

The Kinetics and Mechanism of Liver Alcohol Dehydrogenase with Primary and Secondary Alcohols as Substrates

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1. The activity of liver alcohol dehydrogenase with propan-2-ol and butan-2-ol has been confirmed. The activity with the corresponding ketones is small. Initial-rate parameters are reported for the oxidation of these secondary alcohols, and of propan-1-ol and 2-methylpropan-1-ol, and for the reduction of propionaldehyde and 2-methylpropionaldehyde. Substrate inhibition with primary alcohols is also described. 2. The requirements of the Theorell-Chance mechanism are satisfied by the data for all the primary alcohols and aldehydes, but not by the data for the secondary alcohols. A mechanism that provides for dissociation of either coenzyme or substrate from the reactive ternary complex is described, and shown to account for the initial-rate data for both primary and secondary alcohols, and for isotope-exchange results for the former. With primary alcohols, the rapid rate of reaction of the ternary complex, and its small steady-state concentration, result in conformity of initial-rate data to the requirements of the Theorell-Chance mechanisms. With secondary alcohols, the ternary complex reacts more slowly, its steady-state concentration is greater, and therefore dissociation of coenzyme from it is rate-limiting with non-saturating coenzyme concentrations. 3. Substrate inhibition with large concentrations of primary alcohols is attributed to the formation of an abortive complex of enzyme, NADH and alcohol from which NADH dissociates more slowly than from the enzyme-NADH complex. The initial-rate equation is derived for the complete mechanism, which includes a binary enzyme-alcohol complex and alternative pathways for formation of the reactive ternary complex. This mechanism would also provide, under suitable conditions, for substrate activation or substrate inhibition in a two-substrate reaction, according to the relative rates of reaction through the two pathways.

Liver alcohol dehydrogenase (alcohol-NAD⁺ oxidoreductase, EC 1.1.1.1), has an unusually wide specificity. It has been reported to catalyse the oxidation of a variety of alcohols, primary, secondary and cyclic, and the reduction of several aldehydes and ketones (cf. Sund & Theorell, 1963). However, detailed kinetic studies with widely varied substrate and coenzyme concentrations have been made only with ethanol and butan-1-ol and the corresponding aldehydes (Dalziel, 1962*b*). Merrit & Tomkins (1959) reported that, with concentrations of 1 mM-alcohol and 100 μ M-NAD⁺ at pH 9.5, the rate of oxidation of butan-2-ol was 36% of that of ethanol, whereas propan-2-ol was inactive. Witter (1960) observed activity with propan-2-ol at higher concentration. From such measurements at single concentrations, it appears that primary alcohols and aldehydes are better substrates than secondary alcohols and ketones, but it is not known, for example, whether this is due to differences in the maximum rates, or the

Michaelis constants, or both. Van Eys (1961) showed that liver alcohol dehydrogenase also catalyses the isomerization of lactaldehyde to acetol, and of glyceraldehyde 3-phosphate to dihydroxyacetone phosphate. These reactions involve oxidation of a secondary alcohol group and reduction of an adjacent aldehyde group, and require only catalytic amounts of coenzyme.

In the present work, we have confirmed the activity of the enzyme with propan-2-ol and butan-2-ol and estimated initial-rate parameters for their oxidation by detailed kinetic studies with wide ranges of substrate and coenzyme concentrations. The oxidation of the primary alcohols propan-1-ol and 2-methylpropan-1-ol and the reduction of the corresponding aldehydes have been studied similarly. The main objectives of the work were to compare the mechanisms of catalysis with primary and secondary alcohols and to obtain data for secondary alcohols that would aid interpretation of kinetic studies of the isomerase reactions.

It also seemed desirable to attempt a more rigorous and detailed theoretical interpretation of data for this enzyme, including substrate inhibition, in terms of both abortive complex-formation and alternative pathways of reaction, for which evidence has been adduced from isotope-exchange experiments (Silverstein & Boyer, 1964).

MATERIALS AND METHODS

Materials. Reagent solutions and sodium phosphate buffers were made up in glass-distilled water.

Crystalline alcohol dehydrogenase was prepared from horse liver and assayed as described by Dalziel (1961a). The substrates were obtained commercially and fractionally distilled. NAD⁺ was purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, and purified by chromatography (Dalziel, 1963b). NADH was prepared as described by Dalziel (1962a).

Initial-rate measurements. The technique of initial-rate measurements and the recording fluorimeter employed were described by Dalziel (1962b). All measurements were made at 23.5° and the reaction mixtures were 10.1 with respect to sodium phosphate buffer, pH 7.0. Kinetic coefficients in the initial-rate equation:

$$\frac{e}{v_0} = \phi_0 + \frac{\phi_1}{[S_1]} + \frac{\phi_2}{[S_2]} + \frac{\phi_{12}}{[S_1][S_2]} \quad (1)$$

were obtained from primary and secondary plots of initial-rate data in the usual way, and are functions of rate constants in the mechanism (Dalziel, 1957, 1962b). In this equation, e is the concentration of enzyme active centres (twice the molar concentration) and S_1 and S_2 are coenzyme and substrate respectively. The symbols ϕ_0 etc. are used for kinetic coefficients for alcohol-NAD⁺ reactions, and ϕ'_0 etc. for those for aldehyde-NADH reactions. (This and the corresponding use of primed and unprimed velocity constants is the reverse of the convention adopted in previous papers, and is justified by typographical convenience in this paper, which refers mainly to the alcohol-NAD⁺ reactions.)

All initial-rate measurements were made at least in duplicate with a reproducibility of 5% in general, and at worst 15% with the smallest concentrations of both substrate and coenzyme. Three separate complete experiments (cf. Figs. 1 and 2) were made with each of the two secondary alcohols, and two with each of the other substrates, with different samples of coenzyme and substrate. ϕ_0 was reproducible in every case to within 5%, and the other parameters to within 20%.

RESULTS

Secondary alcohols. The results of initial-rate measurements with propan-2-ol in one experiment are shown in the primary plots of Figs. 1 and 2. Secondary plots of the intercepts and slopes are shown in Figs. 3(a) and (b), the intercepts and slopes of which are values for the kinetic coefficients in eqn. (1). The results of one experiment with butan-2-ol are similarly shown in Figs. 4-6. The

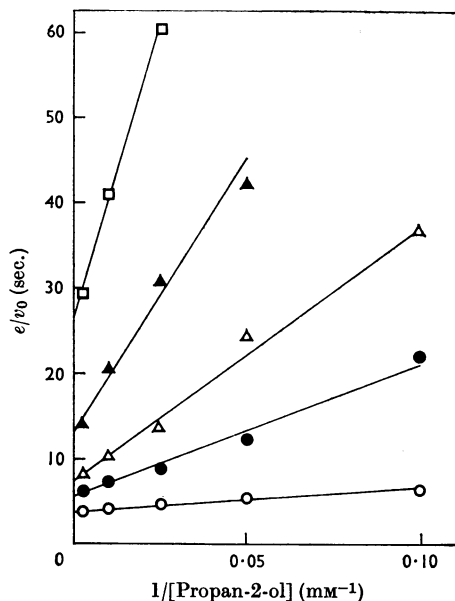


Fig. 1. Primary plots: variation of the reciprocal of the specific initial rate at pH 7.0 and 23.5° with the reciprocal of the propan-2-ol concentration for several constant NAD⁺ concentrations (μM): O, 517; ●, 51.7; Δ , 15.8; \blacktriangle , 10.3; \square , 4.3.

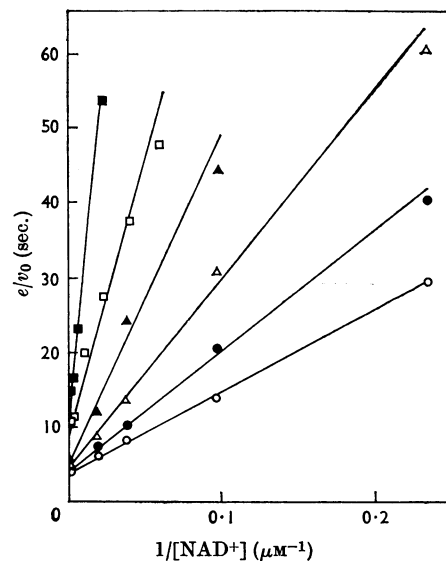


Fig. 2. Primary plots: variation of the reciprocal of the specific initial rate at pH 7.0 and 23.5° with the reciprocal of the NAD⁺ concentration for several constant propan-2-ol concentrations (mm): O, 400; ●, 100; Δ , 40; \blacktriangle , 20; \square , 10; \blacksquare , 5.

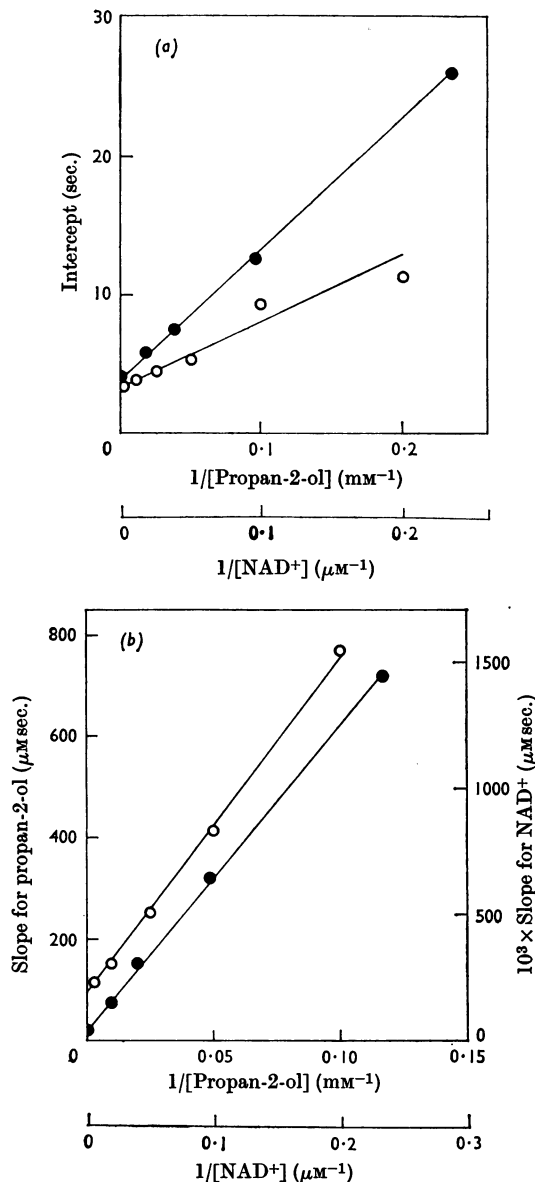


Fig. 3. Secondary plots: variations of the intercepts (a) and slopes (b) of the Lineweaver-Burk plots in Figs. 1 and 2 with the reciprocals of: the NAD⁺ concentration (●) and of the propan-2-ol concentration (○).

average values for the kinetic coefficients obtained from three such experiments with each substrate are given in Table 1.

The ranges of concentration of coenzyme and substrates in these experiments were: NAD⁺, 1–580 μM; propan-2-ol, 5–400 mM; butan-2-ol, 18–375 mM. The primary and secondary plots are

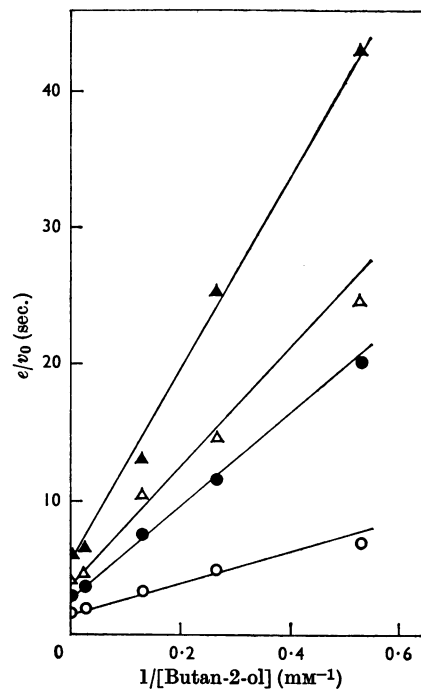


Fig. 4. Primary plots: variation of the reciprocal of the specific initial rate at pH 7.0 and 23.5° with the reciprocal of the butan-2-ol concentration for several constant NAD⁺ concentrations (μM): ○, 45.7; ●, 45.7; △, 22.8; ▲, 12.4.

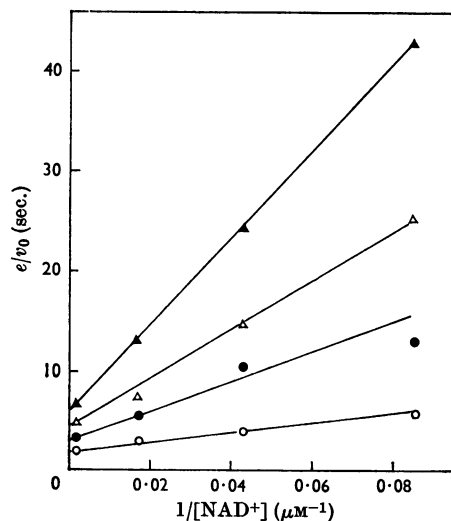


Fig. 5. Primary plots: variation of the reciprocal of the specific initial rate at pH 7.0 and 23.5° with the reciprocal of the NAD⁺ concentration for several constant butan-2-ol concentrations (mM): ○, 375; ●, 7.5; △, 3.8; ▲, 1.88.

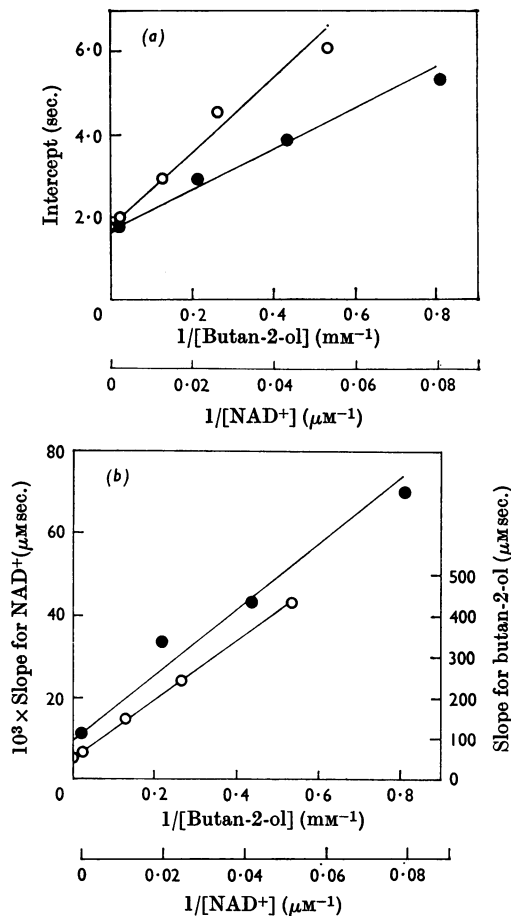


Fig. 6. Secondary plots: variations of the intercepts (a) and slopes (b) of the Lineweaver-Burk plots in Figs. 4 and 5 with the reciprocals of the NAD^+ concentration (\bullet) and of the butan-2-ol concentration (\circ).

linear, to within the experimental error, over these ranges. The absence of substrate inhibition with secondary alcohol concentrations up to 0.4M is noteworthy.

Primary alcohols. The results of initial-rate measurements with 0.1–2mm-2-methylpropan-1-ol are shown in the primary plots of Figs. 7 and 8 and the secondary plots of Fig. 9. Again the plots are linear within the experimental error. With alcohol concentrations greater than 2mm, substrate inhibition occurs (see below).

The results obtained with 0.025–2mm-propan-1-ol (Figs. 10–12) show small but significant deviations from linearity in the primary plots of reciprocal initial rate against reciprocal NAD^+

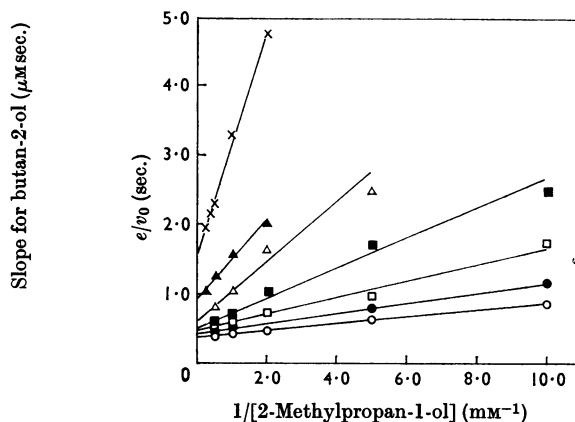


Fig. 7. Primary plots: variation of the reciprocal of the specific initial rate at pH 7.0 and 23.5° with the reciprocal of the 2-methylpropan-1-ol concentration for several constant NAD^+ concentrations (μM): \circ , 530; \bullet , 53; \triangle , 26.5; \blacktriangle , 12.9; \square , 6.6; \blacksquare , 3.3; \times , 1.3.

Table 1. Kinetic coefficients for the oxidation of primary and secondary alcohols and aldehydes by NAD^+ with liver alcohol dehydrogenase at 23.5° and pH 7

The kinetic coefficients are those in the initial-rate equation:

$$\frac{e}{v_0} = \phi_0 + \frac{\phi_1}{[S_1]} + \frac{\phi_2}{[S_2]} + \frac{\phi_{12}}{[S_1][S_2]}$$

where S_1 is NAD^+ and S_2 the substrate. The data for ethanol and butanol are those of Dalziel (1962b), the data for the aldehydes those of Dalziel & Dickinson (1965). Other kinetic data were obtained as described in the text. ϕ_1/ϕ_0 is the Michaelis constant for NAD^+ , and ϕ_2/ϕ_0 that for the substrate.

Substrates	ϕ_0 (sec.)	ϕ_1 ($\mu\text{Msec.}$)	ϕ_2 ($\mu\text{Msec.}$)	ϕ_{12} ($\text{mm}^2\text{sec.}$)	ϕ_1/ϕ_0 (μM)	ϕ_2/ϕ_0 (μM)	ϕ_{12}/ϕ_2 (μM)
Ethanol	0.37	1.1	66	0.0072	3.0	180	109
Propan-1-ol	0.31	1.2	19	0.0012	3.9	61	68
Butan-1-ol	0.35	1.1	4	0.0004	3.1	11	100
2-Methylpropan-1-ol	0.34	1.7	40	0.0032	5.0	118	80
Propan-2-ol	3.9	82	49000	6.2	21	12600	126
Butan-2-ol	1.7	41	9000	0.95	24	5300	105
Acetaldehyde	0.08	1.4	8000	0.26	18	100000	33
Butyraldehyde	0.025	0.6	400	0.015	24	16000	38

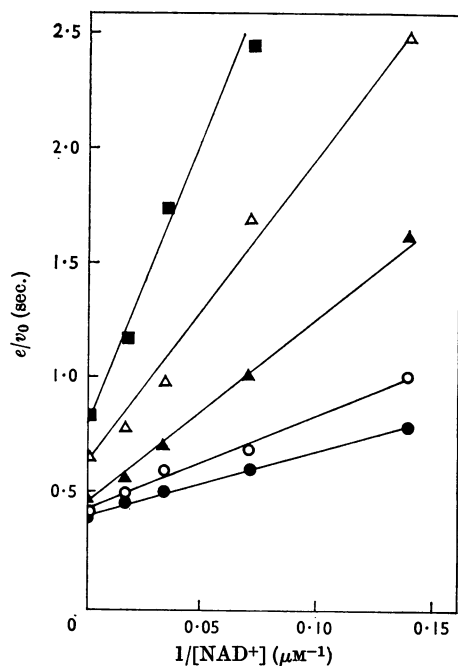


Fig. 8. Primary plots: variation of the reciprocal of the specific initial rate at pH 7.0 and 23.5° with the reciprocal of the NAD⁺ concentration for several constant 2-methylpropan-1-ol concentrations (mM): ●, 2; ○, 1; ▲, 0.5; △, 0.2; ■, 0.1.

concentration (Fig. 11), and in the secondary plot of intercepts against reciprocal NAD⁺ concentration (Fig. 12a). The deviations are towards greater activity with the largest concentrations of propanol and NAD⁺, and make the estimates of kinetic coefficients more uncertain than usual. The smallness of the deviations, and the fact that substrate inhibition occurs with still greater propanol concentrations, make it difficult to draw any firm conclusions about the apparent activation.

Average values for the kinetic coefficients from the experiments with each of these primary alcohols are given in Table 1, together with those for ethanol and butan-1-ol reported by Dalziel (1962b, 1963a).

Substrate inhibition with primary alcohols. With all four primary alcohols, the rate decreases with increase of alcohol concentration above about 2mM. The plots of reciprocal rate against substrate concentration (Fig. 13) show that inhibition is partial, the rate approaching a constant value with large alcohol concentration in each case. Estimates of these limiting rates, and of the alcohol concentrations at which the greatest rates are obtained with a saturating NAD⁺ concentration of 580 μM, are given in Table 2.

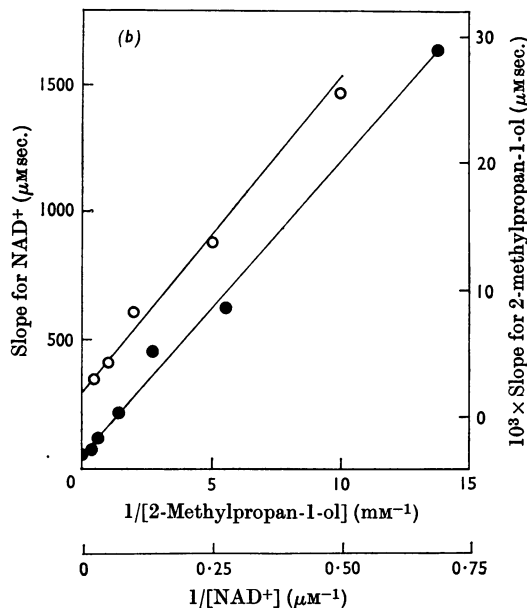
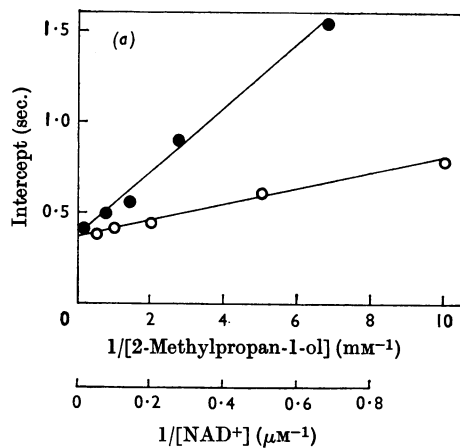


Fig. 9. Secondary plots: variations of the intercepts (a) and slopes (b) of the Lineweaver-Burk plots in Figs. 7 and 8 with the reciprocals of the NAD⁺ concentration (●) and of the 2-methylpropan-1-ol concentration (○).

Aldehydes. Secondary plots of intercepts obtained in the normal way from initial-rate measurements with 2-methylpropionaldehyde and propionaldehyde are shown in Figs. 14 and 15, and the kinetic coefficients obtained from them are compared in Table 3 with those previously reported for acetaldehyde and butyraldehyde. ϕ'_{12} was not estimated because at pH 7 it is small and can only be estimated approximately from very detailed measurements with small concentrations of both substrate and coenzyme (Dalziel, 1962b).

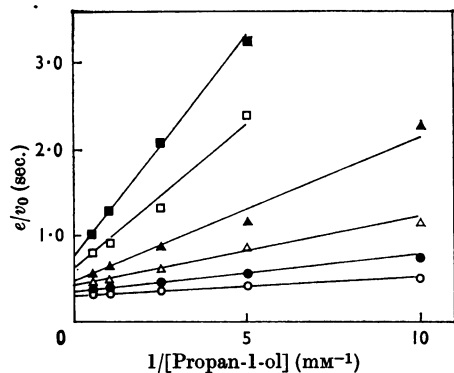


Fig. 10. Primary plots: variation of the reciprocal of the specific initial rate at pH 7.0 and 23.5° with the reciprocal of the propan-1-ol concentration for several constant NAD^+ concentrations (μM): \circ , 580; \bullet , 58; \triangle , 18.3; \blacktriangle , 9.6; \square , 4.5; \blacksquare , 2.42.

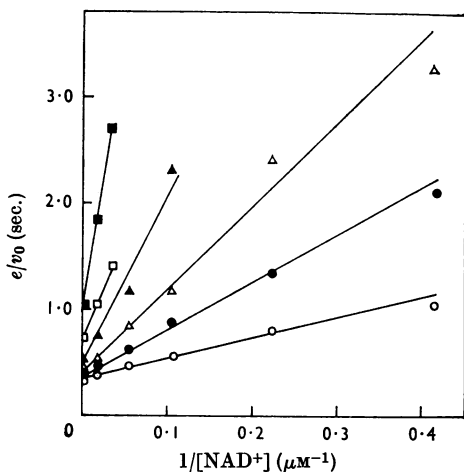
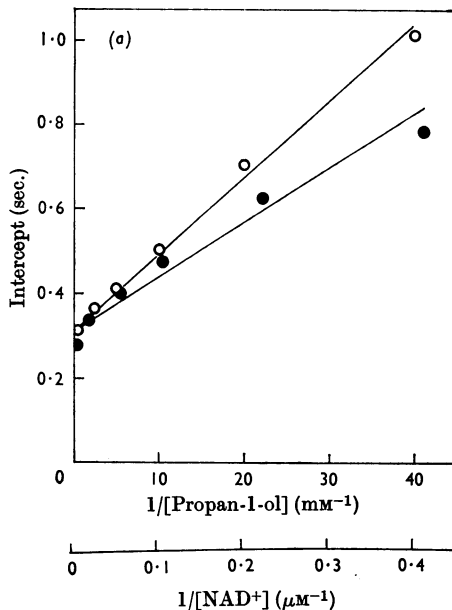


Fig. 11. Primary plots: variation of the reciprocal of the specific initial rate at pH 7.0 and 23.5° with the reciprocal of the NAD^+ concentration for several constant propan-1-ol concentrations (mm): \circ , 2; \bullet , 0.4; \triangle , 0.2; \blacktriangle , 0.1; \square , 0.05; \blacksquare , 0.025.

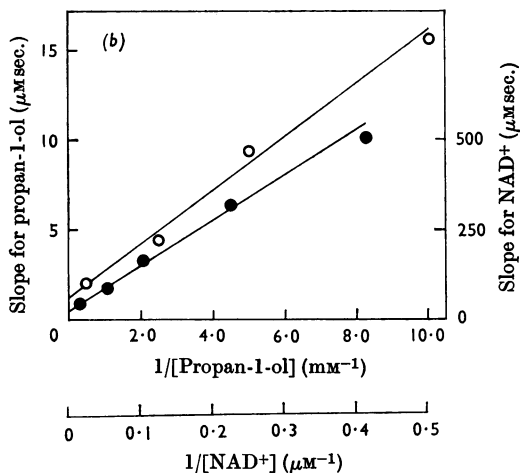


Fig. 12. Secondary plots: variations of the intercepts (a) and slopes (b) of the Lineweaver-Burk plots in Figs. 10 and 11 with the reciprocals of the NAD^+ concentration (\bullet) and of the propan-1-ol concentration (\circ).

Ketones. With $40\mu\text{M-NADH}$, 10mm-acetone and $0.15\mu\text{M-enzyme}$, no oxidation of NADH could be detected in the fluorimeter. The rate must therefore be less than $1.5\mu\text{M-NADH}$ oxidized/min., and the turnover number less than 10/min.

With butan-2-one, NADH oxidation was observed but the progress curves were biphasic, a rapid oxidation being followed by a slower linear decrease of fluorescence. Repeated fractional distillation of the ketone showed the rapid initial phase to be due to an impurity, presumably an

aldehyde, and the amount of NADH oxidized in the rapid phase indicated that the commercial ketone preparations contained about 0.1% of this impurity. By repeated fractional distillation ketone was obtained that showed no significant rapid phase, and gave a constant rate of NADH oxidation, with $0.75\mu\text{M-enzyme}$ and $40\mu\text{M-NADH}$, of $5\mu\text{M/min.}$ with 50mm-ketone and $10\mu\text{M/min.}$ with 930mm-ketone . This very slow reaction could

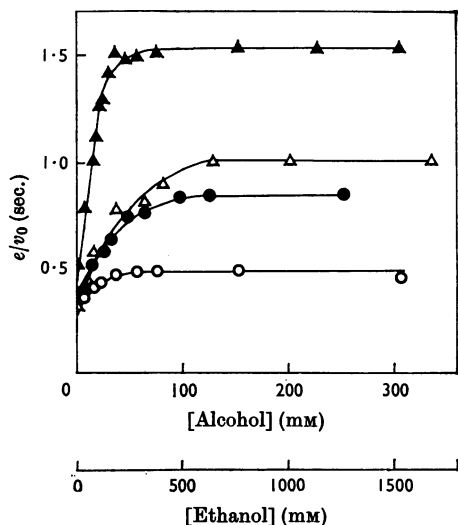


Fig. 13. Variation of the reciprocal of the specific initial rate of primary alcohol oxidation at pH 7.0 and 23.5° and at constant NAD^+ concentration ($580 \mu\text{M}$) with the primary alcohol concentrations: O, 2-methylpropan-1-ol; ●, ethanol; Δ , propan-1-ol; \blacktriangle , butan-1-ol.

Table 2. Maximum specific rates, limiting rates with infinitely large substrate concentrations, and optimum substrate concentrations for primary alcohols at 23.5° and pH 7

Maximum specific rates, $V = 1/\phi_0$, are from Table 1. Optimum substrate concentrations were estimated from the minima of plots of initial rate against reciprocal alcohol concentrations, with 0.58 mM-NAD^+ . Limiting rates with infinitely large substrate concentrations, V_1 , are from Fig. 13.

Substrate	V (sec.^{-1})	V_1 (sec.^{-1})	$[S]_{\text{opt.}}$ (mM)
Ethanol	2.7	1.2	8.0
Propan-1-ol	3.2	1.0	2.0
Butan-1-ol	2.9	0.65	1.0
2-Methylpropan-1-ol	2.9	2.1	3.0

be due to another impurity in the ketone, and detailed kinetic studies would be unprofitable. Evidently butan-2-one is a very poor substrate.

DISCUSSION

These experiments confirm that propan-2-ol and butan-2-ol are substrates for liver alcohol dehydrogenase. The data of Table 1 show that they are poorer substrates than the isomeric primary alcohols, and not only because they have larger Michaelis constants (ϕ_2/ϕ_0). All four kinetic

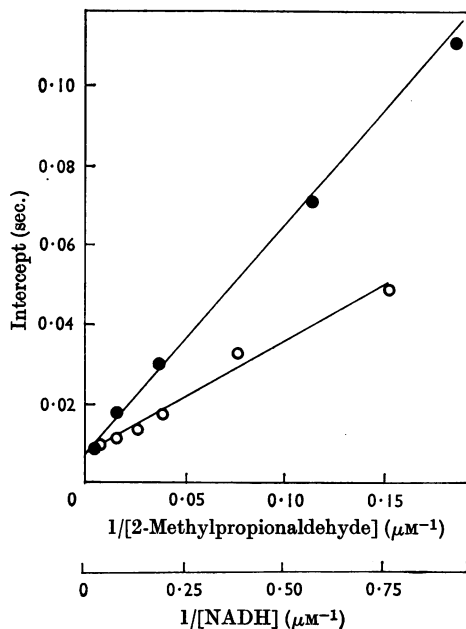


Fig. 14. Secondary plots showing the variation of intercepts of primary Lineweaver-Burk plots with the reciprocal of the NADH concentration (●) and of the 2-methylpropionaldehyde concentration (○).

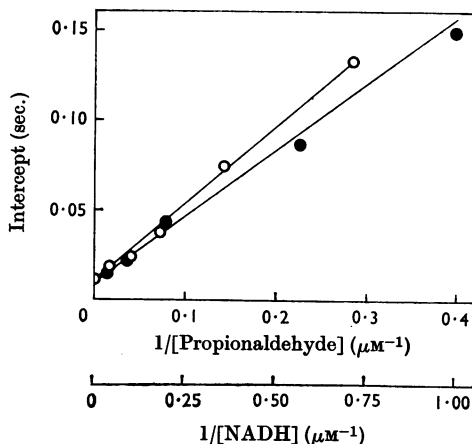


Fig. 15. Secondary plots showing the variation of intercepts of primary Lineweaver-Burk plots with the reciprocals of the NADH concentration (●) and of the propionaldehyde concentration (○).

coefficients in eqn. (1), and the Michaelis constants for the coenzyme (ϕ_1/ϕ_0), are substantially greater with secondary alcohols than with primary alcohols.

For the four primary alcohols ϕ_0 and ϕ_1 are

Table 3. Kinetic coefficients for the reduction of aldehydes by NADH with liver alcohol dehydrogenase at 23.5° and pH 7

The kinetic coefficients are those in the initial-rate equation:

$$\frac{e}{v_0'} = \phi'_0 + \frac{\phi'_1}{[S'_1]} + \frac{\phi'_2}{[S'_2]} + \frac{\phi'_{12}}{[S'_1][S'_2]}$$

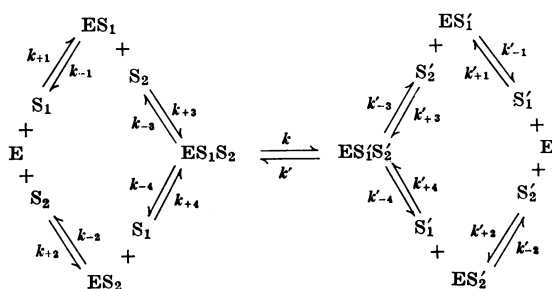
where S'_1 is NADH and S'_2 is aldehyde. The data for acetaldehyde and butyraldehyde are from Dalziel (1962b), and the other data were obtained as described in the text.

Substrate	ϕ'_0 (sec.)	ϕ'_1 (μ Msec.)	ϕ'_2 (μ Msec.)
Acetaldehyde	0.0075	0.10	3.3
Propionaldehyde	0.0095	0.14	0.43
Butyraldehyde	0.0075	0.10	0.17
2-Methylpropion- aldehyde	0.009	0.12	0.28

reasonably constant. The branched-chain 2-methylpropan-1-ol is a poorer substrate than propan-1-ol, but among the three straight-chain alcohols ϕ_2 , and therefore the Michaelis constant for the substrate, decreases with increase of chain length. Similarly, butan-2-ol is a better substrate than propan-2-ol, but in contrast with the primary alcohols these secondary alcohols give different values for ϕ_0 and ϕ_1 as well as for ϕ_2 .

Previous conclusions that the behaviour of primary alcohols and aldehydes could be described by the Theorell-Chance mechanism were partly based on the identity of ϕ_0 , ϕ_1 , ϕ'_0 , and ϕ'_1 for different alcohols and aldehydes, and on relations between the eight kinetic coefficients for forward and reverse reactions (Dalziel, 1962b, 1963b). The most striking feature of the present work is that secondary alcohols do not satisfy several of these criteria, and that the enzyme therefore appears to exhibit different mechanisms with different substrates. Before attempting to interpret the data for secondary alcohols, it is pertinent to summarize previous evidence of the mechanism for ethanol and butan-1-ol oxidation and the reverse reactions, and to consider the extent to which the new data for propan-1-ol and 2-methylpropan-1-ol and the corresponding aldehydes, and the experiments on substrate inhibition, support the previous work.

Two-substrate or coenzyme-substrate reactions may be considered as group-transfer reactions, for which there are two general types of mechanism. In the 'ternary-complex' or 'single-displacement' mechanism (Haldane, 1930; Koshland, 1955) both substrates are simultaneously bound to the enzyme, and group transfer takes place directly within the ternary complex. In the 'substituted-enzyme' or 'double-displacement' mechanism (Doudoroff, Barker & Hassid, 1947; Koshland, 1955) the group



Scheme 1.

is transferred from the donor substrate to the enzyme, and then, after dissociation of the first product, from enzyme to acceptor substrate. These two types of mechanism may be distinguished by isotope-exchange studies, by studies of the reaction between stoichiometric amounts of enzyme and one substrate, and by initial-rate kinetics with rate-limiting concentrations of both substrates. The latter distinction depends on the fact that, because ternary complexes are not formed, the initial-rate equation for the substituted-enzyme mechanism lacks the last term of eqn. (1), i.e. $\phi_{12}=0$ (Dalziel, 1957). This mechanism is therefore not satisfactory for liver alcohol dehydrogenase with either primary (Dalziel & Theorell, 1957) or secondary alcohols or for other nicotinamide nucleotide-linked dehydrogenases (cf. Massey & Veeger, 1963).

Steady-state treatment of the ternary-complex mechanism with alternative pathways (Scheme 1) gives an initial-rate equation of the second degree in each substrate concentration (Dalziel, 1958). Non-linear reciprocal-rate plots (Lineweaver & Burk, 1934) of the substrate activation or inhibition type, or even of more complex forms, are therefore inherent in this general mechanism, and do not necessarily indicate the presence of two or more different, or identical but interacting, substrate-binding sites (Dalziel, 1957, 1958). These points are worth re-emphasizing in view of the current interest in such behaviour by enzymes concerned in metabolic control.

One of several special cases of this mechanism (Dalziel, 1958) that does give a linear reciprocal initial-rate equation of the form of eqn. (1) is the rapid-equilibrium random-order mechanism, in which it is assumed that the interconversions of the ternary complexes (k, k') are rate-limiting, and binary and ternary complexes are in equilibrium (Haldane, 1930). The kinetic coefficients are functions of k and the dissociation constants of the complexes ($K_1 = k_{-1}/k_{+1}$ etc.) as shown in Table 4.

Table 4. *Kinetic coefficients in the initial-rate equation for mechanisms for two-substrate reactions*

The initial rate equation is:

$$\frac{e}{v_0} = \phi_0 + \frac{\phi_1}{[S_1]} + \frac{\phi_2}{[S_2]} + \frac{\phi_{12}}{[S_1][S_2]}$$

The mechanisms are special cases of the general alternative-pathway mechanism shown in Scheme 1, to which the rate constants refer, as described in the text. $A = 1 + k'/k'_{-3}$.

Mechanism	ϕ_0	ϕ_1	ϕ_2	ϕ_{12}
1. Equilibrium random-order	$\frac{1}{k}$	$\frac{K_4}{k}$	$\frac{K_3}{k}$	$\frac{K_1 K_3}{k}$
2. General compulsory-order	$\frac{1}{k'_{-1}} + \frac{1}{k'_{-3}} + \frac{A}{k}$	$\frac{1}{k_{+1}}$	$\frac{Ak_{-3} + k}{kk_{+3}}$	$\frac{k_{-1}(Ak_{-3} + k)}{k_{+1}kk_{+3}}$
3. Theorell-Chance	$\frac{1}{k'_{-1}}$	$\frac{1}{k_{+1}}$	$\frac{Ak_{-3} + k}{kk_{+3}}$	$\frac{k_{-1}(Ak_{-3} + k)}{k_{+1}kk_{+3}}$

If the lower alternative pathways in Scheme 1 are omitted steady-state treatment yields eqn. (1) with kinetic coefficients as shown in Table 4. A further special case of this compulsory-order mechanism arises when $k'_{-1} \ll k$, k'_{-3} and kk'_{-3}/k' (Dalziel, 1957). Ternary complexes are then not kinetically significant, and the maximum specific rate is $1/\phi_0 = k'_{-1}$. This is equivalent to the mechanism first proposed by Theorell & Chance (1951) for liver alcohol dehydrogenase. Although these workers did not actually show ternary complexes in their reaction scheme, the probable existence of such intermediates in rapid steps was recognized (cf. Theorell, Nygaard & Bonnichsen, 1955). The stereospecificity of nicotinamide nucleotide-linked dehydrogenases (Vennesland, 1955) makes the formation of such complexes virtually certain, and the fact that the aldehyde-mutase cycle catalysed by liver alcohol dehydrogenase exhibits a maximum rate is direct evidence for their presence (Dalziel & Dickinson, 1965). This limiting case of a compulsory-order mechanism, in which ternary complexes are formed but are not kinetically significant, is referred to here, as in previous papers, as the Theorell-Chance mechanism. Some authors, however, restrict the term to a peroxidase-type mechanism in which ternary complexes are not formed (Bloomfield, Peller & Alberty, 1962; Cleland, 1963; Wong & Hanes, 1964; Sanwal, Stachow & Cook, 1965).

Primary alcohols and aldehydes. The main evidence in support of the Theorell-Chance mechanism for the ethanol-acetaldehyde and butanol-butylaldehyde systems is as follows.

(1) Initial-rate data over wide ranges of substrate and coenzyme concentrations and pH satisfy eqn. (1) (Theorell & McKinley-McKee, 1961a; Dalziel, 1962b, 1963a).

(2) The velocity constant k'_{-1} for combination

of enzyme with NADH measured directly by rapid-reaction technique (Theorell & Chance, 1951) agrees reasonably well with $1/\phi'_1$ (cf. Table 4). Confirmation by the more sensitive rapid-reaction technique now available is desirable.

(3) Provided that pure coenzymes are used, the maximum specific rate in each direction ($1/\phi_0$, $1/\phi'_0$) is equal to the rate of dissociation of the product coenzyme calculated from initial-rate parameters for the reverse reaction on the assumption of a compulsory-order mechanism ($k_{-1} = \phi_{12}/\phi_1\phi_2$, $k'_{-1} = \phi'_{12}/\phi'_1\phi'_2$). If the ternary complexes were rate-limiting, the maximum specific rate would be smaller than the latter function (Table 4). Previous evidence that the opposite inequality held (Dalziel & Theorell, 1957; Dalziel, 1962b), which was interpreted as evidence that isomeric enzyme-coenzyme complexes were formed (Mahler, Baker & Shiner, 1962; Bloomfield *et al.* 1962; Wratten & Cleland, 1963), was explained by the effects of competitive inhibitors in commercial coenzyme preparations (Dalziel, 1961).

(4) The two alcohols gave the same values for ϕ_0 and for ϕ_1 , but different values for ϕ_2 and ϕ_{12} . The two aldehydes showed similar behaviour in the reverse reaction (Dalziel, 1962b). The identity of the ϕ_1 values is expected for the general compulsory-order mechanism, that of the maximum rates only for the Theorell-Chance mechanism. Neither would be expected for a random-order or alternative-pathway mechanism.

(5) Values for the dissociation constants of enzyme-coenzyme complexes calculated from initial-rate data ($K_1 = \phi_{12}/\phi_2 = \phi_1/\phi'_0$; $K'_1 = \phi'_{12}/\phi'_2 = \phi'_1/\phi_0$) were the same for both pairs of substrates (Dalziel, 1962b, 1963b) and agreed reasonably well with those obtained by direct equilibrium measurements (Theorell & Winer, 1959; Theorell & McKinley-McKee, 1961b).

A direct value for K_1 by a more accurate method is desirable.

The results now reported for propan-1-ol and 2-methylpropan-1-ol and for the corresponding aldehydes (Tables 1 and 3) provide further support for the Theorell-Chance mechanism by substantiating these observations. In particular, the constancy of ϕ_0 and ϕ_1 is in striking contrast with the differing values obtained for these parameters with the secondary alcohols.

Substrate inhibition with primary alcohols and aldehydes. Inhibition with ethanol concentrations greater than 8mM has been attributed to the formation of the abortive ternary complex $E \cdot NADH \cdot ROH$ (Theorell & McKinley-McKee, 1961c), and direct fluorimetric evidence for the formation of this complex, and analogous complexes with other dehydrogenases, has been reported (Winer & Schwert, 1958; Theorell & McKinley-McKee, 1961b). This interpretation is supported by the fact that the inhibition is most marked with saturating NAD^+ concentrations (Dalziel, 1962b). Theorell & McKinley-McKee (1961c) considered that $NADH$ dissociates from the abortive complex, at a lower rate than from $E \cdot NADH$, to give a binary enzyme-alcohol complex. The present results with four primary alcohols substantiate this conclusion by showing that the alcohols behave as partial inhibitors, i.e. that $E \cdot NADH \cdot ROH$ is not a 'dead-end' complex; the limiting rate approached with very large alcohol concentrations can be identified with the rate of dissociation of $NADH$ from this complex, and decreases with increase of chain length of the alcohol (Table 2). The quantitative treatment of this inhibition mechanism is considered below.

Substrate inhibition has also been observed in the reverse reaction with butyraldehyde and 2-methylpropionaldehyde concentrations greater than about 1mM, and is explained by the formation of the analogous complex $E \cdot NAD^+ \cdot RCHO$, which is, however, the active ternary complex of the aldehyde-dehydrogenase reaction catalysed by this enzyme (Dalziel & Dickinson, 1965).

Secondary alcohols. The large values of ϕ_2 for secondary alcohols could be due to their relatively small affinity for $E \cdot NAD^+$, or slow intramolecular reaction in the ternary complex (k). The constancy of the ratio ϕ_{12}/ϕ_2 for both primary and secondary alcohols and for aldehydes in the aldehyde-dehydrogenase reaction (Dalziel & Dickinson, 1965) is striking in view of the enormous variations of the values of each of these parameters for the three sets of oxidizable substrates (Table 1). A satisfactory mechanism for secondary alcohols must clearly retain the relation $\phi_{12}/\phi_2 = K_1$. The low values of this ratio for aldehydes compared with those for alcohols is explained by the smaller

phosphate buffer concentrations used in the aldehyde-mutase studies (Dalziel & Dickinson, 1965).

The different values for ϕ_1 for the two secondary alcohols are not consistent with a simple compulsory-order mechanism, and suggest that with these substrates the dependence of rate on NAD^+ concentration is determined not by the rate of combination of NAD^+ with enzyme but by dissociation of NAD^+ from the active ternary complex $E \cdot NAD \cdot R_1R_2CHOH$. Evidence for such dissociation of coenzyme, without prior dissociation of substrate, was presented by Silverstein & Boyer (1964), and is discussed below. Supporting evidence is the analogous dissociation of the abortive complex $E \cdot NADH \cdot RCH_2OH$ indicated by the partial character of substrate inhibition, and kinetic evidence from studies of the mutase cycle that the active ternary complex of the aldehyde-dehydrogenase reaction, $E \cdot NAD^+ \cdot RCHO$, can dissociate in this way (Dalziel & Dickinson, 1965). With primary alcohols, this alternative pathway of reactant dissociation would not be rate-limiting in initial-rate studies because the ternary-complex interconversion and aldehyde-product dissociation are fast, and therefore with saturating alcohol concentrations the ternary-complex concentration in the steady state will be negligible. However, with secondary alcohols the greater values of ϕ_0 suggest that the intramolecular reaction of the ternary complex is slower than with primary alcohols, and is rate-limiting under maximum-rate conditions. The different values for ϕ_0 obtained with the two secondary alcohols support this view, as in the aldehyde-dehydrogenase reaction (Dalziel & Dickinson, 1965). With saturating concentrations of secondary alcohols the steady-state concentration of the ternary complex would then be large, and dissociation of NAD^+ from it could be the limiting factor that determines ϕ_1 .

Thus a consistent account of the kinetics of oxidation of primary and secondary alcohols (and of aldehydes) can be obtained on the assumption of alternative pathways of dissociation of reactants from the ternary complex, and different rates of oxidation within the ternary complex.

Evidence of alternative pathways and enzyme-alcohol complexes. Silverstein & Boyer (1964) adduced evidence for the existence of alternative pathways from the observation that isotope exchange between NAD^+ and $NADH$ persisted at a significant rate, in the presence of liver alcohol dehydrogenase, up to very large equilibrium concentrations of ethanol and acetaldehyde. If alternative pathways of dissociation of ternary complexes were forbidden, as in a compulsory-order mechanism involving only the upper pathways of Scheme 1, the enzyme would be entirely

in the form of the ternary complexes under these conditions, and $\text{NAD}^+ \rightleftharpoons \text{NADH}$ exchange would be blocked. The abortive complex $\text{E} \cdot \text{NADH} \cdot \text{ROH}$ and the active ternary complex of the aldehyde-dehydrogenase reaction, $\text{E} \cdot \text{NAD} \cdot \text{RCHO}$, would also be present, however, and it was recognized that these complexes, and not the active complexes of the alcohol-dehydrogenase reaction, might be responsible for the exchange (Wong & Hanes, 1964; Dalziel & Dickinson, 1965). However, the evidence presented above for dissociation of NAD^+ from the ternary complex $\text{E} \cdot \text{NAD} \cdot \text{R}_1\text{R}_2\text{CHOH}$ makes it likely that this can also occur with primary alcohols.

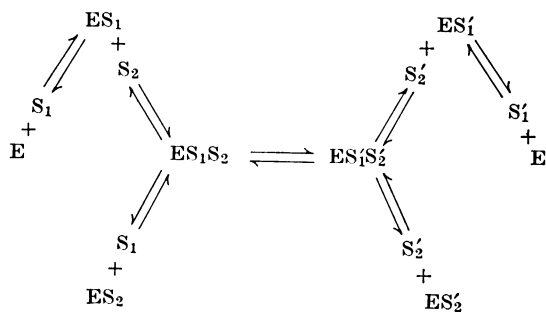
Silverstein & Boyer (1964) found the rate of acetaldehyde \rightleftharpoons ethanol exchange, R' , to be 160 times that of the $\text{NAD}^+ \rightleftharpoons \text{NADH}$ exchange, R , with excess of reactants at equilibrium, and concluded that ternary-complex interconversion could not be a slow step in the catalysis, in accordance with conclusions from initial-rate studies. From the relations given by these authors, this result shows that, in Scheme 1, $k \gg k_{-4}$ or $k' \gg k'_{-4}$, or both, and that $k_{-3} \gg k_{-4}$ or $k'_{-3} \gg k'_{-4}$ or both. However, it sets no restriction on the relative values of k_{-3} and k or k'_{-3} and k' , and therefore, though ternary-complex interconversion cannot be rate-limiting in the lower pathway of reaction, it could be in the upper pathway.

The data do, however, provide other evidence, which seems to have been overlooked, that the dissociation of the product complex $\text{E} \cdot \text{NADH}$ (k'_{-1}) is in fact rate-limiting in the overall ethanol- NAD^+ reaction. The maximum initial rate of the NADH -acetaldehyde reaction, V , was 1.44 times the rate of the acetaldehyde \rightleftharpoons ethanol exchange, R' . At the pH of these experiments, the maximum initial rate of the reverse reaction, V' , is only one-fiftieth of V (Dalziel, 1963a) and therefore one-thirtieth of R' . From the relations given by Silverstein & Boyer (1964) for R' , this shows that

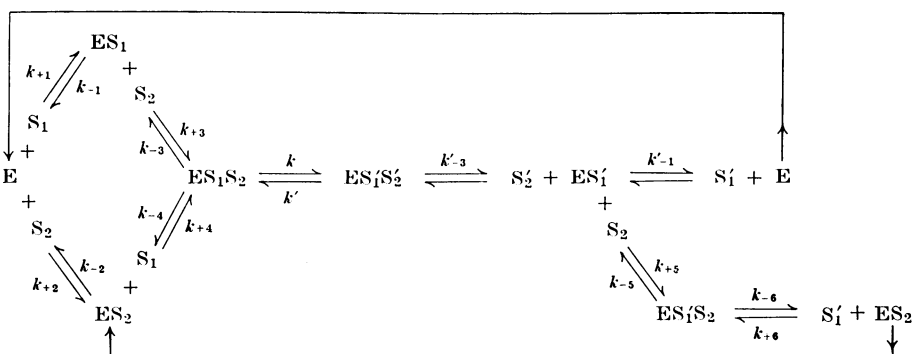
k'_{-1} determines V' , in accordance with conclusions from initial-rate studies.

Wong & Hanes (1964) have pointed out that the isotope-exchange results could be accounted for by a compulsory-order mechanism with dead-end branches (Scheme 2). The exchange rates do not depend on the rates of reaction of free enzyme with coenzyme or substrate. It follows that the relative values of R and R' give no information about the relative rates of net reaction through the upper and lower reactant pathways of Scheme 1. Thus, though the existence of enzyme-alcohol complexes is indicated by the isotope-exchange experiments, by the partial character of substrate inhibition and by the large values of ϕ_1 for secondary alcohols, there is no evidence that they are kinetically significant in the reaction. It remains to show that a mechanism that provides for the formation of such complexes will account for all the experimental results.

Proposed mechanism for alcohol dehydrogenase. The preceding qualitative interpretation of initial-rate data for primary and secondary alcohols can be given quantitative expression by the general mechanism of Scheme 3, of which the mechanisms



Scheme 2.



Scheme 3.

for the two types of alcohol are special cases. This differs from Scheme 1 by inclusion of the abortive complex ES_1S_2 and omission of the alternative pathway of product dissociation through ES_2' . The latter omission is justified for primary alcohols by evidence from isotope-exchange experiments and initial-rate studies that $k'_{-3} \gg k'_{-4}$ and that the maximum specific rate (excluding substrate inhibition) is k'_{-1} . The upper pathway of product dissociation is also likely for secondary alcohols, especially as ketones are such poor substrates. On similar grounds it may be assumed that (i) $k_{-3} \gg k_{-4}$. The initial-rate equation in the absence of products, derived by steady-state treatment of Scheme 3, is then:

$$\frac{e}{v_0} = \left(\frac{A}{k} + \frac{1}{k'_{-3}} + \frac{B}{k'_{-1}} \right) + \left(\frac{Ak_{-4}k_{+1}}{kk_{+4}} + \frac{k_{-2} + (C+D/E)k_{+4}[S_1] + k_{+2}[S_2]}{k_{-2} + k_{+4}[S_1] + Ek_{+2}[S_2]} \right) \frac{1}{k_{+1}[S_1]} + \left(\frac{Ak_{-3}}{k} + \frac{k_{-2} + Ck_{+4}[S_1]}{k_{-2} + k_{+4}[S_1] + Ek_{+2}[S_2]} \right) \frac{1}{k_{+3}[S_2]} + \left(\frac{Ak_{-3}}{k} + \frac{k_{-2} + Ck_{+4}[S_1] + k_{+2}[S_2]}{k_{-2} + k_{+4}[S_1] + Ek_{+2}[S_2]} \right) \times \frac{k_{-1}}{k_{+1}k_{+3}[S_1][S_2]} \quad (2)$$

with:

$$A = \frac{k' + k'_{-3}}{k'_{-3}}, \quad B = \frac{k_{+5}[S_2] + k_{-5} + k_{-6}}{(k_{-6}/k'_{-1})k_{+5}[S_2] + k_{-5} + k_{-6}},$$

$$C = \frac{k_{-5} + k_{-6}}{(k_{-6}/k'_{-1})k_{+5}[S_2] + k_{-5} + k_{-6}},$$

$$D = \frac{(k_{-6}/k'_{-1})k_{+5}[S_2]}{(k_{-6}/k'_{-1})k_{+5}[S_2] + k_{-5} + k_{-6}},$$

$$C + D = 1 \text{ and } E = \frac{k_{+4}}{k_{+1}}$$

If $k_{-6} < k'_{-1}$, B , C and D are substrate-inhibition factors arising from ES_1S_2 formation with large primary alcohol concentrations. With smaller concentrations (less than 8mM) such that (ii) $k_{+5}[S_2] \ll k_{-5} + k_{-6}$ then $B = C = 1$ and $D = 0$. The resulting equation [which can also be derived from that given by Dalziel (1958) for Scheme 1] is not of the linear reciprocal form of eqn. (1) because of the alternative pathways for formation of ES_1S_2 .

If also (iii) $Ek_{+2}[S_2] \ll k_{-2} + k_{+4}[S_1]$ and (iv) $k_{+2}[S_2] \ll k_{-2} + k_{+4}[S_1]$, a linear reciprocal equation of the form of eqn. (1) is obtained, namely:

$$\frac{e}{v_0} = \left(\frac{A}{k} + \frac{1}{k'_{-3}} + \frac{1}{k'_{-1}} \right) + \left(\frac{Ak_{-4}}{kk_{+4}} + \frac{1}{k_{+1}} \right) \frac{1}{[S_1]} + \frac{\phi_2}{[S_2]} + \frac{\phi_{12}}{[S_1][S_2]} \quad (3)$$

where ϕ_2 and ϕ_{12} are the same functions of velocity constants as for a compulsory-order mechanism in Table 4. Condition (iii) together with condition (i) means that the rate of reaction through the bottom pathway (v_b) is negligible compared with that through the top pathway (v_t), since it can be shown by steady-state analysis that:

$$\frac{v_b}{v_t} = \frac{(k_{-4}/k_{-3})k_{-2} + (k_{+4}/k_{+1})k_{+2}[S_2]}{k_{-2} + k_{+4}[S_1]}$$

If $k_{+4} \gg k_{+1}$, condition (iv) is implicit in condition (iii). However, if $k_{+4} < k_{+1}$, condition (iii) may be satisfied but not condition (iv), with sufficiently large substrate concentrations. In that case the coefficients of the second and fourth terms of eqn. (3) would include the inhibition factors $1 + k_{+2}[S_2]/(k_{-2} + k_{+4}[S_2])$, indicating competitive inhibition of S_1 by S_2 . This is another possible cause of substrate inhibition, arising from the inefficiency of the pathway through ES_2 (Dalziel, 1957). It may also be mentioned that a more restrictive assumption than conditions (iii) and (iv), which also yields eqn. (3), is that $k_{+2} = k_{-2} = 0$. This corresponds to a dead-end-complex mechanism in which ES_2 cannot dissociate and is formed only via ES_1S_2 (cf. Scheme 2).

Eqn. (3) accounts for the data for primary and secondary alcohols and that for aldehydes in the aldehyde-dehydrogenase reaction. The constancy of ϕ_{12}/ϕ_2 for all these substrates is predicted. For primary alcohols, $k \gg k_{-4}$ and $\phi_1 = 1/k_{+1} = 1.1 \mu\text{Msec.}$, and $\phi_0 = 1/k'_{-1} = 0.35 \text{sec.}^{-1}$, as discussed above. For secondary alcohols, the larger values of ϕ_0 indicate that $1/k'_{-1}$ is small compared with $A/k + 1/k'_{-3}$. It also seems likely that k'_{-3} will be at least as great for ketone dissociation as for aldehyde dissociation and that $\phi_0 = A/k$ for secondary alcohols, i.e. ternary-complex interconversion is rate-limiting. Similarly, the large values for ϕ_1 indicate that $\phi_1 = Ak_{-4}/kk_{+4}$, $1/k_{+1}$ being negligible.

The very different values for ϕ_0 and ϕ_1 obtained previously for the aldehyde-dehydrogenase reaction can be interpreted in the same way as for secondary alcohols (Dalziel & Dickinson, 1965). According to this interpretation, $\phi_1/\phi_0 = k_{-4}/k_{+4}$, i.e. the Michaelis constant for the coenzyme is equal to K_4 , the dissociation constant for NAD^+ from the ternary complex. The constancy of this ratio for different secondary alcohols and aldehydes is striking in view of the variation of each of these parameters (Table 1), and indicates that $K_4 = 20 \mu\text{M}$ independent of the nature of the substrate. This

is smaller than $K_1 = \phi_{12}/\phi_2$, the dissociation constant of $E \cdot NAD^+$ (Table 1), i.e. these substrates increase the affinity of the enzyme for the coenzyme. The similarity between the behaviour of secondary alcohols and aldehydes may be correlated with their structural similarity if the aldehydes react in the hydrated form. Thus the different values of ϕ_0 and ϕ_1 are explained by different rates of oxidation within the ternary complex (k), which increase in the order propan-2-ol, butan-2-ol, acetaldehyde, butyraldehyde. The aldehydes give bigger maximum rates than the primary alcohols because the product complex $E \cdot NADH$ is rapidly reoxidized by the reverse alcohol-dehydrogenase reaction, whereas the dissociation velocity of $E \cdot NADH$, k'_{-1} , limits the maximum rate for primary alcohols. For the latter, the Michaelis constant for the coenzyme, $\phi_1/\phi_0 = k'_{-1}/k_{+1}$, is not a dissociation constant.

Substrate inhibition with large concentrations (greater than 8mm) of primary alcohols and of NAD^+ is explained by increase of the first term of eqn. (3) from $\phi_0 = 1/k'_{-1}$ to $\phi_0 = B/k'_{-1}$, the other terms being negligible under these conditions. The limiting value of B/k'_{-1} with very large alcohol concentrations is $1/k_{-6}$. The absence of substrate inhibition with secondary alcohol concentrations up to 400mm will be partly due to the fact that A/k and not B/k'_{-1} is the dominant term in ϕ_0 . In addition, the affinity of $E \cdot NADH$ for secondary alcohols could be smaller, or k_{-6} larger, than for primary alcohols.

It has been pointed out above that another cause of substrate inhibition could operate if $k_{+4} < k_{+1}$; it would affect the second and fourth terms of eqn. (3) and therefore be most apparent with small concentrations of NAD^+ . If $k_{+4} > k_{+1}$, substrate activation would be possible. The importance of the relative values of k_{+1} and k_{+4} in explaining such deviations from linear reciprocal plots has been pointed out previously in connexion with a simpler alternative-pathway mechanism (Dalziel, 1957). Substrate activation would also occur if $k_{-6} > k'_{-1}$, and would be most marked with large coenzyme concentrations. This is found experimentally for yeast alcohol dehydrogenase with ethanol as substrate (Nygaard & Theorell, 1955), and also for liver alcohol dehydrogenase with cyclohexanol as substrate (K. Dalziel & F. M. Dickinson, unpublished work). It is apparent that the alternative-pathway and abortive-complex mechanism of Scheme 3 for a two-substrate reaction will readily account for substrate activation and inhibition, and possibly also for the more complex types of behaviour reported for citrate-condensing enzyme (Kosicki & Srere, 1961) and various regulatory enzymes.

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