Substrate Activation and Inhibition in Coenzyme-Substrate Reactions

CYCLOHEXANOL OXIDATION CATALYSED BY LIVER ALCOHOL DEHYDROGENASE

By K. DALZIEL AND F. M. DICKINSON
Department of Biochemistry, University of Oxford

(Received 28 February 1966)

1. The activity of liver alcohol dehydrogenase with cyclohexanol and cyclohexanone as substrates was studied, and the initial-rate parameters were determined from measurements at low substrate concentrations. In contrast with aliphatic ketones, cyclohexanone is a fairly good substrate, although less active than aliphatic aldehydes. The Michaelis constant for cyclohexanol is of the same order as that for ethanol, and the maximum rate and Michaelis constant for NAD^+ obtained with cyclohexanol are very similar to those obtained with primary aliphatic alcohols. The data for this substrate at low concentrations are therefore consistent with a compulsory-order mechanism in which ternary complexes are not rate-limiting. 2. With large concentrations of NAD^+ , substrate activation is observed with increasing concentrations of cyclohexanol, whereas with small NAD^+ concentrations substrate inhibition is observed. This complex behaviour is explained by a mechanism previously proposed for this enzyme, which also satisfactorily described the kinetics of oxidation of primary and secondary aliphatic alcohols and aldehydes, including the substrate inhibition exhibited by primary alcohols, and the reduction of aldehydes. The activation with large concentrations of both NAD^+ and cyclohexanol is attributed to the formation of an abortive complex, $\text{E} \cdot \text{NADH} \cdot \text{ROH}$, from which NADH dissociates more rapidly than from the normal product complex $\text{E} \cdot \text{NADH}$. Substrate inhibition in the presence of small NAD^+ concentrations is attributed to the formation of an active complex $\text{E} \cdot \text{ROH}$, with which NAD^+ reacts more slowly than with the free enzyme. 3. Some support for these mechanisms of substrate activation and inhibition is obtained by approximate theoretical calculations, and their applicability to other two-substrate reactions that exhibit complex initial-rate behaviour, as a more likely alternative to the postulate of a second binding site for the substrate, is suggested.

Merritt & Tomkins (1959) reported that cyclohexanol and cyclohexanone are good substrates for liver alcohol dehydrogenase (alcohol- NAD^+ oxidoreductase, EC 1.1.1.1) with partial maximum rates and apparent Michaelis constants at fixed coenzyme concentrations similar to those for ethanol and acetaldehyde. Aliphatic secondary alcohols and ketones, on the other hand, are much poorer substrates for this enzyme than primary alcohols and aldehydes (Dalziel & Dickinson, 1966). A mechanism has been proposed for liver alcohol dehydrogenase that satisfactorily describes the kinetics of oxidation of primary alcohols, secondary alcohols and aldehydes, and the reduction of aldehydes, as well as the substrate inhibition observed with large concentrations of primary alcohols and NAD^+ (Dalziel & Dickinson, 1966). It was therefore decided to study the kinetics of the reversible oxidation of cyclohexanol in more detail,

and to attempt to account for the results in terms of this mechanism.

It was found that cyclohexanol exhibits complex initial-rate behaviour. Large concentrations of cyclohexanol cause substrate activation in the presence of saturating coenzyme concentrations and substrate inhibition in the presence of rate-limiting coenzyme concentrations. The data can be satisfactorily accounted for on the basis of the mechanism proposed previously, which provides for the formation of an abortive ternary complex of enzyme, product coenzyme (NADH) and alcohol and a binary enzyme-alcohol complex. The results emphasize again that complex initial-rate data for two-substrate reactions can be accounted for on the basis of a steady-state alternative-pathway mechanism without postulating more than one binding site for each substrate (Dalziel, 1957, 1958; Ferdinand, 1966).

MATERIALS AND METHODS

Cyclohexanol and cyclohexanone were obtained commercially, and fractionally distilled before use. Enzyme and coenzymes were prepared as described by Dalziel & Dickinson (1966).

Initial-rate measurements were made at 23.5° with a recording fluorimeter (Dalziel, 1962). Except where other-

wise stated, reaction mixtures were 1/0.1 with respect to sodium phosphate buffer, pH 7.0. All initial-rate measurements were made at least in duplicate, and two separate complete experiments were made with each substrate for the evaluation of the coefficients in the initial-rate equation:

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

The kinetic coefficients, which are functions of rate constants in the mechanism, were evaluated in the usual way from primary and secondary plots of initial-rate data (Dalziel, 1957). In eqn. (1), e is the concentration of active centres (twice the molar concentration), and S_1 and S_2 are coenzyme

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 cyclohexanone concentration for several constant NADH concentrations (μM): $\circ$, 100; $\bullet$, 38; $\triangle$, 12.7; $\blacktriangle$, 5.6; $\square$, 1.9; $\blacksquare$, 0.93.

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 NADH concentration for several constant cyclohexanone concentrations (mm): $\circ$, 116; $\bullet$, 22; $\triangle$, 11; $\blacktriangle$, 5.5; $\square$, 2.7.

Fig. 3. Secondary plots: variation of the slopes (a) and intercepts (b) of the Lineweaver-Burk plots in Figs. 1 and 2 with the reciprocals of the NADH concentration ($\bullet$) and of the cyclohexanone concentration ($\circ$).

Table 1. *Kinetic coefficients for the cyclohexanol oxidation and cyclohexanone reduction catalysed by liver alcohol dehydrogenase at pH 7.0 and 23.5°*

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 coenzyme and S_2 is substrate. For the cyclohexanol reaction, kinetic coefficients were evaluated from measurements with small substrate concentrations below the region of substrate activation and inhibition. Details are given in the text. Kinetic coefficients for the ethanol-acetaldehyde systems are from Dalziel (1963), and those for butan-2-ol oxidation from Dalziel & Dickinson (1966).

Reaction	Alcohol oxidation				Aldehyde or ketone reductions			
	ϕ_0 (sec.)	ϕ_1 ($\mu\text{Msec.}$)	ϕ_2 ($\mu\text{Msec.}$)	ϕ_{12} ($\mu\text{M}^2\text{sec.}$)	ϕ'_0 (sec.)	ϕ'_1 ($\mu\text{Msec.}$)	ϕ'_2 ($\mu\text{Msec.}$)	ϕ'_{12} ($\mu\text{M}^2\text{sec.}$)
Cyclohexanol-cyclohexanone	0.31	3.3	230	17800	0.017	0.12	285	350
Ethanol-acetaldehyde	0.37	1.1	66	7200	0.008	0.10	3.3	1.4
Butan-2-ol oxidation	1.7	41	9000	950000	—	—	—	—

Fig. 4. Variation of the reciprocal of the specific initial rate at pH 7.0 and 23.5° with the reciprocal of the cyclohexanone concentration. The NADH concentration was 100 μM .

and substrate respectively. The symbols ϕ_0 etc. are used to denote kinetic coefficients for the cyclohexanol-NAD⁺ reaction, and primed symbols ϕ'_0 etc. those for the reverse reaction.

RESULTS

The results of initial-rate measurements with cyclohexanone as substrate in one experiment are shown in the primary plots of Figs. 1 and 2. There is evidence of substrate inhibition with large cyclohexanone concentrations in the presence of small coenzyme concentrations (Fig. 1), but apart from this the plots are linear over the concentration ranges used, namely 2.7–116 mm-cyclohexanone and 0.93–108 μM -NADH. Secondary plots of slopes and intercepts are linear (Fig. 3). Average values of

the kinetic coefficients obtained from Fig. 3 and from similar data in another experiment are shown in Table 1. The variation of initial rate with a wider range of cyclohexanone concentrations, down to 0.2 mm, and a constant NADH concentration of 100 μM , is shown in Fig. 4. The plot is linear, and shows no sign of the sharp change of slope at 1 mm-cyclohexanone recorded by Merritt & Tomkins (1959).

Initial-rate data for the oxidation of cyclohexanol by NAD⁺ are shown in the primary plots of Figs. 5 and 6. The primary plots with the reciprocal of the cyclohexanol concentration as variable (Fig. 5) are linear from 0.83 mm- to about 5 mm-cyclohexanol. With larger cyclohexanol concentrations there are deviations from linearity, towards higher activity when the fixed NAD⁺ concentration is large (475 μM) and towards lower activity when the fixed NAD⁺ concentration is small (11 μM). With intermediate NAD⁺ concentrations, increase of cyclohexanol concentration first causes slight substrate activation and then, with still larger cyclohexanol concentrations, substrate inhibition occurs. It is striking that the alternative primary plots of the same initial-rate data in Fig. 6, with the reciprocal NAD⁺ concentration as variable and different fixed cyclohexanol concentrations, are linear within the experimental error, even those for large cyclohexanol concentrations (Fig. 6b), which cause substrate activation in the presence of large NAD⁺ concentrations and substrate inhibition in the presence of small NAD⁺ concentrations (Fig. 5). As a result of the latter effects, the slopes of Fig. 6(b) increase with increase of the fixed cyclohexanol concentration from 33 to 270 mm, whereas with smaller cyclohexanol concentrations (0.83–16.5 mm) the slopes decrease with increase of the cyclohexanol concentration in the usual way (Fig. 6a).

Fig. 5. Primary plots: Variation of the specific initial rate at pH 7.0 and 23.5° with the reciprocal of the cyclohexanol concentration for several fixed concentrations of NAD^+ (μM): (a): $\circ$, 475; $\bullet$, 43.2; Δ , 22.6; (b): $\circ$, 11.3; $\bullet$, 5.7.

Fig. 6. Primary plots: variation of the specific initial rate at pH 7.0 and 23.5° with the reciprocal of the NAD^+ concentration for several fixed concentrations of cyclohexanol (mm): (a): $\circ$, 33; $\bullet$, 16.5; Δ , 6.7; $\blacktriangle$, 3.3; $\square$, 1.7; $\blacksquare$, 0.83; (b): $\circ$, 33; $\bullet$, 50; Δ , 83; $\blacktriangle$, 270.

Slopes and intercepts were obtained from Figs. 5 and 6 by extrapolation of the linear parts of the plots, and their variations with the reciprocals of the fixed substrate and coenzyme concentrations are shown in the secondary plots of Fig. 7. Kinetic coefficients were obtained in the usual way from these secondary plots, by extrapolation of the linear portions where necessary. Average values from two experiments are given in Table 1.

The value of $1/\phi_0$, 3.2 sec.^{-1} , represents the theoretical maximum specific rate, V/e , according to eqn. (1), obtained by extrapolation from small cyclohexanol concentrations, as for example in the intercept plot of Fig. 7(a) with the reciprocal cyclo-

hexanol concentration as variable. The actual specific rate approached with infinitely large cyclohexanol concentration may be estimated from the real ordinate intercept of this same plot as $V'/e = 1/0.14 = 7 \text{ sec.}^{-1}$. As shown below, this may be identified as the rate constant k_{-3} for the dissociation of NADH from the abortive complex $\text{E} \cdot \text{NADH} \cdot \text{ROH}$ (Scheme 1). The evaluation of this constant, and the fact that a constant rate is indeed approached with very large cyclohexanol concentrations, is shown more clearly in Fig. 8, by a plot of e/v_0 against cyclohexanol concentration. The data

Fig. 7. Secondary plots: variations of the intercepts (a) and slopes (b) of the Lineweaver-Burk plots of Figs. 5 and 6 with the reciprocals of the NAD^+ concentration (●) and of the cyclohexanol concentration (○).

were obtained in a separate experiment with a constant NAD^+ concentration of $500 \mu\text{M}$. A value of e/v_0 of 0.16 sec. is approached with increasing cyclohexanol concentration, which gives $k_{-6} = 6.2 \text{ sec.}^{-1}$. This will be slightly smaller than the true value because the NAD^+ concentration is not quite saturating.

DISCUSSION

The activity of liver alcohol dehydrogenase with cyclohexanol and cyclohexanone as substrates reported by Merritt & Tomkins (1959) has been confirmed. The deviations from simple Michaelis-Menten behaviour observed by these workers in

Fig. 8. Variation of the reciprocal of the specific initial rate at $\text{pH } 7.0$ and 23.5° with the cyclohexanol concentration. The NAD^+ concentration was $500 \mu\text{M}$.

Scheme 1.

studies of cyclohexanone reduction, which were not detected in the present experiments (or in similar experiments with commercial NADH preparations), might have been caused by an active impurity, possibly an aldehyde, in the cyclohexanone. Merritt & Tomkins (1959) did not use sufficiently large cyclohexanol concentrations in their experiment at pH 9.5, with a large constant concentration of NAD^+ , to detect the substrate activation demonstrated in the present experiments at pH 7.0. We have found that similar substrate activation does occur at pH 9.5.

This cyclic secondary alcohol and its ketone resemble primary alcohols and aldehydes, rather than aliphatic secondary alcohols and ketones, in their activity with liver alcohol dehydrogenase (Table 1). Cyclohexanone gives the same value for ϕ'_1 as acetaldehyde and other aldehydes, consistent with a compulsory-order mechanism, but the maximum rate ($1/\phi_0$) is half that obtained with aldehydes (Dalziel & Dickinson, 1966). Possibly ternary-complex interconversion limits the maximum rate of cyclohexanone reduction. The coefficient ϕ'_2 and the Michaelis constant for cyclohexanone (ϕ_2/ϕ_0) are considerably bigger than those for acetaldehyde and butyraldehyde. The aliphatic ketones acetone and ethyl methyl ketone, however, are almost inactive (Dalziel & Dickinson, 1966).

The maximum rate of cyclohexanol oxidation is almost the same as that of primary alcohols, and the other parameters ϕ_1 , ϕ_2 and ϕ_{12} are much closer to the values obtained with primary alcohols than those obtained with aliphatic secondary alcohols. It must be noted that the occurrence of substrate activation and inhibition makes it difficult to estimate ϕ_0 and ϕ_1 accurately, and (as shown below) it is likely that ϕ_0 will be underestimated and ϕ_1 overestimated. To a first approximation, therefore, the data for low concentrations of cyclohexanol, like those for primary alcohols, are consistent with the Theorell-Chance mechanism. This requires the $1/\phi_0 = k'_{-1}$, the rate constant for the dissociation of the product complex $\text{E} \cdot \text{NADH}$, and that $1/\phi_1 = k_{+1}$, the rate constant for the combination of enzyme and NAD^+ in the first step of the reaction. These parameters should therefore be independent of the nature of the substrate (Dalziel, 1962; Dalziel & Dickinson, 1966). The ratio ϕ_{12}/ϕ_2 should also be independent of the nature of the substrate. The value of 77 mM for cyclohexanol agrees reasonably well with those obtained with primary and secondary alcohols (Dalziel & Dickinson, 1966). The ratio $\phi'_{12}[\text{H}^+]/\phi_{12} = 2 \times 10^{-9} \text{ M}$ should be equal to the equilibrium constant at 23.5° for the overall reaction, $K_{\text{eq}} = [\text{cyclohexanol}] [\text{NADH}] [\text{H}^+]/[\text{cyclohexanol}] [\text{NAD}^+]$. Merritt & Tomkins (1959) obtained a value of $5.5 \times 10^{-9} \text{ M}$ from equilibrium measurements at room temperature and low ionic strength. The equi-

librium constant for the ethanol- NAD^+ reaction is 0.85×10^{-11} (Bäcklin, 1958).

A satisfactory explanation of the data for cyclohexanol oxidation, including substrate activation and inhibition at different coenzyme concentrations, can be obtained on the basis of the mechanism (Scheme 1) previously shown to account for the oxidation of primary alcohols, secondary alcohols and aldehydes, including substrate inhibition with primary alcohols (Dalziel & Dickinson, 1966). [It should be noted that the reverse reaction is not completely represented in this scheme. The formation of the complex ES_1S_2 , where S_1 is NAD^+ and S_2 is aldehyde, and the aldehyde-dehydrogenase reaction of which it is the active ternary complex (Dalziel & Dickinson, 1965), are omitted for simplicity.]

Scheme 1 is an elaboration of the Theorell-Chance mechanism which includes an abortive complex $\text{ES}'_1\text{S}_2$ (where S'_1 is NADH and S_2 is alcohol), and a binary enzyme-alcohol complex ES_2 formed by dissociation of NADH from the abortive complex and by direct reaction of enzyme with alcohol. There are therefore alternative pathways for the formation of the reactive ternary complex ES_1S_2 , but these additional reactions are assumed to occur only with large alcohol concentrations. The most significant finding with cyclohexanol is substrate activation in the presence of large coenzyme concentrations, in contrast with the inhibition observed with primary alcohols. The activation results in a rate greater than the rate of the last step of the Theorell-Chance mechanism, the dissociation of the product complex $\text{E} \cdot \text{NADH}$ (k'_{-1}). Coupled with the finding that substrate activation is replaced by substrate inhibition when the coenzyme concentration is rate-limiting, this strongly suggests that the abortive complex $\text{ES}'_1\text{S}_2$ is formed and that NADH dissociates from it more quickly than from ES'_1 , i.e. $k_{-6} > k'_{-1}$. A similar explanation was suggested by Theorell & McKinley-McKee (1961) for the behaviour of imidazole as an activator of the alcohol-dehydrogenase reaction with large substrate concentrations and an inhibitor with low substrate concentrations. With large concentrations of both cyclohexanol and NAD^+ , and assuming that the Theorell-Chance mechanism is the basic one, the enzyme will be entirely in the form of this abortive complex, and activation will be observed. On the other hand, with small and rate-limiting concentrations of NAD^+ and large cyclohexanol concentrations, the enzyme will be present largely in the free form and as ES_2 . The latter will be formed by dissociation of the abortive complex, and possibly also by direct reaction of enzyme with substrate. Then if ES_2 reacts more slowly than the free enzyme with NAD^+ , i.e. if $k_{+1} > k_{+4}$, substrate inhibition would be expected (Dalziel, 1957).

*Theoretical treatment of substrate activation
and inhibition*

The initial-rate equation in the absence of products derived by steady-state treatment of Scheme 1 is given in eqn. (2).

For primary alcohols conformity of initial-rate data with low substrate concentrations to the Theorell-Chance mechanism, and isotope-exchange experiments, indicate that $k'_{-1} \ll k$, k'_{-3} and kk'_{-3}/k' , and that $k_{-4} \ll k$ and k_{-3} (Dalziel & Dickinson, 1966). With these inequalities eqn. (2) simplifies to eqn. (3).

As was shown previously (Dalziel & Dickinson, 1966) this complex initial-rate equation will simplify further to the linear reciprocal form of eqn. (1), with initial-rate parameters $\phi_0 = 1/k'_{-1}$, $\phi_1 = 1/k_{+1}$, $\phi_2 = (Ak_{-3} + k)/kk_{+3}$ and $\phi_{12} = (Ak_{-3} + k)k_{-1}/kk_{+1}k_{+3}$ as for the Theorell-Chance mechanism, if the substrate concentration $[S_2]$ is sufficiently small for the following conditions to hold:

- (i) $(k_{-5} + k_{-6}) \gg k_{+5}[S_2]$ and $(k_{-6}/k'_{-1})k_{+5}[S_2]$
 (ii) $k_{-2} + k_{+4}[S_1] \gg k_{+2}[S_2]$ and $(k_{+4}/k_{+1})k_{+2}[S_2]$

Condition (i) precludes formation of abortive complex and gives $B = C = 1$ and $D = 0$. Condition (ii) precludes both significant formation of ES_1S_2 through ES_2 by the bottom pathway of Scheme 1, and a significant concentration of ES_2 in the steady state (Dalziel & Dickinson, 1966). Thus this mechanism accounts for conformity of initial-rate data for primary alcohols and for cyclohexanol to

the requirements of the Theorell-Chance mechanism if the substrate concentration is too small for formation of abortive complex or reaction through the bottom pathway. It may be noted that if $k_{+1} = k_{+4}$ the second and fourth terms of eqn. (3) simplify in the required manner without need for condition (ii), but the third term does not. Therefore it appears that a strictly linear reciprocal initial-rate equation is only obtained if reaction through the bottom pathway is negligible, even when $k_{+1} = k_{+4}$ (Dalziel, 1962).

Failure of conditions (i) and (ii) with increasing values of $[S_2]$ will produce deviations in reciprocal plots towards higher or lower activity, according to whether the predominant term of eqn. (3) decreases or increases.

In the presence of large coenzyme concentrations, as well as large substrate concentrations, the first term of eqn. (3) will predominate. With increase of $[S_2]$, B will either increase or decrease from the value of unity at low values of $[S_2]$, according to the relative values of k_{-6} and k'_{-1} . The limiting value of B/k'_{-1} at infinitely large values of $[S_2]$ will be $1/k_{-6}$. For cyclohexanol a value of 7 sec.^{-1} was estimated for k_{-6} , which may be compared with 1.2 sec.^{-1} and 0.65 sec.^{-1} for dissociation velocity constants of the abortive ethanol and butanol complexes respectively, and $k'_{-1} = 2.8 \text{ sec.}^{-1}$ for that for $E \cdot \text{NAD}^+$, obtained from $1/\phi_0$ for primary alcohols (Dalziel & Dickinson, 1966).

As the coenzyme concentration $[S_1]$ is decreased, the second term of eqn. (3) will become increasingly

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

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

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

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

predominant. The replacement of substrate activation by substrate inhibition can be explained if this term increases with increase of $[S_2]$ as a result of failure of conditions (i) or (ii). Failure of condition (i) will result in decrease of C from unity at low substrate concentrations to zero at very large substrate concentrations, while D will increase from zero to unity. If $k_{+1} = k_{+4}$, then $C + k_{+1}D/k_{+4}$ will remain constant and equal to unity, but if $k_{+1} > k_{+4}$ this factor will increase from unity to a limiting value at very large substrate concentrations of k_{+1}/k_{+4} . Thus substrate inhibition at small co-enzyme concentrations as a result of ES_2 formation by dissociation of abortive complex requires $k_{+1} > k_{+4}$, i.e. that ES_2 so formed reacts more slowly than E with S_1 . Failure of condition (ii), i.e. intervention of the lower pathway of Scheme 1, will also result in increase of the second term of eqn. (3) provided that $k_{+1} > k_{+4}$; the substrate inhibition in this case arises from direct formation of ES_2 from free enzyme and substrate. Neither mechanism of inhibition could operate if $k_{+1} = k_{+4}$. Activation will result if $k_{+1} < k_{+4}$.

Inspection of eqn. (3) shows that the third term can only decrease with increase of $[S_2]$, but the kinetic parameters of Table 1 show that this term will be negligible in the region of substrate inhibition. The fourth term of eqn. (3) will decrease owing to formation of abortive complex ($C \rightarrow 0$), but will increase if direct formation of ES_2 occurs provided that $k_{+1} > k_{+4}$. These conclusions were outlined previously in connexion with a simpler alternative-pathway mechanism (Dalziel, 1957).

The validity of the theory can be tested to some extent by approximate theoretical calculations of initial rates from eqn. (3) with certain simplifying assumptions. Experimental values for initial rates with the largest cyclohexanol concentrations and the smallest NAD^+ concentrations will be used to estimate constants in the equation, and initial rates will then be calculated for the whole range of concentrations used experimentally. It can then be seen whether the shapes of the plots of e/v_0 against the reciprocal cyclohexanol concentration (Fig. 5) are reproduced, and whether the values for ϕ_0 and ϕ_1 obtained by extrapolation of the approximately linear parts of the theoretical plots agree with the experimental values.

Since the first two terms of eqn. (3) will predominate when the cyclohexanol concentration is large, the initial-rate equation will approximate to eqn. (4), with $\phi_2 = 230 \mu M \text{ sec.}$ and $\phi_{12} = 17800 \mu M^2 \text{ sec.}$ from Table 1. According to the proposed

mechanism, $k'_{-1} = 2.8 \text{ sec.}^{-1}$ and $k_{+1} = 0.91 \mu M^{-1} \text{ sec.}^{-1}$, the mean values of $1/\phi_0$ and $1/\phi_1$ respectively for primary alcohols (Dalziel & Dickinson, 1966).

The intercept plot against the reciprocal cyclohexanol concentration in Fig. 7(a), which represents rates with infinitely large NAD^+ concentrations, should be fitted by the equation:

$$\text{Intercept} = \frac{B}{k'_{-1}} + \frac{\phi_2}{[S_2]} = \frac{1 + (k_{-5} + k_{-6})/k_{+5}[S_2]}{(k_{-6}/k'_{-1}) + (k_{-5} + k_{-6})/k_{+5}[S_2]} + \frac{\phi_2}{[S_2]}$$

With cyclohexanol concentrations greater than 5mm, $\phi_2/[S_2]$ will be negligible (Table 1), and, substituting the values $k'_{-1} = 2.8 \text{ sec.}^{-1}$ and $k_{-6} = 7 \text{ sec.}^{-1}$, this equation gives a calculated value of 0.20 for the intercept when $[S_2] = (k_{-5} + k_{-6})/k_{+5}$. From Fig. 7(a), the cyclohexanol concentration for which the intercept has this value is 25mm. Hence B , C and D may be calculated for any other value of $[S_2]$.

The inhibition observed with large cyclohexanol concentrations and small NAD^+ concentrations is attributed to the second term of eqn. (4) with $k_{+1} > k_{+4}$, and values for the coefficient of this term may be obtained from the experimental values of e/v_0 with the largest cyclohexanol concentration of 83mm and the smallest NAD^+ concentrations (Fig. 5b). To calculate the value of this coefficient for other concentrations requires values of k_{-2} , k_{+2} and k_{+1}/k_{+4} , which are not available. The calculation may be made, however, for two extreme cases.

(1) If it is assumed that inhibition is entirely due to failure of condition (i), and that condition (ii) remains satisfied up to the largest cyclohexanol concentrations, and also that $k_{-2} \ll k_{+4}[S_1]$, then the coefficient of the second term of eqn. (4) simplifies to $(C + k_{+1}D/k_{+4})/k_{+1}$, and the ratio k_{+1}/k_{+4} may be calculated from the experimental values of e/v_0 obtained with the largest cyclohexanol concentration and the smallest NAD^+ concentrations (Fig. 5b). A value of 7 was obtained for this ratio. It should be noted that, if these assumptions are not valid, the true value of k_{+1}/k_{+4} will be greater than this.

(2) If inhibition is due to failure of both conditions (i) and (ii) and it is assumed that the terms in $k_{+4}[S_1]$ are negligible, then the coefficient of the second term of eqn. (4) simplifies to $(1 + [S_2]/K_2)/k_{+1}(1 + k_{+4}[S_2]/k_{+1}K_2)$. On this assumption, values of $K_2 = k_{-2}/k_{+2} = 4 \text{ mm}$ and $k_{+1}/k_{+4} = 8$ were calculated from the experimental values of e/v_0 with

$$\frac{e}{v_0} = \frac{B}{k'_{-1}} + \left\{ \frac{k_{-2} + (C + k_{+1}D/k_{+4})k_{+4}[S_1] + k_{+2}[S_2]}{k_{-2} + k_{+4}[S_1] + (k_{+4}/k_{+1})k_{+2}[S_2]} \right\} \frac{1}{k_{+1}[S_1]} + \frac{\phi_2}{[S_2]} + \frac{\phi_{12}}{[S_1][S_2]} \quad (4)$$

83mm- and 50mm-cyclohexanol and $5.7\mu\text{M-NAD}^+$, by using eqn. (4).

Values for e/v_0 were estimated for the whole range of substrate and coenzyme concentrations from eqn. (4) for each of these two cases, and the theoretical primary plots against the reciprocal cyclohexanol concentrations are shown in Fig. 9. The broken lines represent theoretical plots for case (1) and the full lines those for case (2). Though both sets of curves have the same general character-

istics as the experimental plots of Fig. 5, particularly with regard to the range over which the plots are approximately linear, the calculated plots for case (2) agree more closely with the experimental plots, and reproduce the activation that precedes inhibition with intermediate coenzyme concentrations. From secondary plots of the slopes and intercepts obtained from the approximately linear parts of the theoretical plots for small cyclohexanol concentrations, values of 0.29sec. for ϕ_0 and $3.0\mu\text{Msec.}$ for ϕ_1 were obtained for case (1), and 0.29sec. for ϕ_0 and $2.4\mu\text{Msec.}$ for ϕ_1 for case (2). These agree quite well with the experimental values in Table 1, and confirm the earlier prediction that the occurrence of substrate activation and inhibition results in apparent values of ϕ_0 smaller than the assumed value of 0.35sec.^{-1} and an apparent value of ϕ_1 greater than the assumed value of $1.1\mu\text{Msec.}$ These admittedly approximate calculations provide some support for the proposed mechanism, including the formation of ES_2 by direct reaction of enzyme and substrate when the latter is present in large concentrations.

It has been noted that the true value of k_{+1}/k_{+4} will be greater than the value of 7-8 estimated on the basis of simplifying assumptions. This is confirmed by the fact that the slope of the plot of e/v_0 against the reciprocal NAD^+ concentration for the largest cyclohexanol concentration of 270mm (Fig. 6b) is $16.3\mu\text{Msec.}$ From eqn. (4), the limiting value of this slope with infinitely large cyclohexanol concentration would be $1/k_{+4}$, and thus the maximum value of k_{+4} is $0.06\mu\text{M}^{-1}\text{sec.}^{-1}$ and the minimum value of k_{+1}/k_{+4} is 15. The limiting solubility of cyclohexanol prevented measurements with still larger concentrations.

In the reverse reaction, with large NADH concentrations there was no evidence of substrate activation or inhibition by cyclohexanone, but substrate inhibition was observed in the presence of small concentrations of NADH (Fig. 1). This suggests that the inhibition is due to intervention of the less efficient alternative pathway through an enzyme-cyclohexanone complex; either an abortive complex $\text{E}\cdot\text{NAD}^+\cdot\text{cyclohexanone}$ is not formed, or, if it is, NAD^+ dissociates from it at the same rate as from the normal product complex $\text{E}\cdot\text{NAD}^+$. The relatively large value of ϕ'_2 for cyclohexanone, and of the Michaelis constant for this substrate ($\phi'_2/\phi'_0 = 17\text{mm}$), is consistent with a relatively small affinity of this substrate for $\text{E}\cdot\text{NADH}$, and presumably a still smaller affinity for $\text{E}\cdot\text{NAD}^+$.

The two mechanisms of substrate activation and inhibition suggested here, namely the formation of abortive ternary complexes, and the intervention of a less efficient alternative pathway to the active ternary complex, seem to be the likeliest explanations of the rather common deviations from the

Fig. 9. Theoretical plots of the reciprocal of the cyclohexanol concentration, for several constant NAD^+ concentrations. The broken lines and the continuous lines represent values of e/v_0 calculated on the basis of different assumptions, as described in the text. The NAD^+ concentrations (μM) are: (1), 475; (2), 43.2; (3), 22.6; (4), 11.3; (5), 5.7.

simple form of initial-rate equation for two-substrate reactions, and especially for coenzyme-substrate reactions for which a preferred pathway mechanism may be the general one at low substrate concentrations. The existence of two mechanisms for such deviations is particularly clear-cut for cyclohexanol with liver alcohol dehydrogenase, because formation of abortive complex results in substrate activation in the presence of large concentrations of the fixed coenzyme concentration, and the occurrence of substrate inhibition in the presence of small coenzyme concentrations demands another explanation in terms of alternative pathways. Yeast alcohol dehydrogenase shows substrate activation with ethanol in the presence only of large NAD^+ concentrations, which may be explained by formation of abortive complex, but more detailed studies of this enzyme are needed (Nygaard & Theorell, 1955). The possibility of formation of abortive complex as one cause of substrate activation or inhibition emphasizes the importance of considering the complete mechanism of Scheme 1, rather than the simplified form in which all reactions after the formation of the ternary complex are grouped together as a single irreversible step (cf. Ferdinand, 1966). Substrate inhibition of heart malate dehydrogenase by oxaloacetate was interpreted by Raval & Wolfe (1963) in terms of an abortive complex of enzyme, NAD^+ and oxaloacetate but the possibility that this complex could dissociate the product NAD^+ and yield an active enzyme-

oxaloacetate complex, which could react with NADH , was not considered. Instead an additional inactive enzyme-oxaloacetate complex was invoked to explain the complex form of the inhibition. Substrate inhibition of lactate dehydrogenase by pyruvate was also interpreted in terms of an inactive enzyme-pyruvate complex (Hakala, Glaid & Schwert, 1956). The mechanisms considered here seem to be more likely.

We thank the Medical Research Council for financial aid, and Miss Joyce Holland for technical assistance.

REFERENCES

- Bäcklin, K. I. (1958). *Acta chem. scand.* **12**, 1279.
Dalziel, K. (1957). *Acta chem. scand.* **11**, 1706.
Dalziel, K. (1958). *Trans. Faraday Soc.* **54**, 1247.
Dalziel, K. (1962). *Biochem. J.* **84**, 244.
Dalziel, K. (1963). *J. biol. Chem.* **238**, 2851.
Dalziel, K. & Dickinson, F. M. (1965). *Nature, Lond.*, **206**, 255.
Dalziel, K. & Dickinson, F. M. (1966). *Biochem. J.* **100**, 34.
Ferdinand, W. (1966). *Biochem. J.* **98**, 278.
Hakala, M. T., Glaid, A. J. & Schwert, G. W. (1956). *J. biol. Chem.* **221**, 191.
Merritt, A. D. & Tomkins, G. M. (1959). *J. biol. Chem.* **234**, 2778.
Nygaard, A. P. & Theorell, H. (1955). *Acta chem. scand.* **9**, 1300.
Raval, D. N. & Wolfe, R. G. (1963). *Biochemistry*, **2**, 220.
Theorell, H. & McKinley-McKee, J. S. (1961). *Acta chem. scand.* **15**, 1834.