This has been found both with adenosine diphosphate ribose and with different NADH₂ preparations (Dalziel, 1961*b*).

SUMMARY

1. NADH₂ prepared by enzymic reduction of pure NAD by the method of Rafter & Colowick (1957), and isolated as the sodium salt, gives higher maximum rates of reduction of acetaldehyde with liver alcohol dehydrogenase at pH 6 than a number of commercial preparations of high purity. Maximum values of 2.4 for the extinction ratio E_{280}/E_{340} and 2% for the proportion of inactive material absorbing at 340 m μ are taken as criteria of a satisfactory preparation, and are lower than those for most commercial preparations and for products obtained by chemical reduction of NAD.

2. Methods involving enzyme inactivation by heat or isolation by drying aqueous solutions are not satisfactory for the preparation of pure NADH₂. The compound is not stable at room temperature, nor in concentrated solutions. It appears that an inhibitor of liver alcohol dehydrogenase which competes with the coenzyme is formed, and is present in some commercial preparations.

3. Kinetic studies of the inhibition of liver alcohol dehydrogenase by adenosine diphosphate ribose are briefly reported, and the results are discussed with reference to the possible magnitude

of the effects of a competitive inhibitor present as an impurity in coenzyme preparations.

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Kinetic Studies of Liver Alcohol Dehydrogenase

By K. DALZIEL*

Department of Biochemistry, University of Sheffield, Sheffield 10

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Theorell, Nygaard & Bonnichsen (1955) studied the kinetics of liver alcohol dehydrogenase (alcohol-NAD oxidoreductase, EC 1.1.1.1), with acetaldehyde and ethanol as substrates, by fluorimetric measurements in the steady state. The results at several pH values were considered to support the mechanism proposed by Theorell & Chance (1951). The enzyme was prepared by the method of Bonnichsen (1950). Later methods of preparation gave products of higher specific activity (Dalziel, 1958*a*, 1961*a*) and redetermination of the molecular weight also gave a higher

value than that previously accepted (Ehrenberg & Dalziel, 1958). The rate constants calculated by Theorell *et al.* (1955) therefore require correction (cf. Theorell, 1958; Dalziel, 1961*a*), but equilibrium constants calculated from the kinetic data are not affected.

Theorell *et al.* (1955) used high concentrations of either substrate or coenzyme in all their experiments. However, accurate determination of kinetic parameters other than maximum rates, and critical distinction between the Theorell-Chance mechanism and other plausible mechanisms for coenzyme-substrate reactions, require more detailed experiments, including measurements with

* Sorby Research Fellow of the Royal Society.

low concentration of both substrate and coenzyme (Dalziel, 1957, 1958*b*). Experiments of this kind in alkaline solution gave results which agreed reasonably well with the previous results, but the kinetic parameters obtained in neutral and acid solution differed from those reported by Theorell *et al.* (1955) and were inconsistent with the requirements of the Theorell-Chance mechanism (Dalziel, 1957; Dalziel & Theorell, 1957; cf. Theorell, 1958). The measurements of initial rates with low concentrations of both substrate and coenzyme, on which tests of the mechanism mainly depend, were not accurate enough to give more than an approximate value for certain kinetic parameters, however, and the need for further experiments with a more sensitive method was indicated. Nevertheless, the most serious discrepancies, in the maximum rate of aldehyde reduction, were clearly significant. At pH 6, the maximum rate was 4 times and at pH 7.15 more than twice that reported by Theorell *et al.* (1955). These results have since been confirmed, and the lower values reported previously explained by the presence in NADH₂ preparations of variable amounts of a potent inhibitor of liver alcohol dehydrogenase, which is most active in acid solution (Dalziel, 1961*a, b*). Previous conclusions about the mechanisms of the reaction are therefore equivocal.

The present work is an attempt to get reasonably reliable values for all the kinetic coefficients in the initial-rate equation for the reaction, in both directions, at pH 6 and 7.1. The fluorimetric method has been improved. Several experiments have been made under the same conditions, but with different samples of enzymes and coenzymes. Two pairs of substrates have been used. Evidence of the mechanism has been sought from: (1) the Haldane relation between kinetic coefficients and the overall equilibrium constant; (2) relations between the kinetic coefficients; (3) relations between kinetic coefficients and dissociation constants of enzyme complexes; (4) comparison of kinetic coefficients obtained with different substrates.

MATERIALS AND METHODS

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

Enzyme. Crystalline alcohol dehydrogenase was prepared from horse liver and assayed as described by Dalziel (1961*a*). The molecular weight is 84 000 and 1 molecule binds 2 molecules of coenzyme (Theorell & Bonnichsen, 1951; Ehrenberg & Dalziel, 1958); the concentration of active centres, *e*, is twice the molar concentration.

Substrates. Ethanol, butan-1-ol, acetaldehyde and butyraldehyde were redistilled. The aldehydes were stored at -15°.

Coenzymes. NAD was 'β-DPN' from Sigma Chemical Co., and assayed as 98% NAD,4H₂O. The preparation

and assay of NADH₂ was as described by Dalziel (1962*a*). Samples of 'β-DPNH₂' from Sigma Chemical Co. were also used.

Recording fluorimeter. The cell compartment and optical parts of the fluorimeter (Fig. 1*A*) are simplified forms of those described by Theorell & Nygaard (1954): instead of rectangular cells test tubes, 1.5 cm. (diam.) × 7 cm., are used with light tunnels 2 cm. high and 1 cm. wide; variable apertures and lenses are omitted. The cell compartment *C* is a cylindrical Dural block which can be rotated within the fixed thermostatically-controlled jacket *J*. One of the four vertical holes is permanently occupied by a Perspex cylinder *P* which serves as a fluorescent reference standard, the adjacent hole *T* is used for kinetic measurements, and the other two are for temperature equilibration of the reaction mixtures. The source *H* is an air-cooled mercury lamp (Neon 'HgS', 50 w), with relatively low intensity but

Fig. 1. *A*, Sketch plan of the fluorimeter. *B*, Photo-multiplier load circuit with compensating potentiometer. Resistances (kΩ): *R*₁, 1000 (7-step potentiometer); *R*₂, 2000; *R*₃, 22 000; *R*₄, 10 × 22 000; *R*₅, 10 × 2200; *S*, constant-voltage transformer. For explanation of other letters see text.

great stability and little red emission. The Corning 5840 filter F_1 has maximum transmission at 365 m μ .

The detector M is a 13-stage photomultiplier (Model 9502B, Electrical and Musical Industries Ltd.) with a 1 cm. diameter cathode. It is screened by a Corning 3389 filter F_2 and a liquid filter of m-NaNO₂ N to absorb stray ultraviolet light. The stabilized voltage supply to the dynodes V (Felgett, 1954) can be varied to give a useful range of amplification of 200. The photomultiplier load resistor R_1 is a 1000 k Ω potentiometer with seven steps down to 10 k Ω composed of resistors of accurately known value ($\pm 1\%$). The amplifier A is a Vibron Electrometer (Electronic Instruments Ltd.) which can also be used for direct readings. The Brown recorder R has a 10 in. pen travel for 10 mA in 1 sec. and a chart speed of 4 in./min.

A simple but important new feature of the apparatus is the incorporation of a variable potential into the photomultiplier output circuit to oppose the signal (Fig. 1B). Without this, reasonably constant rates during the first minute of reaction, an essential condition for accurate initial rates estimates, cannot be obtained over the desired concentration ranges. For measurements of the rate of oxidation of NADH₂, part of the initial NADH₂ fluorescence is backed off, so that the amplification can be increased and only a small fraction of the total reaction to equilibrium occupies the whole chart width of the recorder. For the reverse reaction, the equilibrium is very unfavourable, and the high sensitivity needed for approximately constant rates with low NAD and alcohol concentrations can only be reached if the blank fluorescence is backed off. The minimum resistance in the compensating circuit R_2 must be large compared with the maximum load resistance R_1 to avoid shunting effects.

The amplification is adjusted to the desired level for a particular measurement by means of the dynode voltage. The deflexion for the Perspex standard is also recorded with the same dynode voltage, and is kept within the range of the recorder by suitable adjustment of the load resistance R_1 . Calibration of the instrument against NADH₂ has been described by Dalziel (1961a). The Perspex standard is equivalent to a NADH₂ concentration of about 3.4 μ M and does not change during illumination or over short periods of time, although significant changes do occur during several weeks. With the highest useful amplification a deflexion of 2.5 in. is obtained with 0.1 μ M-NADH₂.

Initial-rate measurements. Particulate debris must be completely excluded from the reaction mixture since at high sensitivities a single filter-paper thread moving in the light-path will distort the trace. Stock buffer solutions and distilled water were allowed to settle overnight, and a sintered-glass filter was fused into the outlet of the aspirators. Pipettes were kept under dust shields during the experiments, and to avoid wiping the tapered tips were dipped just under the surface of a solution. Micropipettes for the enzyme delivery were wiped and touched off with hardened filter paper. In spite of these precautions it was necessary to prepare fresh dilute enzyme, coenzyme, substrate and buffer solutions frequently. Care was also needed to avoid contamination of stock buffer solutions and water by traces of volatile reaction product, and in particular to exclude acetaldehyde from the laboratory during experiments on ethanol oxidation.

Stock solutions of mm-coenzymes and approx. m-substrates in water were prepared each day and kept at 0°. Various dilutions in water were made frequently, such that 0.05–1.0 ml. was required in a total volume of 4 ml. of reaction mixture, I 0.1 with respect to phosphate buffer. The stock enzyme solution, 10 μ M in phosphate buffer (I 0.1, pH 7) was stable for several days at 0°. Dilutions down to 0.2 μ M were made in the same buffer, stored on ice, and renewed every hour. All pipettes were calibrated to within 1%.

After temperature equilibration in the fluorimeter, the reaction mixture was shaken and the base-line recorded for about 30 sec. to check the stability of the instrument and the absence of debris. The reaction was then started by the addition of 5–10 μ l. of enzyme solution, and the progress curve was recorded for at least 60 sec. The addition of the enzyme on a stirrer (Theorell *et al.* 1955) was found to be a prolific source of debris; moreover, vigorous stirring sometimes caused initial irregularities in the trace due to air bubbles, and non-reproducibility with low enzyme concentrations, probably because of inactivation. The following technique was adopted. After the base-line had been recorded, the chart motor was switched off, the tube was removed from the cell compartment and the enzyme was delivered as a drop on the tube wall. The enzyme was at once mixed in by shaking and the chart motor was started simultaneously. The tube was replaced in the fluorimeter and the record was continued. The delay between addition of enzyme and resumption of the record was about 5 sec.

A series of measurements was made with a fixed coenzyme concentration and 5–10 substrate concentrations. Most of the measurements were then repeated in the reverse order, and measurements with the highest and lowest concentration were carried out once again. Seven or eight such series were made with different coenzyme concentrations in random order, and finally the series with high and low concentrations were repeated. Duplicate measurements usually agreed to within 3% with high substrate or coenzyme concentrations and 10% with low concentrations. The temperature was 23.5° throughout.

Initial rates (μ M-NADH₂/sec.) were calculated from:

$$v_0 = \frac{dF_T}{dt} \times \frac{C}{F_s}$$

where dF_T/dt is the initial slope of the progress curve, F_s is the deflexion for the Perspex standard with the same amplification and load resistance, and C is the NADH₂ equivalent of the standard in μ M, which varies with the initial NADH₂ concentration and is determined from a calibration curve (Dalziel, 1961a). Reciprocals of the specific rates, e/v_0 (sec.), are plotted against reciprocals of the concentrations of both coenzyme, S_1 , and substrate, S_2 , and the slopes and intercepts of these two primary plots are then plotted against the reciprocals of the concentrations of substrate and coenzyme respectively. The slopes and intercepts of these secondary plots give values of the kinetic coefficients in the initial-rate equation (Dalziel, 1957):

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

The symbols ϕ_0 etc. are used for the kinetic coefficients of the aldehyde-NADH₂ reaction, and ϕ'_0 etc. for those of the alcohol-NAD reaction.

RESULTS

The results of a kinetic experiment on the ethanol-NAD reaction at pH 6 are shown in Figs. 2-4: the reciprocals of the specific initial rates are plotted against the reciprocals of the ethanol concentrations in Fig. 2, and the same initial-rate data are plotted against the reciprocals of the NAD concentrations in Fig. 3. The intercepts of these two sets of primary Lineweaver-Burk plots are plotted against the reciprocals of the NAD and ethanol concentrations respectively in Fig. 4A; the slopes of these secondary plots are equal to ϕ'_1 and ϕ'_2 [cf. equation (1)] and the common intercept is ϕ'_0 , the reciprocal of the maximum rate. The slopes of the primary plots are plotted in an analogous manner in Fig. 4B, which gives ϕ'_2 and ϕ'_1 as intercepts and ϕ'_{12} as the common slope.

Fig. 2. Primary plots: variation of the reciprocal of the specific initial rate of ethanol oxidation at pH 6.0 and 23.5° with the reciprocal of the ethanol concentration, for several constant NAD concentrations (μM). A: $\circ$, 500; $\bullet$, 67; $\triangle$, 33; $\blacktriangle$, 20. B: $\circ$, 13.3; $\bullet$, 6.7; $\triangle$, 3.3; $\blacktriangle$, 2.3.

An experiment on the acetaldehyde-NADH₂ reaction at pH 6 is illustrated in a similar manner in Figs. 5 and 6. In this case the slopes of the

Fig. 3. Primary plots: variation of the reciprocal of the specific initial rate of ethanol oxidation with the reciprocal of the NAD concentration at pH 6.0 and 23.5°, for several constant ethanol concentrations (mm). A: $\circ$, 8.1; $\bullet$, 4.0; $\triangle$, 3.0; $\blacktriangle$, 2.0. B: $\circ$, 1.6; $\bullet$, 0.81; $\triangle$, 0.51; $\blacktriangle$, 0.30.

primary plots (Figs. 5A and 5B) change but little over the accessible concentration range, and it is obvious that the estimate of ϕ_{12} , the average of the small slopes of the secondary plots in Fig. 6B, is approximate.

Within experimental error, and over the concentration ranges shown, all the plots are linear, and

Fig. 4. Secondary plots: variation of the intercepts (A) and slopes (B) of Lineweaver-Burk plots in Figs. 2 and 3 with the reciprocals of the NAD concentration (○) and ethanol concentration (●) respectively.

the results are therefore consistent with the initial-rate equation (1). Values of the kinetic coefficients for the acetaldehyde-ethanol system at pH 6 and 7.1 from several experiments, in which different preparations of enzyme and coenzyme were used, are shown in Table 1. In Table 2 the best estimates from these experiments are compared with values obtained for the butyraldehyde-butanol system.

Spectrophotometric measurements on the butanol-NAD reaction at pH 7.0 and 23.5° gave a value of 0.9×10^{-11} for the overall equilibrium constant, K_0 (i.e. $[S_1][S_2][H^+]/[S'_1][S'_2]$).

Fig. 5. Primary plots: variation of the reciprocal of the specific initial rate of acetaldehyde reduction at pH 6.0 and 23.5° with (A) the reciprocal of the NADH₂ concentration for several constant acetaldehyde concentrations (μM): ○, 1440; ●, 144; □, 72; ■, 43; △, 29; and (B) the reciprocal of the acetaldehyde concentration for several constant NADH₂ concentrations (μM): ○, 20; ●, 5.0; □, 2.0; ■, 1.0; △, 0.5.

Fig. 6. Secondary plots: variations of the intercepts (A) and slopes (B) of Lineweaver-Burk plots in Figs. 5A and 5B with the reciprocal of the NADH₂ concentration (○) and of the acetaldehyde concentration (●) respectively.

Table 1. Kinetic coefficients for the acetaldehyde-ethanol system at 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 S₁ is the coenzyme and S₂ the substrate. Details of the NADH₂ preparations are given in the preceding paper (Dalziel, 1962a). Other details are given in the text.

pH	Expt.	NADH ₂ preparation	Acetaldehyde reduction				Ethanol oxidation			
			ϕ ₀ (sec.)	ϕ ₁ (μM sec.)	ϕ ₂ (μM sec.)	ϕ ₁₂ (μM ² sec.)	ϕ' ₀ (sec.)	ϕ' ₁ (μM sec.)	ϕ' ₂ (μM sec.)	ϕ' ₁₂ (μM ² sec.)
6.0	1	9 (Sigma)	0.022	0.115	3.7	2.0	0.73	—	770	78 000
	2	6 (Sigma)	0.011	0.100	4.6	1.6	0.62	2.7	1000	62 000
	3	2a (Laboratory)	0.009	0.116	3.5	1.5	0.58	2.3	752	68 000
	4	1 (Laboratory)	0.008	0.116	3.7	1.4	—	—	—	—
		Best estimates	0.008	0.115	3.7	1.5	0.62	2.5	800	70 000
7.1	5	6 (Sigma)	0.013	0.098	3.6	1.0	0.36	0.95	90	7 400
	6	6 (Sigma)	0.010	0.097	3.6	1.2	0.37	0.95	110	6 300
	7	2 (Laboratory)	0.0075	0.110	2.8	1.9	0.38	1.3	118	7 200
		Best estimates	0.0075	0.100	3.3	1.4	0.37	1.1	110	7 200

Table 2. Comparison of kinetic coefficients at 23.5° for the acetaldehyde-ethanol and butyraldehyde-butanol systems

The initial-rate equation is given in Table 1. Other details are given in the text.

Reaction	pH	Aldehyde reduction				Alcohol oxidation			
		ϕ ₀ (sec.)	ϕ ₁ (μM sec.)	ϕ ₂ (μM sec.)	ϕ ₁₂ (μM ² sec.)	ϕ' ₀ (sec.)	ϕ' ₁ (μM sec.)	ϕ' ₂ (μM sec.)	ϕ' ₁₂ (μM ² sec.)
Acetaldehyde-ethanol	6.0	0.008	0.115	3.7	1.5	0.62	2.5	800	70 000
	7.1	0.0075	0.100	3.3	1.4	0.37	1.1	110	7 200
Butyraldehyde-butanol	7.1	0.0075	0.100	0.17	0.04	0.35	1.1	7.2	360

DISCUSSION

Except for ϕ_0 , the kinetic coefficients for the ethanol-acetaldehyde system are fairly reproducible in quite separate experiments (Table 1) and the precision of the best estimates is about $\pm 10\%$. However, inspection of the relevant plots suggests that the reproducibility of ϕ_{12} and ϕ'_1 is to some extent fortuitous, since these coefficients are small, and the uncertainties are estimated as $\pm 40\%$ and $\pm 20\%$ respectively. On the other hand, ϕ'_0 and ϕ_1 can be obtained quite accurately with the available concentration ranges, and are reproducible to within $\pm 5\%$ (cf. Dalziel, 1961*a*).

The large variations of ϕ_0 with different NADH₂ preparations are attributed to competitive inhibitor in the coenzyme (Dalziel, 1961*a*, *b*, 1962*a*, *b*). Reasons why the effects are apparently confined to the maximum rate, and are so large even with relatively pure coenzyme preparations, have been discussed by Dalziel (1962*a*, *b*).

Relative activities of different substrates. Evaluation of all the parameters in the initial-rate equation, which has not previously been reported for substrates other than acetaldehyde and ethanol, reveals more concerning relative activities than comparisons of rates at single arbitrary concentrations, as well as being a useful test of mechanism. The kinetic coefficients at pH 7.1 show that the relative rates of reaction of the two pairs of substrates vary with the substrate concentration. Since ϕ_2 , ϕ_{12} , ϕ'_2 and ϕ'_{12} are about 20 times as large for acetaldehyde and ethanol as for butyraldehyde and butanol, the higher homologues react the more rapidly at equal and suboptimum concentrations, by a factor which increases to 20 when the substrate concentrations are small enough to be rate-determining. The other four coefficients are identical for the two systems, and therefore both the true maximum rates and the partial maximum rates with low coenzyme and high substrate concentrations are the same. Thus Winer's (1958) conclusion that the C₄ compounds react much faster than the C₂ compounds requires qualification.

The simplest interpretation of the identity of ϕ_0 and ϕ_1 for the two pairs of substrates is that these coefficients are functions of rate constants for steps

Scheme 1

common to the reactions, involving coenzyme and enzyme only. These results are therefore consistent with a compulsory-order mechanism in which the last step, the dissociation of the product coenzyme-enzyme complex, determines the maximum rate ($1/\phi_0$), as suggested by Theorell & Chance (1951). The compulsory-order mechanism in its most general form, where S₁ is the coenzyme, is shown in Scheme 1. Steady-state treatment of this mechanism gives an initial-rate equation of the same form as equation (1) in which

$\phi_1 = 1/k_{+1}$ and $\phi_0 = (1/k'_{-1} + 1/k'_{-2} + 1/k + k'/kk'_{-2})$ (Dalziel, 1957). Further, if

$k'_{-2} \gg k'_{-1}$, $k \gg k'_{-1}$ and $kk'_{-2} \gg k'k'_{-1}$, then $\phi_0 = 1/k'_{-1}$.

This limiting case of a compulsory-order mechanism is kinetically equivalent to that proposed by Theorell & Chance (1951) which did not include ternary complexes. It also requires that $\phi_1/\phi'_0 = K_1$, the dissociation constant of the enzyme-NADH₂ complex, ES₁, and that $\phi'_1/\phi_0 = K'_1$, the dissociation constant of the enzyme-NAD complex, ES'₁. These relations are tested in Table 3 with recent estimates of the dissociation constants by Theorell & McKinley McKee (1961). The agreement is satisfactory at both pH values and in this respect the work confirms that of Theorell & McKinley McKee (1961) with acetaldehyde and ethanol at pH 7.0, although their values for the individual coefficients ϕ_0 and ϕ'_1 are both twice the present values.

Relations between kinetic coefficients. The compulsory-order mechanism of Scheme 1 with any number of ternary complexes is characterized by the relation: $\phi'_{12}/\phi'_1\phi'_2 = k'_{-1}$. Since this is the specific rate of the last step in the forward reaction it must be at least equal to the maximum specific rate. Such symmetrical mechanisms therefore require the following relations between the kinetic coefficients: (a) $1/\phi_0 \leq \phi'_{12}/\phi'_1\phi'_2$ and (b) $1/\phi'_0 \leq \phi_{12}/\phi_1\phi_2$ (Dalziel, 1957). The equalities apply in the limiting case of the Theorell-Chance mechanism. The data

Table 3. Comparison of dissociation constants for enzyme-coenzyme compounds calculated from kinetic data on the basis of the Theorell-Chance mechanism with directly measured values

pH	NADH ₂		NAD	
	ϕ_1/ϕ'_0 (μM)	K_1 (μM)	ϕ'_1/ϕ_0 (μM)	K'_1 (μM)
6.0	0.19	0.23	310	266
7.1	0.28	0.31*	147	160*

* At pH 7.0.

of Table 4 show that relation (b) is satisfied, and the deviations from equality are within the combined experimental errors of the four kinetic coefficients involved. Relation (a) is not satisfied, however: the maximum rate of aldehyde reduction, $1/\phi_0$, is significantly greater than the rate of the last step calculated on the assumption of a compulsory-order mechanism. This was the chief incompatibility with the Theorell–Chance mechanism reported previously (Dalziel, 1957; Dalziel & Theorell, 1957; Theorell, 1958). It must be emphasized, first, that the discrepancy cannot be explained by postulating additional ternary complexes, or rate-limiting interconversion of these (cf. Theorell & Winer, 1959; Theorell & McKinley McKee, 1961), and secondly that further purification of the NADH_2 could only augment the discrepancy.

Kinetic values for dissociation and overall equilibrium constants. Compulsory-order mechanisms also require that $\phi'_{12}/\phi'_2 = K'_1$, $\phi_{12}/\phi_2 = K_1$ and $\phi_{12}[\text{H}^+]/\phi'_{12} = K_0$, the equilibrium constant for the overall reaction, $[\text{S}_1][\text{S}_2][\text{H}^+]/[\text{S}'_1][\text{S}'_2]$ (Alberty, 1953; Dalziel, 1957; Frieden, 1957). These relations are tested in Table 5. As regards K_1 and K_0 , the differences between the values calculated from the kinetic coefficients and those obtained by direct equilibrium measurements (Theorell & McKinley McKee, 1961; Bäcklin, 1958) are not clearly significant, largely because of the low precision of ϕ_{12} . The discrepancies between ϕ'_{12}/ϕ'_2 and K'_1 appear to be outside the experimental error, and of course reflect those in Table 4.

Comparisons with earlier work. The present experiments include those reported by Dalziel, 1961a, and confirm that the low values for the

maximum specific rate of acetaldehyde reduction in neutral and acid solution and the apparent optimum pH of 7.1 and $\text{p}K'$ of 6.4 (Theorell *et al.* 1955) were due to an inhibitor in the coenzyme preparations. The maximum rate is now the same at pH 6.0 and 7.1.

The kinetic constants reported by Theorell & McKinley McKee (1961) are much closer to the present values at pH 7.1 but there are discrepancies, notably in ϕ_0 , ϕ'_1 and ϕ'_2/ϕ'_{12} , which cannot be explained by the small differences of pH and of specific activity of the enzyme. Consequently, though the data of Theorell & McKinley McKee conform closely to all the requirements of the Theorell–Chance mechanism, the present data do not conform to the required relations involving ϕ'_2/ϕ'_{12} (cf. Tables 4 and 5). The value of ϕ_0 found by Theorell & McKinley McKee is within the range obtained here with commercial samples of NADH_2 and is believed to be about twice the true value. That the relation $\phi'_1/\phi_0 = K_1$ was nevertheless satisfied by their results is perhaps due to compensating error in ϕ'_1 which is difficult to determine accurately at this unfavourable pH value.

Such discrepancies, and the unlooked-for effect of small amounts of a competitive inhibitor in NADH_2 , underline the difficulties of accurately evaluating the complete initial-rate equation for such systems over a wide pH range even with the sensitive fluorimetric method, although some attempt has been made here to test the reproducibility of estimates of the kinetic coefficients. As a means of distinguishing mechanisms, comparisons of kinetic coefficients with different substrates, or coenzyme analogues, are perhaps less susceptible to

Table 4. Comparison of maximum specific rates, $1/\phi_0$, with rates of dissociation of product coenzyme–enzyme compounds ($\phi_{12}/\phi_1\phi_2$) calculated on the assumption of a compulsory-order mechanism

The kinetic data were obtained as described in the text.

Substrates	pH	$1/\phi_0$ (sec. ⁻¹)	$\phi'_{12}/\phi'_1\phi'_2$ (sec. ⁻¹)	$1/\phi'_0$ (sec. ⁻¹)	$\phi_{12}/\phi_1\phi_2$ (sec. ⁻¹)
Acetaldehyde, ethanol	6.0	125	35	1.6	3.5
Acetaldehyde, ethanol	7.1	133	60	2.7	4.2
Butyraldehyde, butanol	7.1	133	46	2.9	2.4

Table 5. Comparison of kinetic and direct values for dissociation constants of coenzyme–enzyme compounds and overall equilibrium constants

Values of K_1 and K'_1 are those of Theorell & McKinley McKee (1961), and K_0 values for the acetaldehyde–ethanol system are those of Bäcklin (1958). Other kinetic data were obtained as described in the text.

Substrates	pH	NADH_2		NAD		Overall	
		ϕ_{12}/ϕ_2 (μM)	K_1 (μM)	ϕ'_{12}/ϕ'_2 (μM)	K'_1 (μM)	$\phi_{12}[\text{H}^+]/\phi'_{12}$ (μM)	K_0 (μM)
Acetaldehyde, ethanol	6.0	0.4	0.23	88	266	21	9
Acetaldehyde, ethanol	7.1	0.4	0.31*	65	160*	19	9
Butyraldehyde, butanol	7.1	0.24	0.31*	50	160*	11	9

* At pH 7.0.

systematic and random error than relations between several coefficients or between functions of several coefficients and independent values for dissociation and equilibrium constants. Such comparisons would be especially useful where accurate estimates of the dissociation constants cannot easily be obtained directly, as may be the case for NAD complexes (Theorell & Winer, 1959; Theorell & McKinley McKee, 1961) and NADH₂ complexes which do not differ greatly in absorption or fluorescence spectrum from free NADH₂.

Discussion of the mechanism. The findings that ϕ_0 , ϕ_1 , ϕ'_0 and ϕ'_1 are independent of the nature of the substrate, and the confirmation of the correlation of these with enzyme-coenzyme dissociation constants (Theorell & McKinley McKee, 1961), strongly suggest that the maximum rates of the enzyme-catalysed reaction may be identified with the rates of dissociation of the coenzyme complexes, and ϕ_1 and ϕ'_1 with the reciprocals of the specific rates of formation of the coenzyme complexes. The original observations of Theorell & Chance (1951) are therefore confirmed. On the other hand, there are significant deviations from relations between the ratio ϕ'_0/ϕ'_{12} and other kinetic coefficients and dissociation constants, which are required by the compulsory-order mechanism suggested by Theorell & Chance (1951), whether or not rate-limiting ternary complexes are also postulated. These discrepancies could not be detected in the previous work in the absence of measurements with low concentrations of both substrate and coenzyme. A satisfactory mechanism must therefore retain the essential feature of the Theorell-Chance mechanism, namely limitation of the maximum rate by dissociation of the product coenzyme-enzyme compound (cf. Dixon & Webb, 1958), but be free from the restrictions on the relative values of the kinetic coefficients imposed by a simple compulsory-order mechanism.

Of other distinct mechanisms which have been considered for coenzyme-substrate reactions (Alberty, 1953), that in which the enzyme itself undergoes intermediate oxidation and reduction,

and to which certain flavoprotein systems conform (Massey, Gibson & Veeger, 1960; Massey, Palmer & Bennett, 1961), requires $\phi_{12} = \phi'_{12} = 0$ (Dalziel, 1957), and is therefore not applicable to liver alcohol dehydrogenase. The observed small value of ϕ_{12} is consistent with the values of the other kinetic coefficients in a compulsory-order mechanism, but the possibility of a contribution from an enzyme-oxidation pathway in a mixed mechanism cannot be excluded.

There remains the more general mechanism in which both coenzyme and substrate form active binary complexes with the enzyme, so that the reaction may proceed by alternative pathways (Scheme 2). Steady-state treatment of this mechanism gives a complex non-linear initial-rate equation (Dalziel, 1958*b*). Special cases, in which it is assumed that the enzyme complexes are in rapid equilibrium and that the intramolecular reaction of the ternary complex is rate-limiting, give an initial rate equation of the form of equation (1) (Haldane, 1930; Segal, Kachmar & Boyer, 1952), but are not consistent with the present results (cf. Dalziel, 1957, 1958*b*).

Theorell & McKinley McKee (1961) also concluded that the liver-alcohol-dehydrogenase reaction follows the mechanism shown in Scheme 2. Nevertheless they suggested that the initial-rate data conform completely to the requirements of the Theorell-Chance mechanism because:

- (i) $k'_{-3} \gg k'_{-4}$
- (ii) k and k' are large
- (iii) $k_{+1} = k_{+4}$.

It is desirable to show whether or not these or other assumptions allow simplification of the complex initial-rate equation, obtained by steady-state treatment of the mechanism shown in Scheme 2 (Dalziel, 1958*b*), to a form consistent with the experimental results. (Only the forward reaction will be considered; the complementary relations for the reverse reaction may be obtained by insertion or deletion of primes on the rate constants.)

If either $[S_2]$ or $[S_1]$ is sufficiently large, the initial-rate equation for the mechanism shown in Scheme 2 reduces to:

$$e/v_0 = \phi_0 + \phi_1/[S_1] \text{ or } e/v_0 = \phi_0 + \phi_2/[S_2]$$

with:

$$\phi_0 = \frac{1}{k} + \frac{1}{k'_{-3} + k'_{-4}} + \frac{k'}{k(k'_{-3} + k'_{-4})} + \frac{k'_{-1}k'_{-4} + k'_{-2}k'_{-3}}{k'_{-1}k'_{-2}(k'_{-3} + k'_{-4})} \quad (2)$$

$$\phi_1 = \frac{k_{-4}(k' + k'_{-3} + k'_{-4}) + k(k'_{-3} + k'_{-4})}{kk_{+4}(k'_{-3} + k'_{-4})} \quad (3)$$

$$\phi_2 = \frac{k_{-3}(k' + k'_{-3} + k'_{-4}) + k(k'_{-3} + k'_{-4})}{kk_{+3}(k'_{-3} + k'_{-4})} \quad (4)$$

Considering first the reciprocal of the maximum rate, with condition (i) and:

$$(iv) \quad k'_{-2}k'_{-3} \gg k'_{-1}k'_{-4}$$

then $\phi_0 = (1/k + 1/k'_{-3} + k'/kk'_{-3} + 1/k'_{-1})$,

which is identical with the expression for ϕ_0 in the compulsory-order mechanism (Scheme 1) (Dalziel, 1957). In the dissociation of products, the reaction is confined to the upper path of the mechanism shown in Scheme 1 by (i), and (iv) ensures that the steady-state concentration of ES'_2 is negligible. If also:

$$(v) \quad k \gg k'_{-1}$$

$$(vi) \quad k'_{-3} \gg k'_{-1}$$

$$(vii) \quad kk'_{-3} \gg k'k'_{-1}$$

then the last step is rate-determining and $\phi_0 = 1/k'_{-1}$. Condition (v), with condition (viii) below, is the required explicit form of condition (ii). Condition (vii) ensures that the reverse reaction of $ES'_1S'_2$ does not limit the maximum rate.

For ϕ_1 , with condition (i), equation (3) becomes:

$$\phi_1 = \frac{1}{k_{+4}} \left(1 + \frac{k_{-4}}{k} + \frac{k'k_{-4}}{kk'_{-3}} \right) \quad (5)$$

Since $[S_2]$ is large, the ternary complex is formed via the lower path through ES_2 , and S_1 is in the role of S_2 in a compulsory-order mechanism: accordingly, equation (5) is precisely analogous to that for ϕ_2 in the compulsory-order mechanism (Scheme 1) (Dalziel, 1957). If also:

$$(viii) \quad k \gg k_{-4}$$

$$(ix) \quad kk'_{-3} \gg k'k_{-4}$$

so that dissociation of S_1 from ES_1S_2 , and the reverse reaction $ES'_1S'_2 \rightarrow ES_1S_2$, are negligible, then $\phi_1 = 1/k_{+4}$. Finally, if the presence of bound substrate does not affect the rate of combination of enzyme with the coenzyme [condition (iii)], then $\phi_1 = 1/k_{+1}$.

Thus, with certain qualifications and additions [notably condition (vi)] to the assumptions suggested by Theorell & McKinley McKee (1961), and

corresponding conditions for the reverse reaction, all of which are compatible with one another and with the relation which must exist between all the rate constants for alternative pathways, the mechanism shown in Scheme 2 is consistent with the experimental results for liver alcohol dehydrogenase so far as ϕ_0 , ϕ_1 , ϕ'_0 and ϕ'_1 are concerned.

For ϕ_2 , with condition (i), equation (4) becomes:

$$\phi_2 = \frac{1}{k_{+3}} \left(1 + \frac{k_{-4}}{k} + \frac{k'k_{-4}}{kk'_{-3}} \right)$$

Inspection shows that, because of the unsymmetrical nature of condition (i), simultaneous simplification to $\phi_2 = 1/k_{+3}$ and $\phi'_2 = 1/k'_{+3}$ would require conditions analogous to, but contradictory with, condition (ix) and its complement for the reverse reaction. Moreover, it has not proved possible to reduce the complete initial-rate equation for the mechanism shown in Scheme 2, with finite concentrations of both S_1 and S_2 , to that deduced for the Theorell-Chance mechanism, or indeed to a linear reciprocal equation of the form of equation (1), except by assumptions

$$(k_{+2}S_2 \ll k_{-2} \text{ and } k_{+4} \leq k_{+1})$$

which effectively confine the reaction to the upper path in the reactant phase. Thus the experimental values for ϕ_{12} and ϕ'_{12} cannot be given a simple interpretation as functions of rate constants, and relations involving these coefficients deduced for the Theorell-Chance mechanism will not necessarily be accurately fulfilled.

Further evidence that binary enzyme-substrate complexes play a significant role in the overall kinetics, preferably by direct estimates of their dissociation constants, is required. Competitive behaviour between inhibitors and substrate (Theorell & McKinley McKee, 1961) could perhaps be as well explained by competition for ES_1 as for the free enzyme. Dalziel (1957) has suggested that the reaction is effectively confined to the upper path over the substrate concentration range for which linear Lineweaver-Burk plots are obtained, and that the inhibition observed with high ethanol concentrations (Theorell *et al.* 1955) is due to intervention of the less efficient lower path. However, this fails to account for the fact that this inhibition is less marked with low than with high NAD concentrations (K. Dalziel, unpublished work), as well as for the discrepancies from the strict requirements of the Theorell-Chance mechanism.

It has been considered that the alcohol dehydrogenases of liver and yeast catalyse the same reaction by quite different mechanisms, but it now appears that the differences may be qualitative only. Initial-rate data for the yeast enzyme (Nygaard & Theorell, 1955a) also conformed to the requirements of the mechanism shown in Scheme 2

but the intermolecular reaction of the ternary complex was assumed to be rate-limiting. The discrepancies from the compulsory-order mechanism shown in Scheme 1 were closely analogous to, although greater than, those recorded in Table 4 for the liver enzyme (Dalziel, 1957). The evidence that the intramolecular reaction of the ternary complex is rate-limiting seems to consist only in an approximate concordance of dissociation constants for enzyme-coenzyme complexes calculated from the kinetic data on this assumption with values obtained by the ultracentrifuge method (Hayes & Velick, 1954) under rather different conditions of temperature and pH (Nygaard & Theorell, 1955*b*). Almost equally good concordance is obtained, however, if the dissociation constants are calculated from ϕ_0 and ϕ'_1 , in accordance with the essential features of the Theorell-Chance mechanism (cf. Table 3). Kinetic data with two or more pairs of substrates and direct estimates of the dissociation constants under comparable conditions are lacking for the yeast enzyme. Similar remarks may be made for lactate dehydrogenase, for which the compulsory-order mechanism (Scheme 1) with rate-limiting ternary complexes has been proposed (Winer & Schwert, 1958), especially as this enzyme may be sensitive to the effects of competitive inhibitors in NADH₂ preparations (N. O. Kaplan, personal communication; Dalziel, 1962*b*).

SUMMARY

1. Initial-rate data are reported for the liver-alcohol-dehydrogenase reaction with substrates ethanol and acetaldehyde at pH 6.0 and 7.1, and butanol and butyraldehyde at pH 7.1.

2. The results for both pairs of substrates are consistent with the essential features of the mechanism proposed by Theorell & Chance (1951) whereby certain parameters of the initial-rate equation are identified with the specific rates of the reversible reaction of the enzyme and the co-enzymes. Deviations from the strict requirements of a compulsory-order mechanism are observed, however, and several other simple mechanisms are inconsistent with the results.

3. In agreement with other workers, it is suggested that a more general mechanism involving alternative active pathways through binary co-enzyme and substrate compounds of the enzyme will best account for the results. Conditions under

which this mechanism approximates to the Theorell-Chance mechanism, and may also account for the discrepancies, are deduced.

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