.54	7.70
10mM-KCN		0.00	5.46	5.46
10μM-Hydroxylamine		0.98	8.26	6.16
100μM-Hydroxylamine		0.00	4.55	4.62

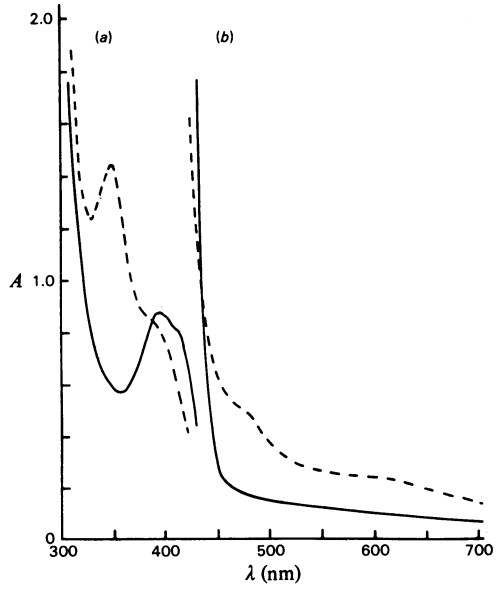

Fig. 6. *Absorption spectra of different forms of methanol dehydrogenase*

The oxidized enzyme form (5 mg/ml) was prepared as described in the Materials and Methods section in 0.1 M-tetrasodium pyrophosphate buffer, pH 9.0. ——, Oxidized enzyme form; ----, oxidized enzyme form with methanol added. (*a*) With the scale as indicated in the Figure; (*b*) scanned at a four-times-higher sensitivity.

test (Table 5). In this context, it is noteworthy that the same blank value was found for enzyme that was 'further' purified by gel filtration or electrophoresis. From these results, we concluded that methanol dehydrogenase becomes oxidized to a labile enzyme form. In that case, competitive inhibitors for methanol might be expected to protect the enzyme. Cyanide and hydroxylamine did, in fact, prevent inactivation (Table 5). It was therefore possible to stabilize and isolate a free intermediate. After oxidation with phenazine methosulphate/2,6-dichlorophenol-indophenol or Wurster's Blue, the oxidized enzyme was subsequently freed from low-molecular-weight substances by gel filtration. The isolated oxidized enzyme was also stable in other buffers, such as 0.1 M-potassium phosphate, pH 7.0.

It was not possible to measure the number of electrons involved in the oxidation of the enzyme. Decomposition of phenazine methosulphate and also of Wurster's Blue resulting in the production of the substrate formaldehyde (McIlwain, 1937), even under anaerobic conditions, and the presence of endogenous substrate made the experiment impossible.

In Fig. 6 the absorption spectrum of the oxidized enzyme is shown. The addition of substrate or a reducing substance such as $NaBH_4$ changed the absorption spectrum of the enzyme immediately, irrespective of the presence of ammonia (Fig. 6). On applying e.s.r. spectroscopy, no signal could be detected in the oxidized enzyme, but on addition of substrate the signal reappeared.

Discussion

The pronounced isotope effect on the maximal rate with a substrate containing a fully deuterated $C_{(1)}$ atom (Table 1), suggests that some hydrogen transfer step is rate determining. However, as the maximal rates for different undeuterated alcohols (Table 1) and different electron acceptors (Table 2) are the same, it remains undecided which step in the overall reaction is rate limiting.

A point that requires comment is the presence or absence of cyanide. Anthony & Zatman (1964*b*) originally included cyanide in their standard assay (but not for the determination of K_D), as is usually done for dehydrogenases that are tested with the electron-acceptor couple phenazine methosulphate/2,6-dichlorophenol-indophenol, to prevent oxidation of reduced electron acceptor (Singer, 1974). However, in more recent work (Anthony & Zatman, 1967), cyanide was omitted without comment. As other authors (Sperl *et al.*, 1974) refer to both of these articles for their test, it is not clear whether they added cyanide.

In the first instance, the behaviour in the test was as expected: in its absence rapid oxidation of reduced 2,6-dichlorophenol-indophenol, resulting in non-linear reaction progress curves, occurred. However, it also appeared that cyanide suppressed the blank value, prevented the inactivation of the enzyme during incubation with electron acceptors (Table 5) and behaved as a competitive inhibitor for methanol (Fig. 5).

In a recent paper (Bamforth & Quayle, 1978) concerning the enzyme from *Rhodopseudomonas acidophila*, it is reported that cyanide is a competitive inhibitor for the activator. In our case, cyanide is a competitive inhibitor for methanol (Fig. 5) and a non-competitive inhibitor for phenazine methosulphate or ammonia. Because the *Rh. acidophila* enzyme has some anomalous properties compared with other methanol dehydrogenases, this might account for the different behaviour towards the inhibitor.

Although the presence of cyanide circumvents serious shortcomings of the assay, it also has a disadvantage. Owing to the competitive substrate inhibition, the K_D value of 0.4 mM obtained for methanol will be too high. As cyanide competes with other substrates in the same way, the mutual

proportions of the K'_D values in Table 1 give a correct indication of the substrate specificity of the enzyme.

Although the remarkably high blank value was noticed by others, no corrections were made for it in the calculation of K_D (Anthony & Zatman, 1964*b*; Goldberg, 1976). However, as long as the quantity and nature of the endogenous substrate in the enyzme are unknown (see below), this blank value cannot be ignored. As the high blank value is always present, irrespective of how far the enzyme was purified, the enzyme itself must contain either reducing groups or a substrate bound to it. The first possibility is unlikely as the blank value is far too high. In view of this observation, it is understandable why the isolated methanol dehydrogenases are always in the reduced form and addition of methanol does not change their absorption spectra. As can be expected, it appeared that the oxidized form of the enzyme is completely devoid of endogenous substrate.

The role of the activators in the reaction mechanism is unclear at present because we could not detect a stable enzyme form in the absence of inhibitors. In any case, ammonia is not consumed in the reaction and the oxidized form of the enzyme requires ammonia after being reduced by substrate.

The absorption spectrum obtained after reaction of the oxidized form of the enzyme with different substrates was always the same (Fig. 6), suggesting a true reduction and not a stable enzyme–substrate adduct. Indeed, the same effect was also produced with $NaBH_4$. Furthermore, the spectral change could also be obtained in small steps by adding portions of cinnamyl alcohol and concomitantly an equivalent amount of cinnamaldehyde was produced. Reduction of the enzyme with substrate was possible at pH 7 in the absence of activator. Clearly, the need for a high pH and an activator concerns the reoxidation step of the enzyme.

As already mentioned, it was not possible to measure the number of electrons involved in the oxidation of the enzyme. Curiously, a free radical is found in the reduced enzyme and it is absent in the oxidized form. Unfortunately, the number of prosthetic groups per enzyme molecule is unknown for, in the case of a number larger than one, the presence of free radical in the reduced enzyme could be explained from an interconversion reaction. Apart from this aspect, it is obvious that the presence of free radical can be related to the oxidation state of the enzyme and to the prosthetic group (Westerling *et al.*, 1979), supporting the concept of an enzyme oxidation proceeding via one-electron steps.

Although Ping Pong kinetics are apparently involved, the particular fact that the sequence of addition and dissociation of activator is obscure makes it impossible to propose a pure Ping Pong mechanism at present. Assuming a sequential ordered mechanism, the observed Ping Pong kinetics can also be explained by the presence of rapid equilibrium conditions or steps separated by e.g. reduced electron acceptors (Cleland, 1970).

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