Biochem. J. (1978) **173**, 483–496
Printed in Great Britain

Kinetic and Mechanistic Studies of Methylated Liver Alcohol Dehydrogenase

By CHISHIUN S. TSAI
Department of Chemistry, Carleton University, Ottawa, Ont. K1S 5B6, Canada

(*Received* 7 *November* 1977)

Reductive methylation of lysine residues activates liver alcohol dehydrogenase in the oxidation of primary alcohols, but decreases the activity of the enzyme towards secondary alcohols. The modification also desensitizes the dehydrogenase to substrate inhibition at high alcohol concentrations. Steady-state kinetic studies of methylated liver alcohol dehydrogenase over a wide range of alcohol concentrations suggest that alcohol oxidation proceeds via a random addition of coenzyme and substrate with a pathway for the formation of the productive enzyme–NADH–alcohol complex. To facilitate the analyses of the effects of methylation on liver alcohol dehydrogenase and factors affecting them, new operational kinetic parameters to describe the results at high substrate concentration were introduced. The changes in the dehydrogenase activity on alkylation were found to be associated with changes in the maximum velocities that are affected by the hydrophobicity of alkyl groups introduced at lysine residues. The desensitization of alkylated liver alcohol dehydrogenase to substrate inhibition is identified with a decrease in inhibitory Michaelis constants for alcohols and this is favoured by the steric effects of substituents at the lysine residues.

Lysine residues have been implicated as essential to the activity of various dehydrogenases (Degani *et al.*, 1974; Fan & Plaut, 1974). Picolinimidylation (Plapp, 1970) and methylation (Tsai *et al.*, 1974) of lysine residues activates liver alcohol dehydrogenase (alcohol–NAD oxidoreductase, EC 1.1.1.1), whereas phosphopyridoxylation (McKinley-McKee & Morris, 1972) inactivates the enzyme. This difference in the effect on modification of liver alcohol dehydrogenase has been attributed to a change in the charge of lysine residues. Kinetic studies of picolinimidylated liver alcohol dehydrogenase indicate that both the native and modified enzymes followed the Ordered Bi Bi mechanism. The activation of picolinimidylated enzyme is associated with an increased dissociation constant of the enzyme–coenzyme complex and a possible change in the rate-limiting step (Plapp *et al.*, 1973). Both picolinimidylation and phosphopyridoxylation modify an identical lysine residue, which interacts with nicotinamide coenzymes (Dworschack *et al.*, 1975), whereas different lysine residue(s) are probably involved in methylation (Jörvall, 1973; Chen & Engel, 1975).

In view of the following observations, steady-state kinetic studies of methylated liver alcohol dehydrogenase were carried out over a wide concentration range of various alcohols to investigate the mechanism for the enhancement of ethanol oxidation after methylation of the enzyme. The observations are: (1) liver alcohol dehydrogenase is subject to substrate inhibition at high ethanol concentrations, and methylation results in partial desensitization to this effect (Tsai *et al.*, 1974); (2) kinetic parameters obtained at low ethanol concentrations do not satisfy relationships required by simple ordered mechanisms; (3) methylation activates liver alcohol dehydrogenase to oxidize primary alcohols, but deactivates the enzyme to catalyse the oxidation of secondary alcohols. A mechanism for the changes in the enzyme activity on methylation is proposed. Kinetic parameters associated with these changes are identified and factors that affect these kinetic parameters are discussed.

Materials and Methods

Chemicals

Alcohol dehydrogenase from horse liver (once crystallized; 1.7×10^{-8}–3.4×10^{-8} kat/mg), NAD^+ and NADH were from Sigma Chemical Co., St. Louis, MO, U.S.A. Deamino-NAD^+ and thionicotinamide-NAD^+ (thionicotinamide–adenine dinucleotide) were from P-L Biochemicals Inc., Milwaukee, WI, U.S.A. Ethanol was the product of Consolidated Alcohol, Toronto, Ont., Canada. Propan-1-ol, propan-2-ol, butan-1-ol, butan-2-ol and cyclohexanol were from Fisher Chemical Co., Ottawa, Ont., Canada, and distilled before use. Aldehydes were from Aldrich Chemical Co., Milwaukee, WI, U.S.A., and liquid aldehydes except formaldehyde were purified by distillation.

Pyrazole was the product of Eastman Organic Chemicals, Rochester, NY, U.S.A., and $NaBH_4$ was from BDH Chemicals, Toronto, Ont., Canada.

Methylation and alkylation of liver alcohol dehydrogenase

Methylation of liver alcohol dehydrogenase was carried out by a slight modification of the procedure previously described (Tsai *et al.*, 1974). First, 0.5ml of freshly prepared $NaBH_4$ solution (5.0mg/ml) was mixed with 4.5ml of solution containing 0.30μmol of the dehydrogenase [based on a mol.wt. of 84000 (Ehrenberg & Dalziel, 1958)] in 0.10M-sodium pyrophosphate buffer, pH9.0, on ice. Formaldehyde solution (6.0μmol in total volume of 0.2ml) was added in six portions over a 60min period. The reaction mixture was kept on ice for an additional 15min, then exhaustively dialysed against water and freeze-dried. The control was prepared by replacing 0.2ml of formaldehyde solution with water. Methylated enzyme protected with NADH was prepared by performing methylation in the presence of 11.4μmol (38-fold excess) of NADH. Alkylation of the enzyme was carried out by the procedure previously described (Tsai, 1977).

Kinetic studies

Kinetic studies were carried out in a Perkin–Elmer spectrophotometer (Coleman model 124) equipped with a variable-output recorder (Coleman model 165) and a water circulator maintained at 30±0.2°C.

For alcohol oxidation, concentrations of alcohols varied from 0.50 to 0.40M and of NAD^+ or its derivatives from 0.050 to 1.0mM in 0.10M-sodium pyrophosphate buffer, pH9.0. For aldehyde reduction, concentrations of acetaldehyde varied from 0.50 to 10mM and of NADH from 0.010 to 0.50mM in 0.10M-sodium pyrophosphate buffer, pH9.0. The reaction was initiated by the addition of 12nM of enzyme and the course of reaction was followed at 340nm for 2.0min. In experiments with deamino-NAD^+ or thionicotinamide-NAD^+ as the coenzyme, changes in A_{338} or A_{395} respectively were carried out in an identical manner.

Kinetic parameters of alkylated enzymes were analysed for substituent effects by the method described previously (Tsai, 1977).

Results

Table 1 lists values of kinetic parameters for ethanol oxidation in the asymptotic region (low ethanol concentrations, 0.50–10mM) according to:

$$v = \frac{V_1[A][B]}{[A][B]+K_a[B]+K_b[A]+K_{ia}K_b} \quad (1)$$

and acetaldehyde reduction according to:

$$v = \frac{V_2[P][Q]}{[P][Q]+K_p[Q]+K_q[P]+K_{iq}K_p} \quad (2)$$

V_1, V_2, K_a, K_b, K_p, K_q, K_{ia} and K_{iq} are maximum velocities, Michaelis constants and inhibition constants respectively [Cleland's (1963*a*) nomenclature]. [A], [B], [P] and [Q] are the NAD^+, ethanol, NADH and acetaldehyde concentrations respectively.

Wratten & Cleland (1963) gave 17.4μM, 0.55mM, 128μM, 0.244μM, 26.8mM and 1.50μM for K_a, K_b, K_{ia}, K_p, K_q and K_{iq} respectively at pH7.15. The corresponding values reported by Plapp (1970) at pH9.0 were 36.3μM, 0.778mM, 40.3μM, 0.520μM, 6.65mM and 6.67μM respectively. These values agree

Table 1. *Kinetic parameters for ethanol oxidation and acetaldehyde reduction catalysed by control and modified enzyme at 30.0°C and pH 9.0*

Initial velocities in the asymptotic region were analysed by the weighted least-squares method according to eqns. (1) and (2) by using a FORTRAN program described previously (Dove & Tsai, 1976).

	Control enzyme	NADH-protected methylated enzyme	Methylated enzyme
V_1 (μM/min)	11.3 ±0.2	14.8 ±0.4	129 ±15
K_a (μM)	45.1 ±6.9	64.7 ±6.0	353 ±34
K_b (mM)	0.793±0.063	0.759±0.065	3.97±0.32
K_{ia} (μM)	50.1 ±12	59.6 ±6.2	447 ±22
$10^{-3}\times V_1/E_t$ (min^{-1})	0.942	1.23	10.8
$10^{-3}\times V_1\cdot K_{ia}/E_t\cdot K_a$ (min^{-1})	1.05	1.13	13.7
V_2 (μM/min)	14.6 ±0.3	16.4 ±0.3	304 ±8.2
K_p (mM)	0.313±0.068	0.368±0.057	1.92±0.37
K_q (μM)	7.56 ±0.06	6.08 ±0.46	37.2 ±6.1
K_{iq} (μM)	10.43 ±2.9	13.1 ±1.1	45.3 ±3.8
$10^{-3}\times V_2/E_t$ (min^{-1})	1.22	1.37	25.3
$10^{-3}\times V_2\cdot K_{iq}/E_t\cdot K_q$ (min^{-1})	1.68	2.95	30.8
V_1K_{ia}/V_2K_a	0.860	0.831	0.537

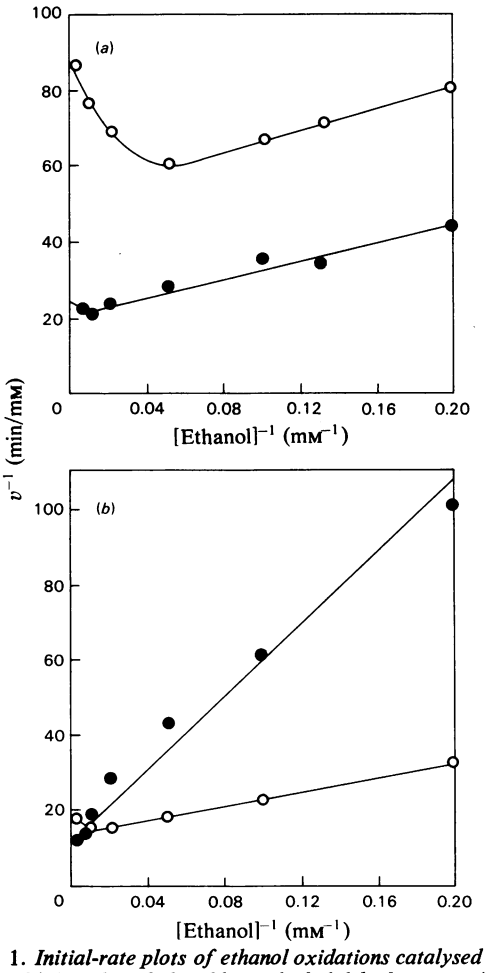


Fig. 1. *Initial-rate plots of ethanol oxidations catalysed by control* (○) *and methylated liver alcohol dehydrogenase* (●) *with* 0.20 mM-*deamino-NAD*$^+$ (*a*) *or* 0.20 mM-*thionicotinamine-NAD*$^+$ (*b*) *as cofactor*

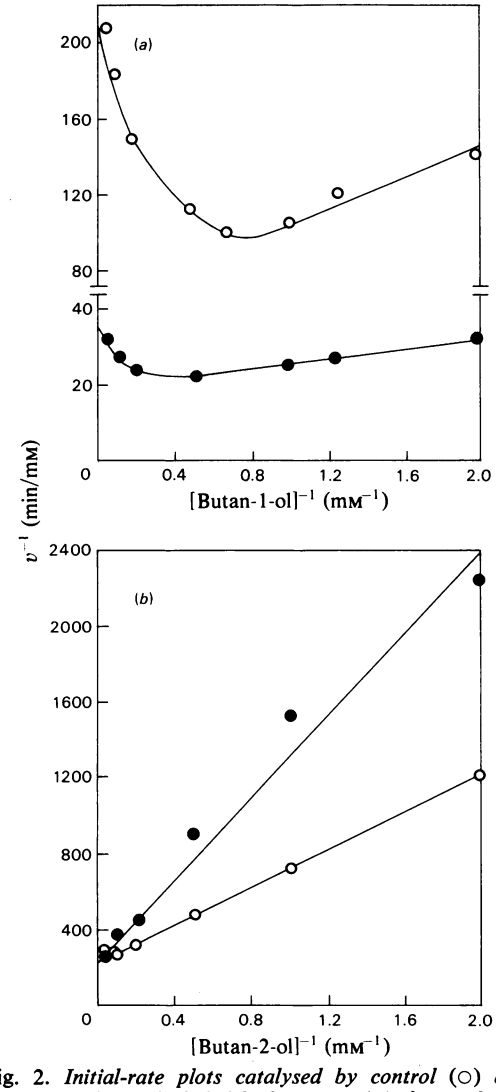


Fig. 2. *Initial-rate plots catalysed by control* (○) *and methylated liver alcohol dehydrogenase* (●) *for oxidation of buton-1-ol* (*a*) *and butan-2-ol* (*b*) *at constant* (0.2 mM) *NAD*$^+$

well with those found in the present study for the control enzyme that had been treated with $NaBH_4$. Methylation results in an increase in the catalytic-centre activity of both forward and reverse reactions. Since all kinetic parameters increase in varying degrees on methylation, it is difficult to identify specific kinetic parameter(s) or step(s) that are associated with the enhanced activity of methylated enzyme to oxidize ethanol. However, unexpected results were observed for reactions with NAD$^+$ derivatives of alcohol analogues. The modified enzyme exhibits an enhanced activity for ethanol oxidation with NAD$^+$ (Tsai *et al.*, 1974) and deamino-NAD$^+$ (Fig. 1*a*), but a lower activity with thionicotinamide-NAD$^+$ (Fig. 1*b*). Similarly, methylated enzyme shows higher activities than the control for the oxidation of primary alcohols, but lower activities for the oxidation of secondary alcohols (Fig. 2). These observations are particularly noteworthy, because they show the necessity for carrying out detailed kinetic studies to gain useful information from chemical modifications of an enzyme.

Liver alcohol dehydrogenase is subject to substrate inhibition at high alcohol concentrations. Within the concentration range used in this study, double-reciprocal plots for ethanol were non-linear, showing

minima at 15±5mM for the control and 100±10mM for methylated enzyme. However, double-reciprocal plots for NAD^+ (primary plots) were linear, from which intercepts and slopes at different fixed alcohol concentrations can be estimated. Replots (secondary plots) of intercepts and slopes versus reciprocal concentrations of alcohol are non-linear (Figs. 3 and 4), consisting of two concentration regions: asymptotic (low alcohol concentrations) region and concave (high alcohol concentrations) region. In the concave region, the concavity approaches the linear asymptote from either above (Fig. 3) or below (Fig. 4), depending on the relative magnitudes of some kinetic coefficients (see the Appendix).

From linear asymptotes, the kinetic parameters V_1, K_a, K_b and K_{ia} were evaluated. To assess the kinetic behaviour of liver alcohol dehydrogenase at high alcohol concentrations, which is profoundly affected by methylation, new operational kinetic parameters, namely maximum velocity (V_1'), Michaelis constant for NAD^+ (K_a'), Michaelis constant for alcohol (K_b') and composite constant for NAD^+ and alcohol (K_{ab}') for the concave region were defined to facilitate analyses of kinetic effects of methylated enzyme at high alcohol concentrations. Since the slopes of some secondary plots (for primary alcohols) are negative (Fig. 3), the inhibitory Michaelis constant, K_{-b}', and composite constant, K_{-ab}', are shown with a subscript negative sign to indicate the negative slope and identify them with an inhibitory effect, such that the larger the constants the greater their inhibitory effect. Consequently K_b' and K_{ab}', associated with the activation, show different kinetic coefficients from K_{-b}' and K_{-ab}', which are associated with substrate inhibition (see the Appendix). These operational kinetic parameters were estimated from the linear segment of the concave region of the secondary plots. Tables 2 and 3 list kinetic parameters for the oxidation of alcohol analogues and ethanol with coenzyme derivatives in both the asymptotic region and the concave region. By comparison, V_1 and V_1' for methylated enzyme are higher for primary

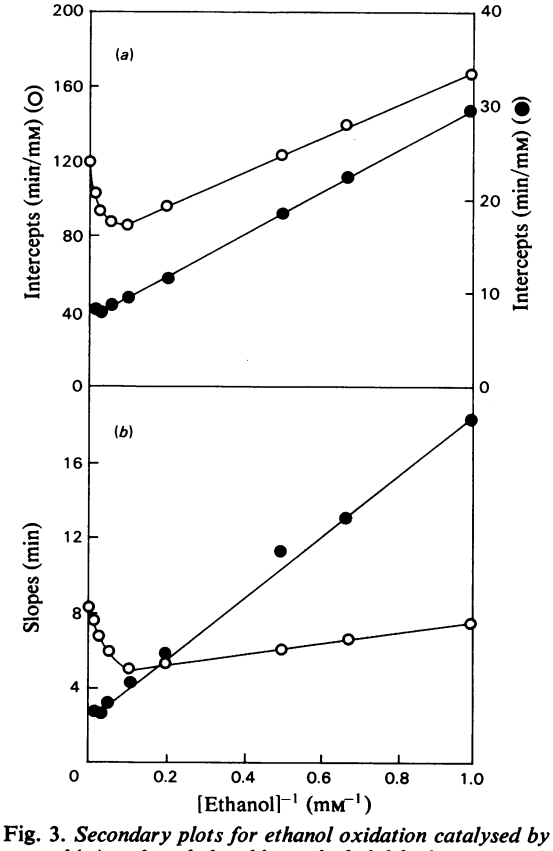


Fig. 3. *Secondary plots for ethanol oxidation catalysed by control* (○) *and methylated liver alcohol dehydrogenase* (●) *showing substrate inhibition*

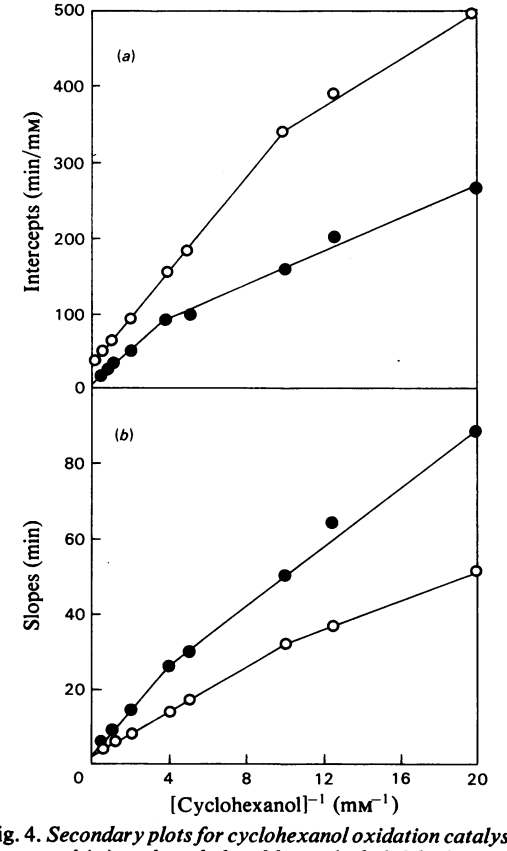


Fig. 4. *Secondary plots for cyclohexanol oxidation catalysed by control* (○) *and methylated liver alcohol dehydrogenase* (●) *showing substrate activation*

Table 2. *Kinetic parameters for reactions catalysed by control liver alcohol dehydrogenase at* 30°*C and pH* 9.0
Kinetic parameters for the asymptotic and concave regions were defined and estimated as described in the Appendix.

Kinetic parameter	Reaction ...	NAD^++ ethanol	Thionicotin-amide-NAD^++ ethanol	Deamino-NAD^++ ethanol	NAD^++ propan-1-ol	NAD^++ propan-2-ol	NAD^++ butan-1-ol	NAD^++ butan-2-ol	NAD^++ cyclohexanol
V_1 (μM/min)		13.5	105	23.3	13.8	0.980	17.7	4.88	5.56
K_a (μM)		62.2	66.3	76.7	26.2	46.1	30.9	18.5	66.7
K_b (mM)		1.27	6.32	0.814	0.210	1.07	0.270	1.84	0.0900
K_{ia} (μM)		29.8	144	100	19.2	63.8	23.8	59.5	122
V_1' (μM/min)		7.41	74.6	11.6	8.00	2.78	7.87	3.23	28.6
K_a' (μM)		63.7	76.1	27.9	76.8	16.7	122	38.7	57.1
K_b' (mM)						2.33			0.829
K'_{-b} (mM)		23.0	29.1	25.6	2.88		5.62	1.50	
K'_{ab} (mM²)				0.640		0.153			0.0423
K'_{-ab} (mM²)		1.27	3.32		0.300		0.888	0.106	

Table 3. *Kinetic parameters for reactions catalysed by methylated liver alcohol dehydrogenase at* 30°*C and pH* 9.0
Kinetic parameters for the asymptotic and concave regions were defined and estimated as described in the Appendix.

Kinetic parameter	Reaction ...	NAD^++ ethanol	Thionicotin-amide–NAD^++ ethanol	Deamino-NAD^++ ethanol	NAD^++ propan-1-ol	NAD^++ propan-2-ol	NAD^++ butan-1-ol	NAD^++ butan-2-ol	NAD^++ cyclohexanol
V_1 (μM/min)		143	102	98.0	111	0.800	132	1.82	18.5
K_a (μM)		271	184	373	167	202	204	136	315
K_b (mM)		3.13	22.3	5.29	0.344	0.416	0.349	0.542	0.191
K_{ia} (μM)		767	135	269	936	283	445	156	325
V_1' (μM/min)		102	182	67.6	83.3	2.78	65.4	6.25	167
K_a' (μM)		263	173	142	255	83.3	190	50.0	583
K_b' (mM)			82.7			7.29		4.63	3.25
K'_{-b} (mM)		8.98		22.9	1.75		1.50		
K'_{ab} (mM²)			14.5	2.28	2.15			0.718	0.868
K'_{-ab} (mM²)		2.45			0.534		0.0359		

alcohols, but lower for secondary alcohols than those of the control. The trends correlate well with the changes in the enzymic activities upon methylation. Furthermore K'_{-b} decreases in all cases that exhibit desensitization to substrate inhibition on methylation of liver alcohol dehydrogenase.

Product-inhibition studies are useful techniques for the differentiation of various mechanisms in enzymic reactions (Cleland, 1963*b*). The control yields product-inhibition patterns that are predictable on the basis of an Ordered Bi Bi mechanism (Fig. 5*a*); however, unexpectedly, acetaldehyde inhibits methylated enzyme competitively with respect to ethanol (Fig. 5*b*). Inhibition constants for intercept effect (K_{ii}) and slope effect (K_{is}) are given in Table 4. A specific dead-end inhibitor, pyrazole (Theorell *et al.*, 1969), inhibits both the control and the methyl-enzyme competitively with respect to ethanol. The inhibition is linear for the control, but non-linear for the methylated enzyme (Fig. 6). Hill plots (Fig. 6 inset) show a change in the interaction coefficient (h) of ethanol from 0.92 in the absence of pyrazole to 1.7 in the presence of 5.0 μM-pyrazole.

To investigate factors that are responsible for the activation of methylated liver alcohol dehydrogenase to oxidize ethanol and its desensitization to substrate inhibition, various substituents were introduced by reductive alkylation of the enzyme, and kinetic parameters from the asymptotic region of activity plots of alkylated enzymes were analysed for the substituent effects (Tsai, 1977). In view of the present kinetic study, which suggests the association of two kinetic parameters in the concave region, V'_1 and K'_{-b}, with the observed methylation effects, these kinetic parameters were determined (Table 5) and analysed for the substituent effects according to:

$$\log V'^{r}_1 \text{ or } K'^{r}_{-b} = d + \rho^*\sigma^* + SE_s + p\pi \qquad (3)$$

as described previously (Tsai, 1977). V'^{r}_1 and K'^{r}_{-b} are ratios of V'_1 and K'_{-b} of alkylated liver alcohol dehydrogenase to NADH-protected alkylated enzymes respectively. All alkylated enzymes show desensitization to ethanol inhibition with decreased K'_{-b}. The activation of alkylated enzymes to oxidize ethanol is associated with increased V_1 and V'_1, whereas the deactivated trichloroethyl-enzyme shows decreased V_1 and V'_1. Stepwise forward regression analyses (Draper & Smith, 1968) of substituent effects at a 95% F level of acceptance according to eqn. (3) indicate that the hydrophobic effect for alkylated enzymes is the most important factor affecting V'_1, whereas the steric effect dominates K'_{-b}, as described by the following expressions:

$$\log V'^{r}_1 = 0.857 - 0.439\pi$$
$$(n = 11,\ r = 0.890,\ s = 0.191)$$

$$\log K'^{r}_{-b} = 0.0643E_s - 0.166$$
$$(n = 11,\ r = 0.906,\ s = 0.0335)$$

where n, r and s are the number of alkylated enzymes analysed, the correlation coefficient and the standard error of estimates respectively. The inclusion of the other two substituents effects generally improves both r and s (Figs. 7 and 8). In spite of the uncertainty in the estimation of V'_1 and K'_{-b}, surprisingly good correlations were observed.

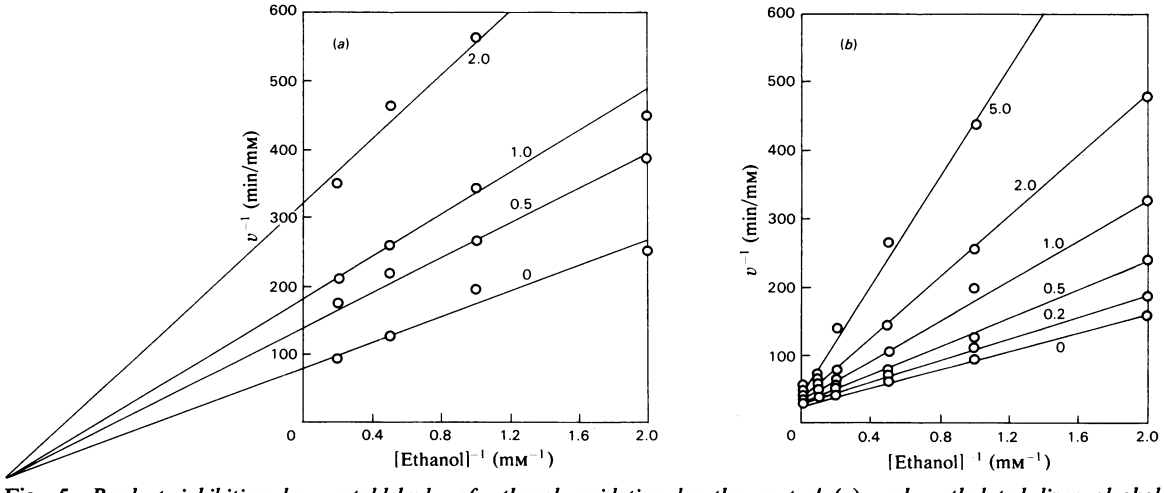


Fig. 5. *Product inhibition by acetaldehyde of ethanol oxidation by the control (a) and methylated liver alcohol dehydrogenase (b) at constant* (0.20 mM) *NAD*$^+$

Because of the substrate inhibition with the control, inhibition studies were carried out in the asymptotic region. However, for methylated enzyme, ethanol concentrations were extended to the concave region. Concentrations of acetaldehyde are shown beside the resulting lines.

Table 4. *Production-inhibition constants for ethanol oxidation*

Kinetic studies of control and modified liver alcohol dehydrogenase catalysed by ethanol oxidations were carried out in the presence of 0.020 and 0.050mM-NADH (at constant ethanol concentration of 2.0mM) or 0.50, 1.0 and 2.0mM-acetaldehyde (at constant NAD^+ concentration of 0.20mM) at pH9.0. K_{ii}, inhibition constant for intercept effect; K_{is}, inhibition constant for slope effect. The determinations were at a constant ethanol concentration of 2.0mM and a constant NAD^+ concentration of 0.2mM.

Enzyme	Product	Inhibition constant	Varied substrate ...	Inhibition constant value (mM)	
				NAD^+	Ethanol
Control	NADH	K_{ii}		—	0.0419
		K_{is}		0.0292	0.0307
	Acetaldehyde	K_{ii}		1.06	0.714
		K_{is}		2.06	1.44
Protected methylated	NADH	K_{ii}		—	0.0677
		K_{is}		0.0285	0.0543
	Acetaldehyde	K_{ii}		0.572	0.398
		K_{is}		1.01	0.579
Methylated	NADH	K_{ii}		—	0.0853
		K_{is}		0.0278	0.0719
	Acetaldehyde	K_{ii}		2.63	—
		K_{is}		1.51	0.788

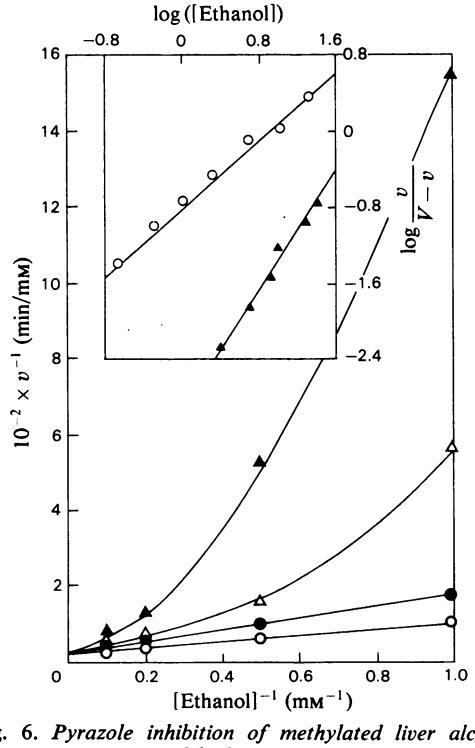


Fig. 6. *Pyrazole inhibition of methylated liver alcohol dehydrogenase*

Kinetic studies of methylated-enzyme-catalysed ethanol oxidation at constant (0.20mM) NAD^+ were carried out in the presence of 0 (○), 1.0 (●), 2.0 (△) and 5.0μM- (▲) pyrazole. In a parallel experiment, the pyrazole inhibition of the control is linearly competitive with respect to ethanol ($K_{is} = 1.79\mu M$), whereas that of methylated enzyme is non-linearly competitive. Insert shows a Hill plot for the pyrazole effect.

Discussion

Two Michaelian kinetic models, Theorell–Chance (Theorell & Chance, 1951) and Ordered Bi Bi (Wratten & Cleland, 1963), are the common mechanisms proposed for catalysis by liver alcohol dehydrogenase. The former requires $V_1K_{ia}/V_2K_a = 1$, whereas for the latter $V_1K_{ia}/V_2K_a > 1$. For catalysis by alcohol dehydrogenase, dissociations of the enzyme–coenzyme complexes are rate-limiting (Dalziel, 1963; Shore & Gutfreund, 1970), and their rate constants, K_{+7} and K_{-1}, can be evaluated from V_2K_{iq}/K_qEt and V_1K_{ia}/K_aEt, but cannot be smaller than the turnover rates, V_1/Et and V_2/Et respectively. These relationships hold reasonably well for the control and NADH-protected methylated enzyme with respect to the Ordered Bi Bi mechanism, but not for methylated enzyme (Table 1). Furthermore, neither the Ordered Bi Bi nor the Theorell–Chance mechanism can adequately explain the kinetic behaviour of methylated enzyme with regard to the methylation effects. Therefore catalysis by methylated liver alcohol dehydrogenase does not seem to follow a simple Michaelian kinetic model.

Dalziel & Dickinson (1966) and Hanes *et al.* (1972) suggest that catalysis by liver alcohol dehydrogenase over a wide range of alcohol concentrations proceeds via a partially random and a partially ordered mechanism with a pathway for the formation of the enzyme–NADH–alcohol complex as shown in Scheme 1. The steady-state rate equation in the

Table 5. *Kinetic parameters for ethanol oxidation catalysed by alkylated liver alcohol dehydrogenases*

Kinetic parameter	Enzyme ... Control	NADH protected	Methyl	Propyl	Trichloroethyl
V_1 (μM/min)	11.3	15.4	129	80.1	10.3
K_a (μM)	52.4	45.1	353	268	48.3
K_b (mM)	0.855	0.793	3.97	3.78	0.647
K_{ia} (μM)	37.7	50.1	466	313	118
V_1' (μM/min)	7.09	10.9	99.2	50.0	6.62
K_a' (μM)	75.1	100	250	165	43.7
K'_{-b} (mM)	20.9	20.4	13.9	10.0	11.5
K'_{-ab} (mM^2)	1.70	1.59	2.79	2.00	0.213

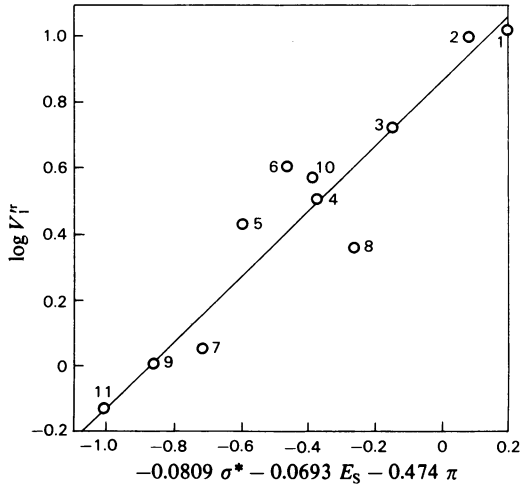


Fig. 7. *Substituent effects on maximum velocities of ethanol oxidation in the concave region catalysed by alkylated liver alcohol dehydrogenase*

Three-parameter analysis gives $r = 0.908$, $s = 0.199$ for $n = 11$. For alkylated liver alcohol dehydrogenases, the alkyl groups (RCH_2–) introduced to the enzymes are: R = H (1), CH_3 (2), CH_3CH_2 (3), $CH_3[CH_2]_2$ (4), $CH_3[CH_2]_3$ (5), $(CH_3)_2CHCH_2$ (6), $(CH_3)_3C$ (7), $CH_3CH{=}CH$ (8), C_6H_5–$CH{=}CH$ (9), C_6H_5 (10) and Cl_3C (11). $V_1^r = V_1'$(alkylated enzyme)/V_1' (corresponding NADH-protected alkylated enzyme).

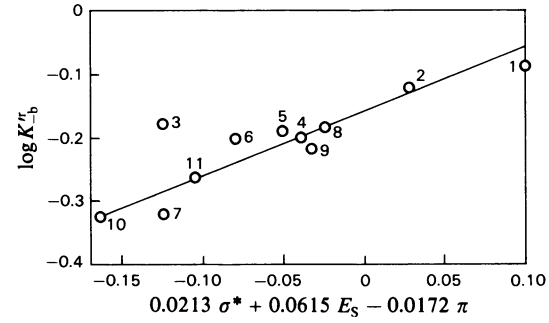
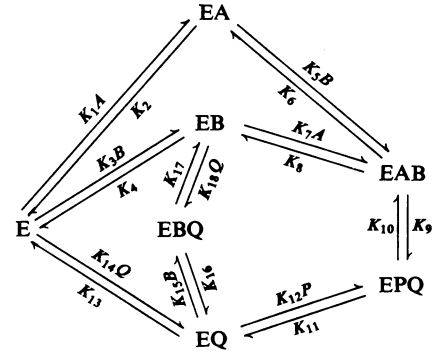


Fig. 8. *Substituent effects on the inhibitory Michaelis constant for ethanol catalysed by alkylated liver alcohol dehydrogenases*

R in RCH_2 groups are indicated by the numbers identical with Fig. 7. Three-parameter analysis gives $r = 0.937$, $s = 0.0315$. $K'^r_{-b} = K'_b$ (alkylated enzyme)/K'_{-b} (corresponding NADH protected enzyme).

absence of products (P and Q) has been derived (Dalziel & Dickinson, 1966). The complete rate equation is given in the Appendix (eqn. 1). For the control, the upper alternative pathway (via EA) of Scheme 1 becomes important at low ethanol concentrations and the mechanism reduces to Ordered Bi Bi. The rate expression obeys eqn. (1). Product inhibitions follow the predicted patterns (Cleland, 1963*b*; Wratten & Cleland, 1963) and pyrazole is a linear competitive inhibitor with respect to ethanol (Theorell *et al.*, 1969).

For methylated liver alcohol dehydrogenase, the



Scheme 1. *Proposed kinetic mechanism for methylated liver alcohol dehydrogenase*

E, A, N, P and Q are the enzyme, NAD^+, alcohol, aldehyde and NADH respectively.

following observations suggest the operation of Scheme 1 without any pathway dominating. Firstly, acetaldehyde (P) inhibits the methylated enzyme competitively with respect to ethanol, in agreement with eqn. (1) of the Appendix. P affects the slope but not the intercept plots of $1/V$ versus $1/B$ according to:

$$\lim_{1/B\to\infty}\frac{d(1/V)}{d(1/B)}=\frac{(d_0+d_aA+d_{a_2}A^2)+(d_p+d_{ap}A+d_{a2p}A^2)P}{n_{ab}A+n_{a_2b}A^2}$$

and

$$\lim_{1/B\to\infty}\frac{1}{V}=\frac{d_{b_3}+d_{ab_3}A}{n_{ab_3}}$$

Secondly, the Hill interaction coefficient (h) for ethanol in the presence of 5.0μM-pyrazole indicates the lower limit for the kinetic molecularity of ethanol to be 1.7. Thirdly, for reactions exhibiting substrate inhibition, $K'_a=K'_{-ab}/K'_{-b}$ within the experimental error (Tables 2 and 3), in agreement with the prediction based on eqn. (4), i.e. for:

$$\frac{n_{ab_3}}{d_{b_3}}\ll\frac{n_{ab_2}}{d_{b_2}}\quad\text{and}\quad\frac{n_{ab_3}}{d_{ab_3}}\ll\frac{n_{ab_2}}{d_{ab_2}}$$

$$K'_a=\frac{d_{b_3}}{d_{ab_3}}=\frac{K'_{-ab}}{K'_{-b}}$$

Although it is difficult from the kinetic study of ethanol oxidation to assign any kinetic parameters associated with the activation and desensitization of methylated enzyme to substrate inhibition, steady-state kinetic studies with coenzyme derivatives and alcohol analogues yield valuable information in this regard. For reactions exhibiting substrate activation, e.g. cyclohexanol, methylation increases K'_b, whereas for reactions exhibiting substrate inhibition, e.g. primary alcohols, the desensitization to inhibition is accompanied by a decrease in K'_{-b}, presumably by facilitating the dissociation of the enzyme–NADH–alcohol complex. The trend indicating the effect of methylation on K'_b (K'_{-b}) can be seen with thionicotinamide-NAD$^+$ and butan-2-ol, which change from K'_{-b} (negative slopes) for the control to K'_b (positive slopes). Methylation either enhances enzyme ability to oxidize primary alcohols by increasing V_1 and V'_1 or decreases its ability to oxidize secondary alcohols by decreasing V_1 and V'_1. Since the rate-limiting steps for the oxidation of primary and secondary alcohols are different (Dalziel & Dickinson, 1966), methylation presumably increases maximum velocities by facilitating the dissociations of the enzyme–NADH and enzyme–NADH–alcohol complexes for primary alcohols while decreasing maximum velocities by retarding the interconversion between enzyme–NAD$^+$–secondary alcohol and enzyme–NADH–ketone complexes and the release of the product from the ternary complex.

The previous study (Tsai, 1977) of substituent effect on alkylated liver alcohol dehydrogenase indicates that the hydrophobicity of alkyl groups is the most important variable affecting V_1. Similar analyses suggest that the hydrophobicity and the steric effect are the chief causes of changes in V'_1 and K'_{-b} respectively. The negative coefficient for π indicates that maximum velocities (V'_1) increase with the hydrophilicity of the alkylated lysine residues that interact with NADH. The positive coefficient for steric constants (E_s) suggests that inhibitory Michaelis constants (K'_{-b}) for ethanol decrease with steric hindrance at the lysine residues.

It is concluded that methylated liver alcohol dehydrogenase catalyses the oxidation of alcohols via a partially random and a partially ordered mechanism with a pathway for the formation of the productive enzyme–NADH–alcohol complex. The facilitation of its formation and dissociation that is favoured by a steric hindrance at the lysine residues desensitizes methylated enzyme to substrate inhibition. Methylation activates liver alcohol dehydrogenase to oxidize primary alcohols but deactivates the enzymes to oxidize secondary alcohols, depending on the rate-limiting step of the reactions, by either increasing or decreasing maximum velocities in the asymptotic and concave regions, which are affected by alkyl groups introduced at the lysine residues.

References

Chen, S.-S. & Engel, P. C. (1975) *Biochem. J.* **149**, 627–635

Cleland, W. W. (1963*a*) *Biochim. Biophys. Acta* **67**, 104–137

Cleland, W. W. (1963*b*) *Biochim. Biophys. Acta* **67**, 188–196

Dalziel, K. (1963) *J. Biol. Chem.* **238**, 1850–2858

Dalziel, K. & Dickinson, F. M. (1966) *Biochem. J.* **100**, 34–46

Degani, Y., Veronese, F. M. & Smith, E. L. (1974) *J. Biol. Chem.* **249**, 7929–7935

Dove, M. J. & Tsai, C. S. (1976) *Can. J. Biochem.* **54**, 432–437

Draper, N. R. & Smith, H. (1968) *Applied Regression Analysis*, pp. 178–195, Wiley, New York

Dworschack, R., Tarr, G. & Plapp, B. V. (1975) *Biochemistry* **14**, 200–203

Ehrenberg, A. & Dalziel, K. (1958) *Acta Chem. Scand.* **12**, 465–469

Fan, C. C. & Plaut, G. W. E. (1974) *Biochemistry* **13**, 52–59
Hanes, C. S., Bronskill, P. M., Gurr, P. A. & Wong, J. T. F. (1972) *Can. J. Biochem.* **50**, 1385–1413
Jörvall, H. (1973) *Biochem. Biophys. Res. Commun.* **51**, 1069–1076
McKinley-McKee, J. S. & Morris, D. L. (1972) *Eur. J. Biochem.* **28**, 1–11
Plapp, B. V. (1970) *J. Biol. Chem.* **245**, 1727–1735
Plapp, B. V., Brooks, R. L. & Shore, J. D. (1973) *J. Biol. Chem.* **248**, 3470–3475
Shore, J. D. & Gutfreund, H. (1970) *Biochemistry* **9**, 4655–4659
Theorell, H. & Chance, B. (1951) *Acta Chem. Scand.* **5**, 1127–1144
Theorell, H., Yonetani, T. & Sjöberg, B. (1969) *Acta Chem. Scand.* **23**, 255–260
Tsai, C. S. (1977) *Bioorg. Chem.* **6**, 117–125
Tsai, C. S., Tsai, Y. H., Lauzon, G. & Cheng, S. T. (1974) *Biochemistry* **13**, 440–443
Wratten, C. C. & Cleland, W. W. (1963) *Biochemistry* **2**, 935–941

APPENDIX

The rate equation for Scheme 1 (main paper) can be derived by the King & Altman (1956) procedure:

$$v = \frac{[n_{ab}AB + n_{a_2b}A^2B + n_{ab_2}AB^2 + n_{a_2b_2}A^2B^2 + n_{ab_3}AB^3 + n_{abq}ABQ + n_{ab_2q}AB^2Q - (n_{pq}PQ + n_{pq_2}PQ^2 + n_{apq}APQ + n_{bpq}BPQ + n_{b_2pq}B^2PQ + n_{bpq_2}BPQ^2 + n_{abpq}ABPQ)]Et}{d_0 + d_aA + d_bB + d_pP + d_qQ + d_{a_2}A^2 + d_{b_2}B^2 + d_{q_2}Q^2 + d_{ab}AB + d_{ap}AP + d_{aq}AQ + d_{bp}BP + d_{bq}BQ + d_{pq}PQ + d_{b_3}B^3 + d_{a_2b}A^2B + d_{ab_2}AB^2 + d_{a_2p}A^2P + d_{b_2p}B^2P + d_{b_2q}B^2Q + d_{bq_2}BQ^2 + d_{pq_2}PQ^2 + d_{abp}ABP + d_{abq}ABQ + d_{apq}APQ + d_{bpq}BPQ + d_{a_2b_2}A^2B^2 + d_{ab_3}AB^3 + d_{b_3q}B^3Q + d_{b_2q_2}B^2Q^2 + d_{a_2bp}A^2BP + d_{ab_2p}AB^2P + d_{ab_2q}AB^2Q + d_{b_2pq}B^2PQ + d_{bpq_2}PBQ^2 + d_{abpq}ABPQ} \quad (1)$$

where Et, A, B, P and Q are enzyme concentration, concentrations of NAD^+, ethanol, acetaldehyde and NADH respectively. The kinetic coefficients n_{x_i} and d_{x_i} are for numerator terms and denominator terms respectively associated with substrate, x, of ith degree such that:

$n_{ab} = k_{+5}k_{+6}k_{+7}(k_{+1}k_{-2}k_{+3} + k_{-1}k_{+2}k_{+4})(k_{-8} + k_{+9})$
$n_{a_2b} = k_{+1}k_{+3}k_{+4}k_{+5}k_{+6}k_{+7}(k_{-8} + k_{+9})$
$n_{ab_2} = k_{+2}k_{+3}k_{+4}k_{+5}k_{+6}k_{+7}(k_{-8} - k_{+9}) + k_{+5}k_{+6}k_{+8}k_{+9}(k_{+1}k_{-2}k_{+3} + k_{-1}k_{+2}k_{+4})$
$n_{a_2b_2} = k_{+1}k_{+3}k_{+4}k_{+5}k_{+6}k_{+8}k_{+9}$
$n_{ab_3} = k_{+2}k_{+3}k_{+4}k_{+5}k_{+6}k_{+8}k_{+9}$
$n_{abq} = k_{+5}k_{+6}(k_{+1}k_{+3}k_{+7}k_{-8}k_{-9} + k_{-1}k_{+4}k_{-7}k_{+8}k_{+9})$
$n_{ab_2q} = k_{+3}k_{+4}k_{+5}k_{+6}k_{-7}k_{+8}k_{+9}$
$n_{pq} = k_{-1}k_{-2}k_{-5}k_{-6}k_{-7}(k_{-3} + k_{-4})(k_{-8} + k_{+9})$
$n_{pq_2} = k_{-1}k_{-5}k_{-6}k_{-7}k_{-8}k_{-9}(k_{-3} + k_{-4})$
$n_{apq} = k_{-1}k_{-3}k_{-4}k_{-5}k_{-6}k_{-7}(k_{-8} + k_{+9})$
$n_{bpq} = k_{-1}k_{+2}k_{-5}k_{-6}k_{-9}(k_{-3} + k_{-4}) + k_{-2}k_{+3}k_{-4}k_{-5}k_{-6}k_{-7}(k_{-8} + k_{+9})$
$n_{b_2pq} = k_{+2}k_{+3}k_{-4}k_{-5}k_{-6}k_{-8}k_{-9}$
$n_{bpq_2} = k_{+3}k_{-4}k_{-5}k_{-6}k_{-7}k_{-8}k_{-9}$
$n_{abpq} = k_{+1}k_{+3}k_{-4}k_{-5}k_{-6}k_{-8}k_{-9}$
$d_0 = k_{-1}k_{-2}k_{+7}(k_{-8} + k_{+9})[(k_{-3} + k_{-4})(k_{-5} + k_{+6}) + k_{+5}k_{+6}]$
$d_a = k_{+7}(k_{-8} + k_{+9})\{k_{+1}k_{-2}[(k_{-3} + k_{-4})(k_{-5} + k_{+6}) + k_{+5}k_{+6}] + k_{-1}k_{+4}[k_{-3}(k_{-5}k_{+6} + k_{+5}k_{+6}]\}$
$d_b = k_{-2}k_{+3}k_{+7}(k_{-8} + k_{+9})[k_{-4}(k_{-5} + k_{+6}) + k_{+5}k_{+6}] + k_{-1}[(k_{-3} + k_{-4})(k_{-5} + k_{+6}) + k_{+5}k_{+6}][k_{-2}k_{+8}k_{+9} + k_{+2}k_{+7}(k_{-8} + k_{+9})]$
$d_p = k_{-1}k_{-2}k_{-5}k_{-6}(k_{-3} + k_{-4})(k_{-8} + k_{+9})$
$d_q = k_{-1}[(k_{-3} + k_{-4})(k_{-5} + k_{+6}) + k_{+5}k_{+6}][k_{+7}k_{-8}k_{-9} + k_{-2}k_{-7}(k_{-8} + k_{+9})]$
$d_{a_2} = k_{+1}k_{+4}k_{+7}(k_{-8} + k_{+9})[k_{-3}(k_{-5} + k_{+5}) + k_{+5}k_{+6}]$
$d_{b_2} = k_{+3}[k_{-4}(k_{-5} + k_{+6}) + k_{+5}k_{+6}][k_{-2}k_{+8}k_{+9} + k_{+2}k_{+7}(k_{-8} + k_{+9})] + k_{-1}k_{+2}k_{+8}k_{+9}[(k_{-3} + k_{-4})(k_{-5} + k_{+6}) + k_{+5}k_{+6}]$
$d_{q_2} = k_{-1}k_{-7}k_{-8}k_{-9}[(k_{-3} + k_{-4})(k_{-5} + k_{+6}) + k_{+5}k_{+6}]$
$d_{ab} = k_{+1}k_{-2}k_{+8}k_{+9}[(k_{-3} + k_{-4})(k_{-5} + k_{+6}) + k_{+5}k_{+6}] + k_{-1}k_{-3}k_{+4}k_{+8}k_{+9}(k_{-5} + k_{+6}) + k_{+3}k_{+4}k_{+5}k_{+6}k_{+7}(k_{-8} + k_{+9}) + k_{+7}(k_{-5} + k_{+6})(k_{-8} + k_{+9})(k_{+1}k_{+3}k_{-4} + k_{+2}k_{-3}k_{+4}) + (k_{+1}k_{-2}k_{+3} + k_{-1}k_{+2}k_{+4})(k_{-8} + k_{+9})[k_{+5}k_{+6} + k_{+7}(k_{+5} + k_{-5} + k_{+6})]$
$d_{ap} = k_{-5}k_{-6}(k_{-8} + k_{+9})[k_{+1}k_{-2}(k_{-3} + k_{-4}) + k_{-1}k_{-3}k_{+4})]$
$d_{aq} = k_{+1}k_{+7}k_{-8}k_{-9}[(k_{-3} + k_{-4})(k_{-5} + k_{+6}) + k_{+5}k_{+6}] + k_{-1}k_{+4}k_{-7}(k_{-8} + k_{+9})[k_{-3}(k_{-5} + k_{+6}) + k_{+5}k_{+6}]$

$$
\begin{aligned}
d_{bp} &= k_{-5}k_{-6}(k_{-8}+k_{+9})[k_{-1}k_{+2}(k_{-3}+k_{-4})+k_{-2}k_{+3}k_{-4}]\\
d_{bq} &= k_{-1}[k_{+2}k_{+9}(k_{+7}+k_{-8})+k_{-7}k_{+8}(k_{-2}+k_{+9})][k_{-3}+k_{-4})(k_{-5}+k_{+6})+k_{+5}k_{+6}]+k_{+3}[k_{-2}k_{-7}(k_{-8}+k_{-9})\\
&\quad +k_{+7}k_{-8}k_{-9}][k_{-4}(k_{-5}+k_{+6})+k_{+5}k_{+6}]\\
d_{pq} &= k_{-6}\{k_{-1}k_{-3}k_{-5}k_{-8}k_{-9}+k_{-7}(k_{-8}+k_{+9})[k_{-1}k_{-2}(k_{-3}+k_{-4}+k_{+5}+k_{-5})+k_{-4}k_{-1}(k_{-1}+k_{-2})]\}\\
d_{b_3} &= k_{+2}k_{+3}k_{+8}k_{+9}[k_{-4}(k_{-5}+k_{+6})+k_{+5}k_{+6}]\\
d_{a_2b} &= k_{+1}k_{+4}\{k_{-3}k_{+8}k_{+9}(k_{-5}+k_{+6})+k_{+3}(k_{-8}+k_{+9})[(k_{+5}+k_{-5})(k_{+6}+k_{+7})+k_{+6}k_{+7}]\}\\
d_{ab_2} &= k_{+2}k_{+4}\{k_{+3}k_{+5}(k_{+6}+k_{+7})(k_{-8}+k_{+9})+k_{-5}+k_{+6})[k_{+3}k_{+7}(k_{-8}+k_{+9})+k_{-3}k_{+8}k_{+9}]\}\\
&\quad +k_{+8}\{k_{+1}k_{+3}k_{+9}[k_{-4}(k_{-5}+k_{+6})+k_{+5}k_{+6}]+(k_{+1}k_{-2}k_{+3}+k_{-1}k_{+2}k_{+4})[k_{+5}k_{+6}+k_{+9}(k_{+5}+k_{-5}+k_{+6})]\}\\
d_{a_2b} &= k_{+1}k_{-3}k_{+4}k_{-5}k_{-6}(k_{-8}+k_{+9})\\
d_{b_2p} &= k_{+2}k_{+3}k_{-4}k_{-5}k_{-6}(k_{-8}+k_{+9})\\
d_{bq_2} &= k_{-7}k_{-9}\{k_{-1}k_{-3}k_{+8}(k_{-5}+k_{+6})+(k_{-1}k_{+8}+k_{+3}k_{-8})[k_{-4}(k_{-5}+k_{+6})+k_{+5}k_{+6}]\}\\
d_{apb} &= k_{-6}(k_{-8}+k_{+9})\{k_{+2}k_{+4}[k_{-1}(k_{+5}+k_{-5})+k_{-3}k_{-5}]+k_{+1}k_{+3}[k_{-2}(k_{+5}+k_{-5})+k_{-4}k_{-5}]\}\\
d_{abq} &= k_{+1}k_{+3}k_{-9}\{k_{+5}k_{-8}(k_{+6}+k_{+7})+(k_{-5}+k_{+6})[k_{-4}(k_{+7}+k_{-8})+k_{+5}k_{-8}]\}+k_{-1}k_{+4}k_{-7}k_{+8}[k_{+5}(k_{+6}+k_{+9})\\
&\quad +(k_{-5}+k_{+6})(k_{-3}+k_{+9})]+k_{+4}k_{-7}[k_{+3}k_{+5}k_{+6}(k_{-8}+k_{+9})+k_{-3}k_{+8}k_{+9}(k_{-5}+k_{+6})]\\
d_{apq} &= k_{-6}\{k_{+1}k_{-3}k_{-5}k_{-8}k_{-9}+k_{+4}k_{-7}(k_{-8}+k_{+9})[k_{-1}(k_{-3}+k_{+5}+k_{-5})+k_{-3}k_{-5}]\}\\
d_{bpq} &= k_{+2}k_{-6}k_{-9}\{k_{-3}k_{-5}k_{-8}+k_{-1}[(k_{-3}+k_{-4})(k_{-5}+k_{-8})+k_{-8}(k_{+5}+k_{-5})]\}+k_{+3}k_{-6}k_{-7}(k_{-8}+k_{+9})[k_{-2}(k_{-4}\\
&\quad +k_{+5}+k_{-5})+k_{-4}k_{-5}]\\
d_{a_2b_2} &= k_{+1}k_{+3}k_{+4}k_{+8}[k_{+5}(k_{+6}+k_{+9})+k_{+9}(k_{-5}+k_{+6})]\\
d_{ab_3} &= k_{+2}k_{+3}k_{+4}k_{+8}[k_{+5}(k_{+6}+k_{+9})+k_{+9}(k_{-5}+k_{+6})]\\
d_{b_3q} &= k_{+2}k_{+3}k_{+8}k_{-9}[k_{-4}(k_{-5}+k_{+6})+k_{+5}k_{+6}]\\
d_{b_2q_2} &= k_{+3}k_{-7}k_{+8}k_{-9}[k_{-4}(k_{-5}+k_{+6})+k_{+5}k_{+6}]\\
d_{a_2bp} &= k_{+1}k_{+3}k_{+4}k_{+5}k_{-6}(k_{-8}+k_{+9})\\
d_{ab_2p} &= k_{+2}k_{+3}k_{+4}k_{-6}(k_{+5}+k_{-5})(k_{-8}+k_{+9})\\
d_{ab_2q} &= k_{+3}k_{+8}\{k_{+4}k_{-7}[k_{+5}(k_{+6}+k_{+9})+k_{+9}(k_{-5}+k_{+6})+k_{+1}k_{-9}[k_{-4}(k_{-5}+k_{+6})+k_{+5}k_{+6}]\}\\
d_{b_2pq} &= k_{+2}k_{+3}k_{-6}k_{-9}[k_{-4}(k_{-5}+k_{-8})+k_{+5}k_{-8}]\\
d_{bpq_2} &= k_{+3}k_{-6}k_{-7}k_{-9}[k_{-4}(k_{-5}+k_{-8})+k_{-8}(k_{+5}+k_{-5})]\\
d_{abpq} &= k_{+3}k_{-6}\{k_{+1}k_{-9}[k_{-4}(k_{-5}+k_{-8})+k_{-8}(k_{+5}+k_{-5})]+k_{+4}k_{-7}(k_{+5}+k_{-5})(k_{-8}+k_{+9})\}
\end{aligned}
$$

For the initial velocity of alcohol oxidation (in the absence of P and Q), eqn. (1) becomes:

$$v=\frac{(n_{ab}AB+n_{a_2b}A^2B+n_{ab_2}AB^2+n_{a_2b_2}A^2B^2+n_{ab_3}AB^3)Et}{d_0+d_aA+d_bB+d_{a_2}A^2+d_{b_2}B^2+d_{ab}AB+d_{b_3}B^3+d_{a_2b}A^2B+d_{ab_2}AB^2+d_{a_2b_2}A^2B^2+d_{ab_3}AB^3}$$

which can be written in the double-reciprocal form at constant concentrations of B:

$$\frac{1}{v}=\frac{\beta_0}{\alpha_1}\left(\frac{1}{A}\right)+\frac{1}{\alpha_1^2}(\alpha_1\beta_1-\alpha_2\beta_0)+\frac{\alpha_1^2\beta_2-\alpha_2(\alpha_1\beta_1-\alpha_2\beta_0)}{\alpha_1^2\left\{\alpha_1\left(\frac{1}{A}\right)+\alpha_2\right\}} \tag{2}$$

where:

$$
\begin{aligned}
\alpha_1 &= (n_{ab}B+n_{ab_2}B^2+n_{ab_3}B^3)Et\\
\alpha_2 &= (n_{a_2b}B+n_{a_2b_2}B^2)Et\\
\beta_0 &= d_0+d_bB+d_{b_2}B^2+d_{b_3}B^3\\
\beta_1 &= d_a+d_{ab}B+d_{ab_2}B^2+d_{ab_3}B^3\\
\beta_2 &= d_{a_2}+d_{a_2b}B+d_{a_2b_2}B^2
\end{aligned}
$$

Since plots of $1/v$ versus $1/A$ are linear in all cases, eqn. (2) is simplified to the Michaelis form for the asymptotic region of A:

$$\frac{1}{v}=\frac{\beta_0}{\alpha_1}\left(\frac{1}{A}\right)+\frac{1}{\alpha_1^2}(\alpha_1\beta_1-\alpha_2\beta_0) \tag{3}$$

Its positive intercepts follow:

$$\lim_{1/A\to 0}\frac{1}{v}=\frac{d_a\left(\frac{1}{B}\right)^3+d_{ab}\left(\frac{1}{B}\right)^2+d_{ab_2}\left(\frac{1}{B}\right)+d_{ab_3}}{\left\{n_{ab}\left(\frac{1}{B}\right)^2+n_{ab_2}\left(\frac{1}{B}\right)+n_{ab_3}\right\}Et} \tag{4}$$

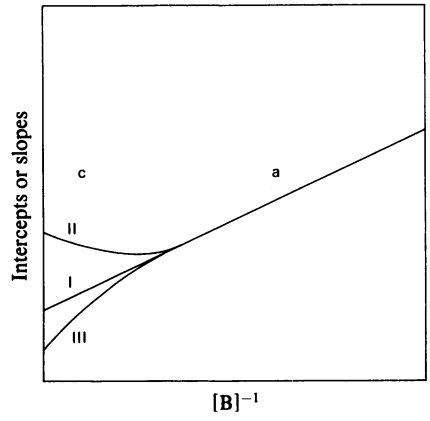


Fig. 1. *Typical secondary plots for non-Michaelian kinetics*

Table 1. *Relationships between kinetic parameters and kinetic coefficients of eqn.* (1)

Region	Kinetic parameter	Kinetic coefficient
Asymptotic	V_1	$n_{ab}Et/d_{ab}$
	K_a	d_b/d_{ab}
	K_b	d_a/d_{ab}
	K_{1a}	d_0/d_a
Concave	V_1'	$n_{ab_3}Et/d_{ab_3}$
	K_a'	d_{b_3}/d_{ab_3}
	K_b'	d_{ab_2}/d_{ab_3}
	K_{-b}'	n_{ab_2}/n_{ab_3}
	K_{ab}'	d_{b_2}/d_{ab_3}
	K_{-ab}'	$n_{ab_2}\cdot d_{b_3}/n_{ab_3}\cdot d_{ab_3}$

and its limiting slopes obey:

$$\lim_{1/A\to\infty}\frac{d(1/v)}{d(1/A)}=\frac{d_0\left(\frac{1}{B}\right)^3+d_b\left(\frac{1}{B}\right)^2+d_{b_2}\left(\frac{1}{B}\right)+d_{b_3}}{\left\{n_{ab}\left(\frac{1}{B}\right)^2+n_{ab_2}\left(\frac{1}{B}\right)+n_{ab_3}\right\}Et} \tag{5}$$

Secondary plots of intercepts versus $1/B$ (Appendix Fig. 1) according to eqn. (4) give the asymptotic region (a) which is expressed by:

$$(\text{intercept})_a=\frac{d_a}{n_{ab}Et}\left(\frac{1}{B}\right)+\frac{n_{ab}d_{ab}-n_{ab_2}d_{b_2}}{(n_{ab})^2Et} \tag{6}$$

and the concave region (c) having the form:

$$(\text{intercept})_c=\frac{n_{ab_3}d_{ab_2}-n_{ab_2}d_{ab_3}}{(n_{ab_3})^2Et}\left(\frac{1}{B}\right)+\frac{d_{ab_3}}{n_{ab_3}Et} \tag{7}$$

which describes linear (I), concave up (II) or down (III) curves depending on whether n_{ab_3}/d_{ab_3} is equal to, smaller than or larger than n_{ab_2}/d_{ab_2} respectively.

Table 2. *Product inhibition predicted from eqn.* (1)
Abbreviations: C and NC, competitive and non-competitive inhibition respectively.

Product inhibitor	Varied substrate	$1/v$ versus $1/A$ or $1/B$		Inhibition pattern
		Intercept	Slope	
P	A	$\frac{(d_{a3}+d_{a2b}B+d_{a2b_2}B^2)+(d_{a2p}+d_{a2bp}B)P}{n_{a\ b}B+n_{a\ b}\ B^2}$	$\frac{(d_0+d_bB+d_{b_2}B^2+d_{b_3}B^3)+(d_p+d_{bp}B+d_{b2p}B^2)P}{n_{ab}B+n_{ab_2}B^2+n_{ab_3}B^3}$	NC
	B	$\frac{d_{b3}+d_{ab3}A}{n_{ab}\ A}$	$\frac{(d_0+d_aA+d_{a_2}A^2)+(d_p+d_{ap}A+d_{a3p}A^2)P}{n_{ab}A+n_{a_2b}A^2}$	C
Q	A	$\frac{d_{a_2}+d_{a_2b}B+d_{a_2b_2}B^2}{n_{a_2b}B+n_{a_2b_2}B^2}$	$\frac{(d_0+d_bB+d_{b_2}B^2+d_{b_3}B^3)+(d_q+d_{bq}B+d_{b2q}B^2+d_{b3q}B^3)Q+(d_{q_2}B+d_{bq2}+d_{q2b_2}B^2)Q^2}{(n_{ab}B+n_{ab_2}B^2+n_{ab_3}B^3)+(n_{abq}B+n_{a2bq}B^2)Q}$	C
	B	$\frac{(d_{b3}+d_{ab_3}A)+d_{b3q}Q}{n_{ab_3}A}$	$\frac{(d_0+d_aA+d_{a_2}A^2)+(d_q+d_{aq}A)Q+d_{q2}Q^2}{(n_{ab}A+n_{a_2b}A^2)+n_{abq}AQ}$	NC

Similarly, secondary plots of slopes versus $1/B$ (Appendix Fig. 1) according to eqn. (5) give the asymptotic region (a), which obeys:

$$(\text{slopes})_a = \frac{d_0}{n_{ab}Et}\left(\frac{1}{B}\right) + \frac{n_{ab}d_b - n_{ab_2}d_{b_2}}{(n_{ab})^2 Et} \tag{8}$$

and the concave region (c):

$$(\text{slopes})_c = \frac{n_{ab_3}d_{b_2} - n_{ab_2}d_{b_3}}{(n_{ab_3})^2 Et}\left(\frac{1}{B}\right) + \frac{d_{b_3}}{n_{ab}Et} \tag{9}$$

which describes linear (I) or concave-up (II) or concave-down (III) plots depending on whether n_{ab_3}/d_{b_3} is equal to, smaller than or larger than n_{ab_2}/d_{b_2} respectively.

From eqns. (6)–(9), kinetic parameters for the asymptotic region and the concave region for catalyses by liver alcohol dehydrogenase can be evaluated (Appendix Table 1). The definition of operational kinetic parameters for the concave region not only facilitates the analyses of non-Michaelian kinetics, but also serves to identify substrate activation ($V_1' > V_1$, $K_b' > K_b$ for B) or inhibition ($V_1' > V_1$, K_{-b}') of enzymic reactions without relying on graphical presentations. Kinetic implications of the two kinetic parameters, V_1' and K_{-b}', that are found to associate with the methylation effects can be readily seen from following expressions:

$$V_1' = k_{+6}\left(\frac{k_{+9}}{k_{+6}+k_{+9}} + \frac{k_{+5}}{k_{-5}+k_{+6}}\right)Et$$

and

$$K_{-b}' = \frac{k_{+7}}{k_{+8}}\left(1 + \frac{k_{-8}}{k_{+9}}\right) + \frac{k_{+1}k_{-2}k_{+3} + k_{-1}k_{+2}k_{+4}}{k_{+2}k_{+3}k_{+4}}$$

Furthermore, eqn. (1) predicts $K_{-ab} = K_a' \cdot K_{-b}'$ for reactions exhibiting substrate inhibition (both the control and methylated enzyme).

If catalysis by methylated enzyme follows eqn. (1) and its double-reciprocal plots over the concentration range used are approximately linear, product-inhibition patterns for ethanol oxidation catalysed by methylated liver alcohol dehydrogenase can be predicted (Appendix Table 2). Notably acetaldehyde (P) is competitive with respect to ethanol (B).

Reference

King, E. L. & Altman, C. (1956) *J. Phys. Chem.* **60**, 1375–1378